WORLD ORGANISATION FOR ANIMAL HEALTH

MANUAL OF DIAGNOSTIC TESTS AND VACCINES
FOR TERRESTRIAL ANIMALS
(mammals, birds and bees)

Seventh Edition
Volume 1

2012
Reference to commercial kits does not mean their endorsement by the OIE. All commercial kits should be validated; tests on the OIE register have already met this condition (the register can be consulted at: www.oie.int).
FOREWORD

The Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual) aims to prevent and control animal diseases, including zoonoses, to contribute to the improvement of animal health services world-wide and to allow safe international trade in animals and animal products. The principal target readership is laboratories carrying out veterinary diagnostic tests and surveillance, along with vaccine manufacturers and users, and regulatory authorities in Member Countries. The main objective is to provide internationally agreed diagnostic laboratory methods and requirements for the production and control of relevant vaccines and other biological products.

This ambitious task has required the cooperation of highly renowned animal health specialists from many OIE Member Countries. The OIE, the World Organisation for Animal Health, received the mandate from its Member Countries to undertake this task on a global level. The main activities of the organisation, which was established in 1924, and in 2012 comprised 178 Member Countries, are as follows:

1. To ensure transparency in the global animal disease and zoonosis situation.
2. To collect, analyse and disseminate scientific veterinary information on animal disease control methods.
3. To provide expertise and encourage international solidarity in the control of animal diseases.
4. Within its mandate under the WTO (World Trade Organization) Agreement on Sanitary and Phytosanitary Measures (SPS Agreement), to safeguard world trade by publishing health standards for international trade in animals and animal products.
5. To improve the legal framework and resources of national Veterinary Services.
6. To provide a better guarantee of the safety of food of animal origin and to promote animal welfare through a science-based approach.

The Terrestrial Manual, covering infectious and parasitic diseases of mammals, birds and bees, was first published in 1989. Each successive edition has extended and updated the information provided. This seventh edition includes over 50 updated chapters and guidelines (including a new guideline on the application of biotechnology to the development of veterinary vaccines, and the addition of epizootic haemorrhagic disease to the relevant chapter). This edition has a slightly different structure from former editions: Part 1 contains ten introductory chapters that set general standards for the management of veterinary diagnostic laboratories and vaccine production facilities; Part 2 comprises chapters on OIE listed diseases and other diseases of importance to international trade; Part 3 comprises four guidelines that have been developed on topics such as biotechnology and antimicrobial susceptibility testing that are intended to give a brief introduction to their subjects (they are to be regarded as background information rather than strict standards); and Part 4 is the list of OIE Reference Centres at the time of publication (the list of OIE Reference Centres is updated by the World Assembly of Delegates (of OIE Member Countries) each year; the revised list is available on the OIE Web site).

As a companion volume to the Terrestrial Animal Health Code, the Terrestrial Manual sets laboratory standards for all OIE listed diseases as well as several other diseases of global importance. In particular it specifies (in blue font) those “Prescribed Tests” that are recommended for use in health screening for international trade or movement of animals. The Terrestrial Manual has become widely adopted as a key reference book for veterinary laboratories around the world. Aquatic animal diseases are included in a separate Aquatic Manual.

The task of commissioning chapters and compiling the Terrestrial Manual was assigned to the OIE Biological Standards Commission by the World Assembly of national Delegates. Manuscripts were requested from specialists (usually the OIE designated experts at OIE Reference Laboratories) in each of the diseases or the other topics covered. Occasionally, an ad hoc Group of experts was convened tasked with updating or developing a chapter. After initial scrutiny by the Consultant Technical Editor, the chapters were sent to scientific reviewers and to experts at OIE Reference Laboratories. They were also circulated to all OIE Member Countries for review and comment. The Biological Standards Commission, elected every 3 years by the World Assembly, and the
Consultant Technical Editor took all the resulting comments into consideration, often referring back to the contributors for further help, before finalising the chapters. The final text has the approval of the World Assembly.

A procedure for the official recognition of commercialised diagnostic tests, under the authority of the Assembly, was finalised in September 2004. Data are submitted using a validation template that was developed by the Biological Standards Commission. Submissions are evaluated by appointed experts, who advise the Biological Standards Commission before the final opinion of the OIE World Assembly is sought. All information on the submission of applications can be found on the OIE Web site.

The Terrestrial Manual continues to expand and to extend its range of topics covered. It is our sincere hope that it will grow in usefulness to veterinary diagnosticians and vaccine manufacturers in all the OIE Member Countries. A new paper edition of the Terrestrial Manual is published every 4 years. It is important to note that annual updates to the Terrestrial Manual will be published on the OIE website once approved by the World Assembly, so readers are advised to check there for the latest information. This new version of the Terrestrial Manual is published in English and Spanish.

Doctor Bernard Vallat
Director General, OIE
September 2012

Professor Vincenzo Caporale
President, OIE Biological Standards Commission
September 2012
I am most grateful to the many people whose combined efforts have gone into the preparation of this Terrestrial Manual. In particular, I would like to express my thanks to:

Dr Bernard Vallat, Director General of the OIE from 2001 to the present, who gave his encouragement and support to the project of preparing the new edition of this Terrestrial Manual,

The Members of the OIE Standards Commission, Prof. Vincenzo Caporale, Dr Beverly Schmitt, Dr Mehdi El Harrak, Dr Paul Townsend, Dr Alejandro Schudel and Dr Hualan Chen who were responsible for commissioning chapters and, with the Consultant Technical Editor, for editing all the contributions so as to finalise this edition of the Terrestrial Manual,

The contributors listed on pages xxii to xxxv who contributed their invaluable time and expertise to write the chapters,

The expert advisers to the Biological Standards Commission’s meeting, Dr Adama Diallo and Dr Peter Wright, the OIE Reference Laboratory experts and other reviewers who also gave their time and expertise to scrutinising the chapters,

Those OIE Member Countries that submitted comments on the draft chapters that were circulated to them. These were essential in making the Terrestrial Manual internationally acceptable,

Ms Sara Linnane who, as Scientific Editor, organised this complex project and made major contributions to the quality of the text,

Prof. Steven Edwards, Consultant Technical Editor of the Terrestrial Manual, who contributed hugely to editing and harmonising the contents, but also in collating and incorporating Member Country comments,

Members of both the OIE Scientific and Technical Department and the Publications Department, for their assistance.

Dr Karin Schwabenbauer
President of the OIE World Assembly

September 2012
## CONTENTS

### VOLUME 1

Introduction (How to use this Terrestrial Manual) .......................................................... ix  
List of tests for International trade .................................................................................. xi  
Common abbreviations used in this Terrestrial Manual .................................................. xv  
Glossary of terms ............................................................................................................. xvii  
Contributors ..................................................................................................................... xxii

### PART 1 GENERAL STANDARDS

#### SECTION 1.1. INTRODUCTORY CHAPTERS

Chapter 1.1.1. Collection and shipment of diagnostic specimens .................................. 3  
Chapter 1.1.2. Transport of specimens of animal origin (under study) ......................... 15  
Chapter 1.1.3. Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities ................................................................. 16  
Chapter 1.1.4. Quality management in veterinary testing laboratories ...................... 27  
Chapter 1.1.5. Principles and methods of validation of diagnostic assays for infectious diseases ................................................................. 34  
Chapter 1.1.6. Principles of veterinary vaccine production ........................................... 52  
Chapter 1.1.7. Tests for sterility and freedom from contamination of biological materials ......................... 67  
Chapter 1.1.8. Minimum requirements for vaccine production facilities (under study) ......................... 77  
Chapter 1.1.9. Quality control of vaccines (under study) ........................................... 78  
Chapter 1.1.10. International standards for vaccine banks ......................................... 79

### PART 2 OIE LISTED DISEASES AND OTHER DISEASES OF IMPORTANCE TO INTERNATIONAL TRADE

#### SECTION 2.1. MULTIPLE SPECIES

Chapter 2.1.1. Anthrax ............................................................................................. 87  
Chapter 2.1.2. Aujeszky's disease ............................................................................. 97  
Chapter 2.1.3. Bluetongue and Epizootic haemorrhagic disease ............................... 112  
Chapter 2.1.4. Echinococcosis/hydatidosis ............................................................... 130  
Chapter 2.1.5. Foot and mouth disease .................................................................. 145  
Chapter 2.1.6. Heartwater ..................................................................................... 174  
Chapter 2.1.7. Japanese encephalitis ....................................................................... 188  
Chapter 2.1.8. Leishmaniosis .................................................................................. 198  
Chapter 2.1.9. Leptospirosis ................................................................................... 209  
Chapter 2.1.10. New World screwworm (Cochliomyia hominivorax) and Old World screwworm (Chrysomya bezziana) ............................................................................ 223  
Chapter 2.1.11. Paratuberculosis (Johne's disease) ................................................... 234  
Chapter 2.1.12. Q fever ......................................................................................... 250  
Chapter 2.1.13. Rabies ......................................................................................... 263  
Chapter 2.1.14. Rift Valley fever ............................................................................ 283  
Chapter 2.1.15. Rinderpest .................................................................................... 294  
Chapter 2.1.16. Trichinellosis ............................................................................... 305  
Chapter 2.1.17. Trypanosoma evansi infections (including surra) ............................. 314  
Chapter 2.1.18. Tularemia ..................................................................................... 329  
Chapter 2.1.19. Vesicular stomatitis ...................................................................... 335  
Chapter 2.1.20. West Nile fever ............................................................................. 346
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.1.</td>
<td>Acarapisis of honey bees</td>
<td>357</td>
</tr>
<tr>
<td>2.2.2.</td>
<td>American foulbrood of honey bees</td>
<td>358</td>
</tr>
<tr>
<td>2.2.3.</td>
<td>European foulbrood of honey bees</td>
<td>365</td>
</tr>
<tr>
<td>2.2.5.</td>
<td>Small hive beetle infestation (Aethina tumida)</td>
<td>375</td>
</tr>
<tr>
<td>2.2.6.</td>
<td>Tropilaelaps infestation of honey bees (Tropilaelaps spp.)</td>
<td>380</td>
</tr>
<tr>
<td>2.2.7.</td>
<td>Varroosis of honey bees</td>
<td>385</td>
</tr>
<tr>
<td>2.4.1.</td>
<td>Bovine anaplasmosis</td>
<td>401</td>
</tr>
<tr>
<td>2.4.2.</td>
<td>Bovine babesiosis</td>
<td>414</td>
</tr>
<tr>
<td>2.4.3.</td>
<td>Bovine brucellosis</td>
<td>427</td>
</tr>
<tr>
<td>2.4.4.</td>
<td>Bovine cysticercosis</td>
<td>436</td>
</tr>
<tr>
<td>2.4.5.</td>
<td>Bovine genital campylobacteriosis</td>
<td>455</td>
</tr>
<tr>
<td>2.4.6.</td>
<td>Bovine spongiform encephalopathy</td>
<td>470</td>
</tr>
<tr>
<td>2.4.7.</td>
<td>Bovine tuberculosis</td>
<td>480</td>
</tr>
<tr>
<td>2.4.8.</td>
<td>Bovine viral diarrhoea</td>
<td>489</td>
</tr>
<tr>
<td>2.4.9.</td>
<td>Contagious bovine pleuropneumonia</td>
<td>500</td>
</tr>
<tr>
<td>2.4.10.</td>
<td>Dermatophilosis</td>
<td>507</td>
</tr>
<tr>
<td>2.4.11.</td>
<td>Enzootic bovine leucosis</td>
<td>514</td>
</tr>
<tr>
<td>2.4.12.</td>
<td>Infectious bursal disease (Gumboro disease)</td>
<td>528</td>
</tr>
<tr>
<td>2.4.13.</td>
<td>Infectious bovine rhinotracheitis/Infectious pustular vulvovaginitis</td>
<td>545</td>
</tr>
<tr>
<td>2.4.14.</td>
<td>Lumpy skin disease</td>
<td>555</td>
</tr>
<tr>
<td>2.4.15.</td>
<td>Malignant catarrhal fever</td>
<td>574</td>
</tr>
<tr>
<td>2.4.16.</td>
<td>Theileriosis</td>
<td>589</td>
</tr>
<tr>
<td>2.4.17.</td>
<td>Trichomonosiosis</td>
<td>601</td>
</tr>
<tr>
<td>2.4.18.</td>
<td>Trypanosomosis (Tsetse-transmitted)</td>
<td>616</td>
</tr>
</tbody>
</table>
INTRODUCTION
(How to use this Terrestrial Manual)

• Arrangement of the Terrestrial Manual

Part 1, the beginning of this Terrestrial Manual, contains ten introductory chapters that set general standards for the management of veterinary diagnostic laboratories and vaccine facilities.

The main part of the Terrestrial Manual (Part 2) covers standards for diagnostic tests and vaccines for specific diseases listed in the OIE Terrestrial Animal Health Code. The diseases are in alphabetical order, subdivided by animal host group. OIE listed diseases are transmissible diseases that have the potential for very serious and rapid spread, irrespective of national borders. They have particularly serious socio-economic or public health consequences and are of major importance in the international trade of animals and animal products.

Four of the diseases in Section 2.9 are included in some individual species sections, but these chapters cover several host species and thus give a broader description. Some additional diseases that may also be of importance to trade but that do not have a chapter in the Terrestrial Code are also included in Section 2.9. This section also includes some important zoonotic infections.

The contributors of all the chapters are listed on pages xxii–xxxv, but the final responsibility for the content of the Terrestrial Manual lies with the World Assembly of the OIE.

There is an alphabetical index of the diseases at the end of Volume 2.

• Format of chapters

Each disease chapter includes a summary intended to provide information for veterinary officials and other readers who need a general overview of the tests and vaccines available for the disease. This is followed by a text giving greater detail for laboratory workers. In each disease chapter, Part A gives a general introduction to the disease, Part B deals with laboratory diagnosis of the disease, and Part C (where appropriate) with the requirements for vaccines or in vivo diagnostic biologicals. The information concerning production and control of vaccines or diagnostics is given as an example; it is not always necessary to follow these when there are scientifically justifiable reasons for using alternative approaches. Bibliographic references that provide further information are listed at the end of each chapter.

• Explanation of the tests described and of the table on pages xi–xiv

The table on pages xi–xiv lists diagnostic tests in two categories: ‘prescribed’ and ‘alternative’. Prescribed tests are those that are required by the Terrestrial Animal Health Code for the testing of animals before they are moved internationally. In the Terrestrial Manual these tests are printed in blue. At present it is not possible to have prescribed tests for every listed disease. ‘Alternative tests’ are those that are suitable for the diagnosis of disease within a local setting, and can also be used in the import/export of animals after bilateral agreement. There are often other tests described in the chapters, which may be useful for specific purposes such as diagnosis or surveillance as indicated in the chapters.
• General guidelines

Four guidelines that have been developed on topics such as biotechnology and antimicrobial susceptibility testing are included in Part 3 of this Terrestrial Manual. These are intended to give a brief introduction to their subjects. They are to be regarded as background information rather than standards.

• List of OIE Reference Laboratories

A list of OIE Reference Laboratories is given in Part 4 of this Terrestrial Manual. These laboratories have been designated by the OIE as centres of excellence with expertise in their particular field. They are able to provide advice to other laboratories on methodology. In some cases standard strains of micro-organisms or reference reagents (e.g. antisera, antigens) can also be obtained from the OIE Reference Laboratories.

The list of OIE Reference Laboratories will be updated by the World Assembly each year. The revised list is available on the OIE Web site.

* 
* *
LIST OF TESTS FOR INTERNATIONAL TRADE

The table below lists diagnostic tests in two categories: ‘prescribed’ and ‘alternative’. Prescribed tests are required by the OIE Terrestrial Animal Health Code for the international movement of animals and animal products and are considered optimal for determining the health status of animals. In the Terrestrial Manual these tests are printed in blue. At present it is not possible to have prescribed tests for every listed disease. Alternative tests are those that are suitable for the diagnosis of disease within a local setting, and can also be used in the import/export of animals after bilateral agreement. There are often other tests described in the chapters that may also be of some practical value in local situations or that may still be under development.

<table>
<thead>
<tr>
<th>Chapter No.</th>
<th>Disease name</th>
<th>Prescribed tests</th>
<th>Alternative tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.1.</td>
<td>Anthrax</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.1.2.</td>
<td>Aujeszky’s disease</td>
<td>ELISA, VN</td>
<td>–</td>
</tr>
<tr>
<td>2.1.3.</td>
<td>Bluetongue</td>
<td>Agent id., ELISA, PCR</td>
<td>AGID, VN</td>
</tr>
<tr>
<td>2.1.4.</td>
<td>Echinococciosis/Hydatidosis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.1.5.</td>
<td>Foot and mouth disease</td>
<td>ELISA*, VN</td>
<td>CF</td>
</tr>
<tr>
<td>2.1.6.</td>
<td>Heartwater</td>
<td>–</td>
<td>ELISA, IFA</td>
</tr>
<tr>
<td>2.1.7.</td>
<td>Japanese encephalitis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.1.8.</td>
<td>Leishmaniosis</td>
<td>–</td>
<td>Agent id.</td>
</tr>
<tr>
<td>2.1.9.</td>
<td>Leptospirosis</td>
<td>–</td>
<td>MAT</td>
</tr>
<tr>
<td>2.1.10.</td>
<td>New World screwworm (Cochliomyia hominivorax) and Old World screwworm (Chrysomya bezziana)</td>
<td>–</td>
<td>Agent id.</td>
</tr>
<tr>
<td>2.1.11.</td>
<td>Paratuberculosis (Johne’s disease)</td>
<td>–</td>
<td>DTH, ELISA</td>
</tr>
<tr>
<td>2.1.12.</td>
<td>Q fever</td>
<td>–</td>
<td>CF</td>
</tr>
<tr>
<td>2.1.13.</td>
<td>Rabies</td>
<td>ELISA, VN</td>
<td>–</td>
</tr>
<tr>
<td>2.1.14.</td>
<td>Rift Valley fever</td>
<td>VN</td>
<td>ELISA, HI</td>
</tr>
<tr>
<td>2.1.15.</td>
<td>Rinderpest</td>
<td>ELISA</td>
<td>VN</td>
</tr>
<tr>
<td>2.1.16.</td>
<td>Trichinellosis</td>
<td>Agent id.</td>
<td>ELISA</td>
</tr>
<tr>
<td>2.1.17.</td>
<td>Trypanosoma evansi infections (including surra)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.1.18.</td>
<td>Tularemia</td>
<td>–</td>
<td>Agent id.</td>
</tr>
<tr>
<td>2.1.19.</td>
<td>Vesicular stomatitis</td>
<td>CF, ELISA, VN</td>
<td>–</td>
</tr>
<tr>
<td>2.1.20.</td>
<td>West Nile fever</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.2.1.</td>
<td>Acarapisosis of honey bees</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.2.2.</td>
<td>American foulbrood of honey bees</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Please refer to Terrestrial Manual chapters to verify which method is prescribed.
<table>
<thead>
<tr>
<th>Chapter No.</th>
<th>Disease name</th>
<th>Prescribed tests</th>
<th>Alternative tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.3.</td>
<td>European foulbrood of honey bees</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.2.4.</td>
<td>Nosemosis of bees</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.2.5.</td>
<td>Small hive beetle infestation (&lt;i&gt;Aethina tumida&lt;/i&gt;)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.2.6.</td>
<td>Tropilaelaps infestation of honey bees (&lt;i&gt;Tropilaelaps spp.&lt;/i&gt;)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.2.7</td>
<td>Varroosis of honey bees</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.3.1.</td>
<td>Avian chlamydiosis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.3.2.</td>
<td>Avian infectious bronchitis</td>
<td>–</td>
<td>ELISA, HI, VN</td>
</tr>
<tr>
<td>2.3.3.</td>
<td>Avian infectious laryngotracheitis</td>
<td>–</td>
<td>AGID, ELISA, VN</td>
</tr>
<tr>
<td>2.3.4.</td>
<td>Avian influenza</td>
<td>Virus isolation with pathogenicity testing</td>
<td>AGID, HI</td>
</tr>
<tr>
<td>2.3.5.</td>
<td>Avian mycoplasmosis (&lt;i&gt;Mycoplasma gallisepticum, M. synoviae&lt;/i&gt;)</td>
<td>–</td>
<td>Agg., HI</td>
</tr>
<tr>
<td>2.3.6.</td>
<td>Avian tuberculosis</td>
<td>–</td>
<td>Agent id., Tuberculin test</td>
</tr>
<tr>
<td>2.3.7.</td>
<td>Duck virus enteritis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.3.8.</td>
<td>Duck virus hepatitis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.3.9.</td>
<td>Fowl cholera</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.3.10.</td>
<td>Fowl pox</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.3.11.</td>
<td>Fowl typhoid and Pullorum disease</td>
<td>–</td>
<td>Agent id., Agg.</td>
</tr>
<tr>
<td>2.3.12.</td>
<td>Infectious bursal disease (Gumboro disease)</td>
<td>–</td>
<td>AGID, ELISA</td>
</tr>
<tr>
<td>2.3.13.</td>
<td>Marek’s disease</td>
<td>–</td>
<td>AGID</td>
</tr>
<tr>
<td>2.3.14.</td>
<td>Newcastle disease</td>
<td>Virus isolation</td>
<td>HI</td>
</tr>
<tr>
<td>2.3.15.</td>
<td>Turkey rhinotracheitis (avian metapneumovirus)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.4.1.</td>
<td>Bovine anaplasmosis</td>
<td>–</td>
<td>CAT, CF</td>
</tr>
<tr>
<td>2.4.2.</td>
<td>Bovine babesiosis</td>
<td>–</td>
<td>CF, ELISA, IFA</td>
</tr>
<tr>
<td>2.4.3.</td>
<td>Bovine brucellosis</td>
<td>BBAT, CF, ELISA, FPA</td>
<td>–</td>
</tr>
<tr>
<td>2.4.4.</td>
<td>Bovine cysticercosis</td>
<td>–</td>
<td>Agent id.</td>
</tr>
<tr>
<td>2.4.5.</td>
<td>Bovine genital campylobacteriosis</td>
<td>Agent id.</td>
<td>–</td>
</tr>
<tr>
<td>2.4.6.</td>
<td>Bovine spongiform encephalopathy</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.4.7.</td>
<td>Bovine tuberculosis</td>
<td>Tuberculin test</td>
<td>Gamma interferon test</td>
</tr>
<tr>
<td>2.4.8.</td>
<td>Bovine viral diarrhoea</td>
<td>Agent id.</td>
<td>–</td>
</tr>
<tr>
<td>2.4.9.</td>
<td>Contagious bovine pleuropneumonia</td>
<td>CF, ELISA</td>
<td>–</td>
</tr>
<tr>
<td>2.4.10.</td>
<td>Dermatophilosis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.4.11.</td>
<td>Enzootic bovine leukosis</td>
<td>AGID, ELISA</td>
<td>PCR</td>
</tr>
<tr>
<td>2.4.12.</td>
<td>Haemorrhagic septicaemia</td>
<td>–</td>
<td>Agent id.</td>
</tr>
<tr>
<td>2.4.13.</td>
<td>Infectious bovine rhinotracheitis/ infectious pustular vulvovaginitis</td>
<td>Agent id. (semen only), ELISA, PCR, VN</td>
<td>–</td>
</tr>
<tr>
<td>Chapter No.</td>
<td>Disease name</td>
<td>Prescribed tests</td>
<td>Alternative tests</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------</td>
<td>------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>2.4.14.</td>
<td>Lumpy skin disease</td>
<td>–</td>
<td>VN</td>
</tr>
<tr>
<td>2.4.15.</td>
<td>Malignant catarrhal fever</td>
<td>–</td>
<td>IFA, PCR, VN</td>
</tr>
<tr>
<td>2.4.16.</td>
<td>Theileriosis</td>
<td>Agent id., IFA</td>
<td>–</td>
</tr>
<tr>
<td>2.4.17.</td>
<td>Trichomonos</td>
<td>Agent id.</td>
<td>Mucus agg.</td>
</tr>
<tr>
<td>2.4.18.</td>
<td>Trypanosomosis (Tsetse-transmitted)</td>
<td>–</td>
<td>IFA</td>
</tr>
<tr>
<td>2.5.1.</td>
<td>African horse sickness</td>
<td>CF, ELISA</td>
<td>Agent id. (real-time PCR), VN</td>
</tr>
<tr>
<td>2.5.2.</td>
<td>Contagious equine metritis</td>
<td>Agent id.</td>
<td>–</td>
</tr>
<tr>
<td>2.5.3.</td>
<td>Dourine</td>
<td>CF</td>
<td>ELISA, IFA</td>
</tr>
<tr>
<td>2.5.4.</td>
<td>Epizootic lymphangitis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.5.5.</td>
<td>Equine encephalomyelitis (Eastern and Western)</td>
<td>–</td>
<td>CF, HI, PRN</td>
</tr>
<tr>
<td>2.5.6.</td>
<td>Equine infectious anaemia</td>
<td>AGID</td>
<td>ELISA</td>
</tr>
<tr>
<td>2.5.7.</td>
<td>Equine influenza</td>
<td>–</td>
<td>HI</td>
</tr>
<tr>
<td>2.5.8.</td>
<td>Equine piroplasmosis</td>
<td>ELISA, IFA</td>
<td>CF</td>
</tr>
<tr>
<td>2.5.9.</td>
<td>Equine rhinopneumonitis</td>
<td>–</td>
<td>VN</td>
</tr>
<tr>
<td>2.5.10.</td>
<td>Equine viral arteritis</td>
<td>Agent id. (semen only), VN</td>
<td>–</td>
</tr>
<tr>
<td>2.5.11.</td>
<td>Glanders</td>
<td>CF</td>
<td>–</td>
</tr>
<tr>
<td>2.5.12.</td>
<td>Horse mange</td>
<td>–</td>
<td>Agent id.</td>
</tr>
<tr>
<td>2.5.13.</td>
<td>Venezuelan equine encephalomyelitis</td>
<td>–</td>
<td>CF, HI, PRN</td>
</tr>
<tr>
<td>2.6.1.</td>
<td>Myxomatosis</td>
<td>–</td>
<td>AGID, CF, IFA</td>
</tr>
<tr>
<td>2.6.2.</td>
<td>Rabbit haemorrhagic disease</td>
<td>–</td>
<td>ELISA, HI</td>
</tr>
<tr>
<td>2.7.1.</td>
<td>Border disease</td>
<td>Agent id.</td>
<td>–</td>
</tr>
<tr>
<td>2.7.2.</td>
<td>Caprine and ovine brucellosis (excluding Brucella ovis)</td>
<td>BBAT, CF, ELISA, FPA</td>
<td>Brucellin test</td>
</tr>
<tr>
<td>2.7.3/4.</td>
<td>Caprine arthritis/encephalitis &amp; Maedi-visna</td>
<td>AGID, ELISA</td>
<td>–</td>
</tr>
<tr>
<td>2.7.5.</td>
<td>Contagious agalactia</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.7.6.</td>
<td>Contagious caprine pleuropneumonia</td>
<td>CF</td>
<td>–</td>
</tr>
<tr>
<td>2.7.7.</td>
<td>Enzootic abortion of ewes (ovine chlamydiosis)</td>
<td>–</td>
<td>CF</td>
</tr>
<tr>
<td>2.7.8.</td>
<td>Nairobi sheep disease</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.7.9.</td>
<td>Ovine epididymitis (Brucella ovis)</td>
<td>CF</td>
<td>ELISA</td>
</tr>
<tr>
<td>2.7.10.</td>
<td>Ovine pulmonary adenocarcinoma (adenomatosis)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.7.11.</td>
<td>Peste des petits ruminants</td>
<td>VN</td>
<td>ELISA</td>
</tr>
<tr>
<td>2.7.12.</td>
<td>Salmonellosis (S. abortusovis)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.7.13.</td>
<td>Scrapie</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.7.14.</td>
<td>Sheep pox and goat pox</td>
<td>–</td>
<td>VN</td>
</tr>
<tr>
<td>2.8.1.</td>
<td>African swine fever</td>
<td>ELISA</td>
<td>IFA</td>
</tr>
<tr>
<td>2.8.2.</td>
<td>Atrophic rhinitis of swine</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Chapter No.</td>
<td>Disease name</td>
<td>Prescribed tests</td>
<td>Alternative tests</td>
</tr>
<tr>
<td>------------</td>
<td>-------------------------------------------------------------------------------</td>
<td>-----------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>2.8.3.</td>
<td>Classical swine fever (hog cholera)</td>
<td>ELISA, FAVN, NPLA</td>
<td>–</td>
</tr>
<tr>
<td>2.8.4.</td>
<td>Nipah virus encephalitis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.8.5.</td>
<td>Porcine brucellosis</td>
<td>BBAT, CFT, ELISA, FPA</td>
<td>–</td>
</tr>
<tr>
<td>2.8.6.</td>
<td>Porcine cysticercosis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.8.7.</td>
<td>Porcine reproductive and respiratory syndrome</td>
<td>–</td>
<td>ELISA, IFA, IPMA</td>
</tr>
<tr>
<td>2.8.8.</td>
<td>Swine influenza</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.8.9.</td>
<td>Swine vesicular disease</td>
<td>VN</td>
<td>ELISA</td>
</tr>
<tr>
<td>2.8.10.</td>
<td>Teschovirus encephalomyelitis (previously enterovirus encephalomyelitis or</td>
<td>–</td>
<td>VN</td>
</tr>
<tr>
<td></td>
<td>Teschen/Talfan disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.8.11.</td>
<td>Transmissible gastroenteritis</td>
<td>–</td>
<td>VN, ELISA</td>
</tr>
<tr>
<td>2.9.1.</td>
<td>Bunyaviral diseases of animals (excluding Rift Valley fever)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.9.2.</td>
<td>Camelpox</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.9.3.</td>
<td>Campylobacter jejuni and C. coli</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.9.4.</td>
<td>Cryptosporidiosis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.9.5.</td>
<td>Cysticercosis</td>
<td>–</td>
<td>Agent id.</td>
</tr>
<tr>
<td>2.9.6.</td>
<td>Hendra and Nipah virus diseases</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.9.8.</td>
<td>Listeria monocytogenes</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.9.8.</td>
<td>Mange</td>
<td>–</td>
<td>Agent id.</td>
</tr>
<tr>
<td>2.9.9.</td>
<td>Salmonellosis</td>
<td>–</td>
<td>Agent id.</td>
</tr>
<tr>
<td>2.9.10.</td>
<td>Toxoplasmosis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.9.11.</td>
<td>Verocytotoxigenic <em>Escherichia coli</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.9.12.</td>
<td>Zoonoses transmissible from non-human primates</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Note:* The tests prescribed by the *Terrestrial Animal Health Code* for the purposes of international trade are printed in blue in this *Terrestrial Manual.*

**Abbreviations**

- **Agent id.** Agent identification
- **HI** Haemagglutination inhibition
- **Agg.** Agglutination test
- **IFA** Indirect fluorescent antibody
- **AGID** Agar gel immunodiffusion
- **IPMA** Immunoperoxidase monolayer assay
- **BBAT** Buffered *Brucella* antigen test
- **MAT** Microscopic agglutination test
- **CAT** Card agglutination test
- **NPLA** Neutralising peroxidase-linked assay
- **CF** Complement fixation
- **PCR** Polymerase chain reaction
- **DTH** Delayed-type hypersensitivity
- **PRN** Plaque reduction neutralisation
- **ELISA** Enzyme-linked immunosorbent assay
- **VN** Virus neutralisation
- **FAVN** Fluorescent antibody virus neutralisation
- **FPA** Fluorescence polarisation assay
- **–** No test designated yet
### COMMON ABBREVIATIONS USED IN THIS TERRESTRIAL MANUAL

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td>2,2'‐azino‐di‐(3‐ethyl‐benzthiazoline)‐6‐sulphonic acid</td>
</tr>
<tr>
<td>AGID</td>
<td>Agar gel immunodiffusion</td>
</tr>
<tr>
<td>ATCC(^1)</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>BBAT</td>
<td>Buffered Brucella antigen test</td>
</tr>
<tr>
<td>BFK</td>
<td>Bovine fetal kidney (cells)</td>
</tr>
<tr>
<td>BGPS</td>
<td>Beef extract‐glucose‐peptone‐serum (medium)</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby hamster kidney (cell line)</td>
</tr>
<tr>
<td>BLP</td>
<td>Buffered lactose peptone</td>
</tr>
<tr>
<td>BPAT</td>
<td>Buffered plate antigen test</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSF</td>
<td>Bovine serum factors</td>
</tr>
<tr>
<td>CAM</td>
<td>Chorioallantoic membrane</td>
</tr>
<tr>
<td>CEF</td>
<td>Chicken embryo fibroblast</td>
</tr>
<tr>
<td>CF</td>
<td>Complement fixation (test)</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony‐forming unit</td>
</tr>
<tr>
<td>CIEP</td>
<td>Counter immunoelectrophoresis</td>
</tr>
<tr>
<td>CK</td>
<td>Calf kidney (cells)</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>CPLM</td>
<td>Cysteine‐peptone‐liver infusion maltose (medium)</td>
</tr>
<tr>
<td>CSY</td>
<td>Casein‐sucrose‐yeast (agar)</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphide</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed‐type hypersensitivity</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra‐acetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetra‐acetic acid</td>
</tr>
<tr>
<td>EID</td>
<td>Egg‐infective dose</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme‐linked immunosorbent assay</td>
</tr>
<tr>
<td>EMTM</td>
<td>Evans’ modified Tobie’s medium</td>
</tr>
<tr>
<td>EYL</td>
<td>Earle’s yeast lactalbumin (balanced salt solution)</td>
</tr>
<tr>
<td>FAT</td>
<td>Fluorescent antibody test</td>
</tr>
<tr>
<td>FAVN</td>
<td>Fluorescent antibody virus neutralisation</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FLK</td>
<td>Fetal lamb kidney (cells)</td>
</tr>
<tr>
<td>FPA</td>
<td>Fluorescence polarisation assay</td>
</tr>
<tr>
<td>g</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>GIT</td>
<td>Growth inhibition test</td>
</tr>
<tr>
<td>HA</td>
<td>Haemagglutination</td>
</tr>
<tr>
<td>HAD</td>
<td>Haemadsorption</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
</tr>
<tr>
<td>HEP</td>
<td>High‐egg‐passage (virus)</td>
</tr>
<tr>
<td>HEPES</td>
<td>N‐2‐hydroxyethylpiperazine, N‐2‐ethanesulphonic acid (buffer)</td>
</tr>
<tr>
<td>HRPO</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IFA</td>
<td>Indirect fluorescent antibody (test)</td>
</tr>
<tr>
<td>IHA</td>
<td>Indirect haemagglutination</td>
</tr>
<tr>
<td>ICFTU</td>
<td>International complement fixation test unit</td>
</tr>
<tr>
<td>ICPI</td>
<td>Intracerebral pathogenicity index</td>
</tr>
<tr>
<td>ID50</td>
<td>Median infectious dose</td>
</tr>
<tr>
<td>IFA</td>
<td>Indirect fluorescent antibody (test)</td>
</tr>
<tr>
<td>IHA</td>
<td>Indirect haemagglutination</td>
</tr>
<tr>
<td>IPMA</td>
<td>Immunoperoxidase monolayer assay</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>IVPI</td>
<td>Intravenous pathogenicity index</td>
</tr>
<tr>
<td>LA</td>
<td>Latex agglutination</td>
</tr>
<tr>
<td>LD</td>
<td>Lethal dose</td>
</tr>
<tr>
<td>LEP</td>
<td>Low egg passage (virus)</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAT</td>
<td>Microscopic agglutination test</td>
</tr>
<tr>
<td>MCS</td>
<td>Master cell stock</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MDC</td>
<td>Madin‐Darby bovine kidney (cell line)</td>
</tr>
<tr>
<td>MDT</td>
<td>Mean death time</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLV</td>
<td>Modified live virus (vaccine)</td>
</tr>
<tr>
<td>m.o.i.</td>
<td>Multiplicity of infection</td>
</tr>
</tbody>
</table>

\(^1\) American Type Culture Collection, P.O. Box 1549, Manassas, Virginia 20108, United States of America.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSV</td>
<td>Master seed virus</td>
</tr>
<tr>
<td>NI</td>
<td>Neutralisation index</td>
</tr>
<tr>
<td>OGP</td>
<td>1-octyl-beta-D-glucopyranoside (buffer)</td>
</tr>
<tr>
<td>OPD</td>
<td>Orthophenyldiamine (chromogen)</td>
</tr>
<tr>
<td>OPG</td>
<td>Oxalase-phenol-glycerin (preservative solution)</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAP</td>
<td>Peroxidase–antiperoxidase (staining procedure)</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid-Schiff (reaction)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Protective dose</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed field gel electrophoresis</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque-forming unit</td>
</tr>
<tr>
<td>PHA</td>
<td>Passive haemagglutination (test)</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified protein derivative</td>
</tr>
<tr>
<td>PPL0</td>
<td>Pleuroneumonia-like organisms</td>
</tr>
<tr>
<td>PRN</td>
<td>Plaque reduction neutralisation</td>
</tr>
<tr>
<td>PSG</td>
<td>Phosphate buffered saline glucose</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RK</td>
<td>Rabbit kidney</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RSA</td>
<td>Rapid serum agglutination</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SAT</td>
<td>Serum agglutination test</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific pathogen free</td>
</tr>
<tr>
<td>SPG</td>
<td>Sucrose phosphate glutamic acid</td>
</tr>
<tr>
<td>SRBC</td>
<td>Sheep red blood cells</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Median tissue culture infective dose</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethyl benzidine</td>
</tr>
<tr>
<td>TSI</td>
<td>Triple sugar iron (medium)</td>
</tr>
<tr>
<td>VB</td>
<td>Veronal buffer</td>
</tr>
<tr>
<td>VBS</td>
<td>Veronal buffered saline</td>
</tr>
<tr>
<td>Vero</td>
<td>African green monkey kidney (cells)</td>
</tr>
<tr>
<td>VN</td>
<td>Virus neutralisation</td>
</tr>
</tbody>
</table>

* *
GLOSSARY OF TERMS

The definitions given below have been selected and restricted to those that are likely to be useful to users of this OIE Terrestrial Manual.

• Absorbance/optical density
Absorbance and optical density are terms used to indicate the strength of reaction. A spectrophotometer is used to measure the amount of light of a specific wave length that a sample absorbs and the absorbance is proportional to the amount of a particular analyte present.

• Accuracy
Nearness of a test value to the expected value for a reference standard reagent of known activity or titre.

• Assay
Synonymous with test or test method, e.g. enzyme immunoassay, complement fixation test or polymerase chain reaction tests.

• Batch
All vaccine or other reagent, such as antigen or antisera, derived from the same homogeneous bulk and identified by a unique code number.

• Cell line
A stably transformed line of cells that has a high capacity for multiplication in vitro.

• Centrifugation
Throughout the text, the rate of centrifugation has been expressed as the Relative Centrifugal Force, denoted by \( g \). The formula is:

\[
\frac{(\text{RPM} \times 0.10472)^2}{980} \times \text{Radius (cm)} = g
\]

where RPM is the rotor speed in revolutions per minute, and where Radius (cm) is the radius of the rotor arm, to the bottom of the tube, in centimetres.

It may be necessary to calculate the RPM required to achieve a given value of \( g \), with a particular rotor. The formula is:

\[
\text{RPM} = \sqrt[2]{\frac{g \times 980}{\text{Radius (cm)}}} \times 0.10472
\]

• Cross-reaction
See ‘False-positive reaction’.

• Cut-off/threshold
Test result value selected for distinguishing between negative and positive results; may include indeterminate or suspicious zone.
• **Dilutions**

Where dilutions are given for making up liquid reagents, they are expressed as, for example, 1 in 4 or 1/4, meaning one part added to three parts, i.e. a 25% solution of A in B.

- **v/v** – This is volume to volume (two liquids).
- **w/v** – This is weight to volume (solid added to a liquid).

• **Dilutions used in virus neutralisation tests**

There are two different conventions used in expressing the dilution used in virus neutralisation (VN) tests. In Europe, it is customary to express the dilution before the addition of the antigen, but in the United States of America and elsewhere, it is usual to express dilutions after the addition of antigen.

These alternative conventions are expressed in the *Terrestrial Manual* as 'initial dilution' or 'final dilution', respectively.

• **Efficacy**

Specific ability of the biological product to produce the result for which it is offered when used under the conditions recommended by the manufacturer.

• **Equivalency testing**

Determination of certain assay performance characteristics of new and/or different test methods by means of an interlaboratory comparison to a standard test method; implied in this definition is that participating laboratories are using their own test methods, reagents and controls and that results are expressed qualitatively.

• **False-negative reaction**

Negative reactivity in an assay of a test sample obtained from an animal exposed to or infected with the organism in question, may be due to lack of analytical sensitivity, restricted analytical specificity or analyte degradation, decreases diagnostic sensitivity.

• **False-positive reaction**

Positive reactivity in an assay that is not attributable to exposure to or infection with the organism in question, maybe due to immunological cross-reactivity, cross-contamination of the test sample or non-specific reactions, decreases diagnostic specificity.

• **Final product (lot)**

All sealed final containers that have been filled from the same homogenous batch of vaccine in one working session, freeze-dried together in one continuous operation (if applicable), sealed in one working session, and identified by a unique code number.

• **Harmonisation**

The result of an agreement between laboratories to calibrate similar test methods, adjust diagnostic thresholds and express test data in such a manner as to allow uniform interpretation of results between laboratories.

• **Incidence**

Estimate of the rate of new infections in a susceptible population over a defined period of time; not to be confused with prevalence.

• **In-house checks**

All quality assurance activities within a laboratory directly related to the monitoring, validation, and maintenance of assay performance and technical proficiency.

• **In-process control**

Test procedures carried out during manufacture of a biological product to ensure that the product will comply with the agreed quality standards.
• **Inter-laboratory comparison (ring test)**

Any evaluation of assay performance and/or laboratory competence in the testing of defined samples by two or more laboratories; one laboratory may act as the reference in defining test sample attributes.

• **Master cell (line, seed, stock)**

Collection of aliquots of cells of defined passage level, for use in the preparation or testing of a biological product, distributed into containers in a single operation, processed together and stored in such a manner as to ensure uniformity and stability and to prevent contamination.

• **Master seed (agent, strain)**

Collection of aliquots of an organism at a specific passage level, from which all other seed passages are derived, which are obtained from a single bulk, distributed into containers in a single operation and processed together and stored in such a manner as to ensure uniformity and stability and to prevent contamination.

• **Performance characteristic**

An attribute of a test method that may include analytical sensitivity and specificity, accuracy and precision, diagnostic sensitivity and specificity and/or repeatability and reproducibility.

• **Potency**

Relative strength of a biological product as determined by appropriate test methods. (Initially the potency is measured using an efficacy test in animals. Later this may be correlated with tests of antigen content, or antibody response, for routine batch potency tests.)

• **Precision**

The degree of dispersion of results for a repeatedly tested sample expressed by statistical methods such as standard deviation or confidence limits.

• **Predictive value (negative)**

The probability that an animal is free from exposure or infection given that it tests negative; predictive values are a function of the DSe (diagnostic sensitivity) and DSp (diagnostic specificity) of the diagnostic assay and the prevalence of infection.

• **Predictive value (positive)**

The probability that an animal has been exposed or infected given that it tests positive; predictive values are a function of the DSe and DSp of the diagnostic assay and the prevalence of infection.

• **Prevalence**

Estimate of the proportion of infected animals in a population at one given point in time; not to be confused with incidence.

• **Primary cells**

A pool of original cells derived from normal tissue up to and including the tenth subculture.

• **Production seed**

An organism at a specified passage level that is used without further propagation for initiating preparation of a production bulk.

• **Proficiency testing**

One measure of laboratory competence derived by means of an interlaboratory comparison; implied in this definition is that participating laboratories are using the same test methods, reagents and controls and that results are expressed qualitatively.
- **Purity**

  Quality of a biological product prepared to a final form and:
  
  a) Relatively free from any extraneous microorganisms and extraneous material (organic or inorganic) as determined by test methods appropriate to the product; and
  
  b) Free from extraneous microorganisms or material which could adversely affect the safety, potency or efficacy of the product.

- **Reference animal**

  Any animal for which the infection status can be defined in unequivocal terms; may include diseased, infected, vaccinated, immunised or naïve animals.

- **Reference Laboratory**

  Laboratory of recognised scientific and diagnostic expertise for a particular animal disease and/or testing methodology; includes capability for characterising and assigning values to reference reagents and samples.

- **Repeatability**

  Level of agreement between replicates of a sample both within and between runs of the same test method in a given laboratory.

- **Reproducibility**

  Ability of a test method to provide consistent results when applied to aliquots of the same sample tested by the same method in different laboratories.

- **Room temperature**

  The term ‘room temperature’ is intended to imply the temperature of a comfortable working environment. Precise limits for this cannot be set, but guiding figures are 18–25°C. Where a test specifies room temperature, this should be achieved, with air conditioning if necessary; otherwise the test parameters may be affected.

- **Safety**

  Freedom from properties causing undue local or systemic reactions when used as recommended or suggested by the manufacturer and without known hazard to in-contact animals, humans and the environment.

- **Sample**

  Material that is derived from a specimen and used for testing purposes.

- **Sensitivity (analytical)**

  Synonymous with ‘Limit of Detection’, smallest detectable amount of analyte that can be measured with a defined certainty; analyte may include antibodies, antigens, nucleic acids or live organisms.

- **Sensitivity (diagnostic)**

  Proportion of known infected reference animals that test positive in the assay; infected animals that test negative are considered to have false-negative results.

- **Sensitivity (relative)**

  Proportion of reference animals defined as positive by one or a combination of test methods that also test positive in the assay being compared.

- **Specific pathogen free (SPF)**

  Animals that have been shown by the use of appropriate tests to be free from specified pathogenic microorganisms, and also refers to eggs derived from SPF birds.
• **Specificity (analytical)**

Degree to which the assay distinguishes between the target analyte and other components in the sample matrix; the higher the analytical specificity, the lower the level of false-positives.

• **Specificity (diagnostic)**

Proportion of known uninfected reference animals that test negative in the assay; uninfected reference animals that test positive are considered to have false-positive results.

• **Specificity (relative)**

Proportion of reference animals defined as negative by one or a combination of test methods that also test negative in the assay being compared.

• **Specimen**

Material submitted for testing.

• **Standard Reagents**

• **International Standard Reagents**
  Standard reagents by which all other reagents and assays are calibrated; prepared and distributed by an International Reference Laboratory.

• **National Standard Reagents**
  Standard reagents calibrated by comparison with International Standard Reagents; prepared and distributed by a National Reference Laboratory.

• **Working Standards (reagents)**
  Standard reagents calibrated by comparison with the National Standard Reagent, or, in the absence of a National Standard Reagent, calibrated against a well-characterised in-house standard reagent; included in routine diagnostic tests as a control and/or for normalisation of test results.

• **Sterility**

Freedom from viable contaminating microorganisms, as demonstrated by approved and appropriate tests.

• **Test method**

Specified technical procedure for detection of an analyte (synonymous with assay).

• **Tests**

• **Prescribed**
  Test methods that are required by the OIE *Terrestrial Animal Health Code* for the international movement of animals and animal products and that are considered optimal for determining the health status of animals.

• **Alternative**
  Test methods considered in this *Terrestrial Manual* to be suitable for the diagnosis of disease in a local situation, and that can also be used for import/export by bilateral agreement.

• **Screening**
  Tests of high diagnostic sensitivity suitable for large-scale application.

• **Confirmatory**
  Test methods of high diagnostic specificity that are used to confirm results, usually positive results, derived from other test methods.

• **Working seed**

Organism at a passage level between master seed and production seed.
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The chapters in the Terrestrial Manual are prepared by invited contributors (OIE Reference Experts, where possible). In accordance with OIE standard procedure, all chapters are circulated to OIE Member Countries and to other experts in the disease for comment. The OIE Biological Standards Commission and the Consultant Editor then modify the text to take account of comments received, and the text is circulated a second time as the final version that will be presented for adoption by the World Assembly of Delegates to the OIE at the General Session in May of each year. The Terrestrial Manual is thus deemed to be an OIE Standard Text that has come into being by international agreement. For this reason, the names of the contributors are not shown on individual chapters but are listed below. The Biological Standards Commission greatly appreciates the work of the following contributors (address at the time of writing):

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2.5.7. Equine influenza  
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2.5.8. Equine piroplasmosis  
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2.5.10. Equine viral arteritis  
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2.5.11. Glanders  
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2.5.12. Horse mange  
See chapter 2.9.8.

2.5.13. Venezuelan equine encephalomyelitis  
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2.6.1. Myxomatosis  
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2.6.2. Rabbit haemorrhagic disease  
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2.7.1. Border disease  
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2.7.2. Caprine and ovine brucellosis (excluding Brucella ovis)5  
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2.7.5. Contagious agalactia  
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2.7.6. Contagious caprine pleuropneumonia  
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2.7.7. Enzootic abortion of ewes (ovine chlamydiosis)  
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2.7.8. Nairobi sheep disease  
See chapter 2.9.1.

5 All four brucellosis chapters were updated by consensus of the OIE ad hoc Group on Brucellosis.
2.7.9. Ovine epididymitis (Brucella ovis) (Dr B. Garin-Bastuji)
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2.7.10. Ovine pulmonary adenocarcinoma (adenomatosis) (Dr M.J. Sharp)
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2.7.12. Salmonellosis (S. abortusovis) (See chapter 2.9.9.)

2.7.13. Scrapie (Dr M.M. Simmons, Mr M. Stack, Dr T. Konold & Dr D. Matthews)
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2.7.14. Sheep pox and goat pox (Dr E. Tuppurainen)
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2.8.2. Atrophic rhinitis of swine (Dr M. Arias)
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2.8.3. Classical swine fever (hog cholera) (Dr K.B. Register)
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2.8.4. Nipah virus encephalitis (See chapter 2.9.6.)

2.8.5. Porcine brucellosis (Dr S. Olsen,)
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2.8.6. Porcine cysticercosis (See chapter 2.9.5.)

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6 All four brucellosis chapters were updated by consensus of the OIE ad hoc Group on Brucellosis.
7 This chapter was updated by consensus of the OIE ad hoc Group on Peste des Petits Ruminants.
8 All four brucellosis chapters were updated by consensus of the OIE ad hoc Group on Brucellosis.
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2.8.8. **Swine influenza**

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2.8.9. **Swine vesicular disease**

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2.8.10. **Teschovirus encephalomyelitis (previously enterovirus encephalomyelitis or Teschen/Talfan disease)**

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2.9.11. Verocytotoxigenic Escherichia coli

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| Guideline 3.2. Biotechnology in the diagnosis of infectious diseases | Dr G. Viljoen, Dr I. Naletoski & Dr A. Diallo  
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|---|---|
| Guideline 3.3. The Application of Biotechnology to the Development of Veterinary Vaccines | Dr A.A. Potter, Dr V. Gerdts, Dr G. Mutwiri, Dr S. Tikoo & De S. van Drunen Littel-van den Hurk  
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PART 1

GENERAL STANDARDS
SECTION 1.1.
INTRODUCTORY CHAPTERS

CHAPTER 1.1.1.
COLLECTION AND SHIPMENT OF DIAGNOSTIC SPECIMENS

INTRODUCTION

The starting point for the laboratory investigation of an animal disease is the taking of samples. This first introductory chapter considers some of the general principles involved in sample collection, submission and storage. Each of the disease chapters of this Terrestrial Manual provides specific information on sample collection for that particular disease. Samples may be taken from animals or the environment for a variety of purposes, such as disease diagnosis, disease surveillance, health certification or monitoring the response to treatment or vaccination. The samples collected should be appropriate for the intended purpose, and adequate in number and amount to provide statistically valid results. Diagnostic laboratories require the submission of appropriate samples that arrive at the laboratory in good condition. For disease diagnosis, the tissues sampled should be representative of the condition being investigated and the lesions observed. Samples should be taken with care, to avoid undue stress or injury to the animal or danger to the operator. Where appropriate, samples should be collected aseptically, and care should be taken to avoid cross-contamination between samples.

The samples should be carefully packaged, labelled, and transmitted to the laboratory by the fastest practicable method, with the appropriate temperature control. There are specific requirements for the packaging and shipping of infectious substances, including diagnostic specimens that must be followed. If material is sent to a laboratory in another country, this laboratory should be consulted in advance to ensure that it is willing to receive the material and to obtain the appropriate import licence. All samples should be accompanied by a letter or submission form, which includes the name and address of the submitter, the origin of the material, the relevant history, animal identification and corresponding specimens, and the tests requested.

A. COLLECTION OF SAMPLES

Before taking samples, careful consideration should be given to the purpose for which they are required. This will determine the type and number of samples needed to provide valid results. When samples are taken from live animals, care should be taken to avoid injury or distress to the animal or danger to the operator and attendants. It may be necessary to use mechanical restraint, tranquillisation or anaesthesia. Whenever handling biological material, from either live or dead animals, the risk of zoonotic disease should be kept in mind and precautions taken to avoid human infection (see also Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities). Post-mortem examinations should be carried out under as aseptic conditions as is practicable. Care should be taken to avoid environmental contamination, or risk of spread of disease through insects or fomites. Arrangements should be made for appropriate safe disposal of animals and tissues.

Considerable skill and care are required to decide on the correct samples to be sent to the laboratory. The samples collected should be representative of the condition being investigated and the lesions observed. Also the stage of the disease and lesion development should be considered, as well as the type of test(s) that will be performed. Frequently, a combination of blood samples for serology and tissues from dead or culled animals for
Chapter 1.1.1. — Collection and shipment of diagnostic specimens

microbiological culture and pathological examination will be required. Recommendations for transport are described later in this chapter.

The disease chapters in this Terrestrial Manual provide guidance on samples that should be collected so that information will not be repeated here. In addition, procedures for sample collection and submission have been prepared by national and international authorities (Cook et al., 1996; NVSL, 2006; AHVLA, 2003; USDA, 2005). These publications provide detailed recommendations of specific samples that should be collected from different species and for a wide variety of suspected diseases. They also provide information on post-mortem procedures, lists of appropriate media, and instructions on submission of samples. The laboratory that is going to perform the assay(s) should be contacted if there are specific questions concerning the type of sample that should be collected.

1. Sample collection from live animals

a) Blood

Blood samples may be taken for haematology or for culture and/or direct examination for bacteria, viruses, or protozoa, in which case it is usual to use anticoagulants, such as ethylene diamine tetra-acetic acid (EDTA) or heparin. They may also be taken for serology, which requires a clotted sample. Blood plasma is also used for some procedures. A blood sample is taken, as cleanly as possible, by venipuncture. In most large mammals, the jugular vein or a caudal vein is selected, but brachial veins and mammary veins are also used. Vena cava veins are also used in pigs. In birds, a wing vein (brachial vein) is usually selected. For techniques for sampling small laboratory animals, see Anon, 1993 and Hem et al., 1998. Blood may be taken by syringe and needle or by needle and vacuum tube (not easy in delicate veins but convenient in strong veins). Small quantities of blood are conveniently obtained by pricking with a triangular, solid-pointed needle. Ideally the skin at the site of venipuncture should first be shaved (plucked) and swabbed with 70% alcohol and allowed to dry.

For samples that are collected with anticoagulant, thorough mixing, using gentle agitation only, is necessary as soon as the sample has been taken. It may also be necessary to make a smear of fresh blood on a microscope slide; both thick and thin smears may be prepared. For polymerase chain reactions, EDTA is the preferred anticoagulant. For serum samples, the blood should be left to stand at ambient temperature (but protected from excessive heat or cold) for 1–2 hours until the clot begins to contract. The clot can then be ringed round with a sterile rod and the bottles placed in a refrigerator at 4°C. After several hours, or overnight, the sample can be centrifuged at about 1000 g for 10–15 minutes and the serum can be decanted or removed with a pipette. In order to establish the significance of antibody titres, paired serum samples will often need to be collected 7–14 days apart. An alternative method for collecting and transporting blood that is to be used for serology is to place a drop of blood on to filter paper, the blood is dried at room temperature and the sample can then be shipped unrefrigerated. Contact the laboratory to enquire if this method of collection is validated for the required tests.

b) Faeces

At least 10 g of freshly voided faeces should be selected. Faeces for parasitology should fill the container and be sent to arrive at the laboratory within 24 hours. If transport times are likely to be longer than 24 hours, the sample should be sent on ice or refrigerated to prevent the hatching of parasite eggs. Screw top containers or sterile plastic bags should be used for shipment; avoid tubes with rubber stoppers as gas generated can result in blowing the stopper off the tube, ruining the integrity of the sample and contaminating other samples in the package. An alternative and sometimes preferable method is to take swabs from the rectum (or cloaca), taking care to swab the mucosal surface. The swabs should be visibly coated with faecal material; however, samples collected with a swab are inadequate for parasitology. Care should be taken when collecting swabs from small, delicate animals or birds to avoid injury to the animal; small swabs are commercially available that should be used. Swabs should be transported in appropriate transport medium. Faeces are best stored and transported at 4°C.

c) Skin

In diseases producing vesicular lesions, collect, if possible, 2 g of affected epithelial tissue as aseptically as possible and place it in 5 ml phosphate buffered glycerine or Tris-buffered tryptose broth virus transport medium at pH 7.6. Additionally, the vesicular fluid should be sampled where unruptured vesicles are present; if possible, vesicular fluid should be aspirated with a syringe and placed in a separate sterile tube. Plucked hair or wool samples are useful for surface-feeding mites, lice and fungal infections. Deep skin scrapings, using the edge of a scalpel blade, are useful for burrowing mites and, in birds, feather tips can be taken for detection of viral antigen where Marek’s disease is suspected.
d) Genital tract and semen

Samples may be taken by vaginal or preputial washing, or by the use of suitable swabs. The cervix or urethra may be sampled by swabbing. Samples of semen are best obtained using an artificial vagina or by extrusion of the penis and artificial stimulation. The sperm-rich fraction should be present in the sample and contamination by antiseptic washing solutions should be avoided. Specific transport media and conditions are often required.

e) Eye

A sample from the conjunctiva can be taken by holding the palpebra apart and gently swabbing the surface. The swab is then put into transport medium. Scrapings may also be taken on to a microscope slide. The handles of metal-handled swabs are useful for this, to ensure that sufficient cells are removed for microscopic examination. Mucopurulent nasal and lacrimal discharges are rarely useful.

f) Nasal discharge (saliva, tears)

Samples may be taken with dacron, cotton or gauze swabs, preferably on wire handles as wood is inflexible and may snap. It may be helpful if the swab is first moistened with transport medium. The swab should be allowed to remain in contact with the secretions for up to 1 minute, then placed in transport medium and sent to the laboratory without delay at 4°C. Long protected nasopharyngeal swabs should be used to collect samples for some suspected viral infections.

g) Milk

Milk samples should be taken after cleansing and drying the tip of the teat, the use of antiseptics should be avoided. The initial stream of milk should be discarded and a tube filled with the next stream(s), a sample of bulk tank milk can be used for some tests. Milk for serological tests should not have been frozen, heated or subjected to violent shaking. If there is going to be a delay in submitting them to the laboratory, preservatives can be added to milk samples that are being collected for serological testing. If necessary, milk for bacterial examination can be frozen.

2. Sample collection at post-mortem

Samples of tissue from a variety of organs can be taken at post-mortem. Detailed procedures for conducting a post-mortem examination and collecting samples are described in most pathology textbooks; a guide to necropsy procedures has been published (Strafuss, 1988). Post-mortem techniques are also included in some of the national guidelines (Cook et al., 1996; NVSL, 2006). A summary of these procedures will be provided here.

Animal health personnel should be trained in the correct procedures for post-mortem examination of the species of animals with which they work. The equipment required will depend on the size and species of animal, but a knife, saw and cleaver will be required, and also scalpel, forceps and scissors, including scissors with a rounded tip on one blade, for opening intestines. A plentiful supply of containers and tubes of transport media appropriate to the nature of the sample required should be available, along with labels and report forms. Containers should be fully labelled with the date, tissue and animal identification. Special media may be required for transport of samples from the field. The operator should wear protective clothing: overalls, washable apron, rubber gloves and rubber boots. Additionally, if potential zoonotic diseases are being investigated, the post-mortem examination should be conducted in a biological safety cabinet; if this is not possible, an efficient face mask and eye protection should be worn. If rabies or transmissible spongiform encephalopathies (TSEs) are suspected, it is usual to detach the animal’s head.

Tissues may be collected for microbiological culture, parasitology, biochemistry, histopathology and/or immunohistochemistry, and for detection of proteins or genome nucleic acids. In addition buccal, oropharyngeal or rectal (cloacal) swabs may be collected. The person conducting the post-mortem examination should have sufficient knowledge of anatomy and pathology to select the most promising organs and lesions for sampling. Each piece of tissue should be placed in a fully labelled separate plastic bag or sterile screw-capped jar. Swabs should always be submitted in appropriate transport media. Sterile instruments should be used for collecting specimens for microbiological culture and care should be taken not to contaminate tissues with intestinal contents. Disinfectants should not be used on or near tissues to be sampled for bacterial culture or virus isolation.

The tissues may be sent to the laboratory dry or in bacterial or virus transport medium, depending on the type of specimen and the examinations required; swabs should be sent in transport medium. After collection, the samples for microbiological examination should be refrigerated until shipped. If shipment cannot be made within 48 hours, the samples should be frozen; however, prolonged storage at –20°C may be detrimental to virus isolation. For histopathology, blocks of tissue not more than 0.5 cm thick and 1–2 cm long are cut and placed in neutral buffered 4–10% formalin, which should be at least ten times the volume of the tissue sample. For certain suspected diseases, larger portions of brain are required; the brain is sectioned using a sagittal cut, half is submitted fresh,
on ice, and the other half is submitted in 10% buffered formalin. For scrapie, bovine spongiform encephalopathy and other TSEs, details of sample collection are provided in the individual disease chapters in this Terrestrial Manual. Store and pack formalin-fixed tissues separately from fresh tissues, blood and smears. Care should be taken to ensure that formalin-fixed tissues are not frozen. Once fixed, tissues can be removed from formalin and, as long as they are kept moist and protected (e.g. by wrapping in formalin-soaked paper towels, then sealed in screw-capped jars), they can be forwarded to the laboratory without formalin.

3. Environmental and feed sampling

Samples may be taken to monitor hygiene or as part of a disease enquiry. Environmental samples are commonly taken from litter or bedding and voided faeces or urine. Swabs may be taken from the surface of ventilation ducts, feed troughs and drains. This kind of sampling is particularly important in hatcheries, artificial insemination centres and slaughterhouses in which specialised equipment is maintained. Samples may also be taken from animal feed, in troughs or bulk containers. Water may be sampled in troughs, drinkers, header tanks or from the natural or artificial supply.

4. Honey bees

Adult bees, either dead or moribund, may be collected in the vicinity of the colonies. Live bees should be killed by freezing. Brood samples are taken by removing a piece of brood comb that shows abnormalities. This should be wrapped in paper and placed in a box for transport to the laboratory. Hive debris may be collected for examination, preferably on a sticky board to trap mobile parasites.

B. SAMPLE SIZE

When investigating a case of clinical disease, the specimens collected should be representative of the condition being investigated and the lesions observed. When developing a programme of surveillance and monitoring for animal health in the absence of clinically evident disease, some general statistical sampling methods should be used. These sampling methods are needed to perform the scientifically based surveys specified in the OIE Terrestrial Animal Health Code (2012). It is possible to calculate how many animals should be sampled from a herd/flock of a certain size, to achieve a 95% probability of detecting infection or previous exposure assumed to be present in a certain percentage of the animals. The following formulae can give approximate numbers, but a specific sampling programme for the planned surveillance programme should be based on complete formulas available in the references (Cameron & Baldock, 1998; Cannon & Roe, 1982) or by the use of a program (FreeCalc) available off the internet: http://www.ausvet.com.au/content.php?page=res_software#freecalc. All calculation examples provided in the following paragraphs can be calculated using FreeCalc. This software also includes “a “pooled prevalence calculator”, which describes the calculation of prevalence using pooled samples.

The following formula could be used to calculate the sample size \( n \) to detect at least one infection with a test that has a 100% sensitivity and specificity; where \( \alpha \) is the significance level and \( 1–\alpha \) is the level of confidence, \( p \) is the prevalence in the population. If disease were present in 5% of a herd of 500 animals, it would be necessary to collect specimens from 56 animals to be 95% confident of finding at least one positive, assuming that both the sensitivity and specificity of the test were 100%. In order to make a prediction of disease prevalence, it is critical that the sample be selected from the population by a formal random sampling procedure. As most diagnostic tests do not have specificity and sensitivity of 100%, the number of specimens collected must be adjusted to the sensitivity and specificity of the test that will be used (see also Chapter 1.1.5 Principles and methods of validation of diagnostic assays for infectious diseases).

\[
\begin{align*}
    n &= \frac{\ln (\alpha)}{\ln (1–p)} \\
    \text{In the above example } \alpha &= 0.05, \ 1–\alpha = 95\%, \ p = 0.05 \text{ and } n = 59
\end{align*}
\]

If the sensitivity (\( Se \)) is less than 100%, the above formula should be modified as follows:

\[
\begin{align*}
    n &= \frac{\ln (\alpha)}{\ln (1–p.\text{Se})} \\
    \text{In the above example with } \alpha = 0.05, \ p = 0.05, \text{ specificity (Sp) = 1 and } Se = 0.95, \text{ a minimum of } n = 62 \text{ animals instead of 59 would need to be sampled to have a probability of at least } 0.95 \text{ of finding a positive animal. The}
\end{align*}
\]
increase in the sample size from 59 to 62 is due to the decrease in the sensitivity of the test from 1 to 0.95. The graph below gives the minimum sample size required for finding at least one positive for several sensitivity and prevalence combinations at $\alpha = 0.05$ and $Sp = 1$.

If the test is known to have a specificity of less than 1, the positive results should be confirmed by a test with a higher specificity. If the prevalence is very low and the test used has a specificity of less than 1, it is very likely that a positive test result is a false positive.

**Sample Size Calculations**

$(\text{Specificity}=1 \text{ Alpha}=0.05)$

![Graph showing minimum sample size required to be 95% confident of finding infection at various sensitivity and prevalence combinations.](image)

**Fig. 1. Minimum sample size required to be 95% confident of finding infection at various sensitivity and prevalence combinations.**

### C. INFORMATION TO BE SENT WITH SAMPLES

It is essential that individual samples be clearly identified using appropriate methods. Marking instruments should be able to withstand the condition of use, i.e. being wet or frozen (use indelible marking pen). Pencil has a tendency to rub off containers and labels attached to plastic will fall off when stored at $-70^\circ$C. Information and case history should always accompany the samples to the laboratory, and should be placed in a plastic envelope on the outside of the shipping container. As outlined in the following section on transport of samples, this information must also be inside the shipping container. The following are suggested items that should be addressed. It would be advisable to contact the receiving laboratory to determine if it has a submission form that it would like to have submitted with the samples or if it needs other information.

i) Name and address of owner/occupier and geolocation (latitude and longitude, if available) where disease occurred, with telephone and fax numbers.

ii) Name, postal and e-mail address, telephone and fax numbers of the sender.

iii) Diseases suspected and tests requested.

iv) The species, breed, sex, age and identity of the animals sampled.

v) Date samples were collected and submitted.

vi) List of samples submitted with transport media used.

vii) A complete history would be beneficial for the laboratory and should be included if possible. Some of the components of the history are:
Chapter 1.1.1. — Collection and shipment of diagnostic specimens

a) A list and description of the animals examined and the findings of the post-mortem examination.
b) The length of time sick animals have been on the farm; if they are recent arrivals, from where did they originate.
c) The date of the first cases and of subsequent cases or losses, with any appropriate previous submission reference numbers.
d) A description of the spread of infection in the herd or flock.
e) The number of animals on the farm, the number of animals dead, the number showing clinical signs, and their age, sex and breed.
f) The clinical signs and their duration including the temperature of sick animals, condition of mouth, eyes and feet, and milk or egg production data.
g) The type and standard of husbandry, including the type of feed available, possible contact with poison or poisonous plants.
h) History of foreign travel by owner or of introduction of animals from other countries or regions.
i) Any medication given to the animals, and when given.
j) Any vaccines given, and when given.
k) Other observations about the disease, husbandry practices and other disease conditions present.

D. PACKAGING AND TRANSPORT OF SAMPLES

1. Approval to ship specimens

The laboratory that is going to receive the samples should be contacted to ensure that it has the capability to do the testing requested and to see if there are any special packaging or shipping requirements. It is essential to contact the receiving laboratory when material is sent to another country. A special import licence will usually be required for shipment of any biological material to other countries and must be obtained in advance. This licence should be placed in an envelope on the outside of the parcel.

Shipments must be made in accordance with the dangerous goods rules for the particular mode of transport. For air transport it is the International Civil Aviation Organization (ICAO) technical instructions for the safe transport of dangerous goods by air. These are reflected in the International Air Transport Association (IATA) Dangerous Goods Regulations which is the interpretation of ICAO instructions applied to shipments by air (IATA, 2006). These regulations have been described in a United Nations World Health Organization publication (WHO, 2005). The shipper is responsible for checking the variations guidelines to insure that restrictions are met.

2. Transportation of specimens

The specimens should be forwarded to the laboratory by the fastest method available. If they can reach the laboratory within 48 hours, samples should be sent refrigerated. If dry ice is used, the additional packaging requirements must be met. Infectious substances, which can include diagnostic specimens, are not permitted to be shipped as checked luggage or as carry on luggage and must be shipped as cargo.

3. Packaging

The shipper should ensure that the specimens are packaged so they arrive at the laboratory in good condition and there is no leakage during shipment. The Dangerous Goods Regulations (DGR) have explicit requirements for packaging and shipment of diagnostic specimens, by all commercial means of air transport (IATA, 2006; WHO, 2005). In some countries, there are similar requirements for ground shipments and the postal service, but these requirements should be reviewed before shipping. These requirements for air transport are covered in detail in the IATA publication, which are updated every year. The shipper is expected to know and follow the procedures outlined in the current DGR. The following is a summary of the regulations at the time that this revision of the Terrestrial Manual was published and it should only be used as a guide for shipping. Shippers must also always check the latest version of the DGR prior to shipping diagnostic specimens. In addition, three of the national guidelines provide explicit directions for packaging and shipping diagnostic specimens and are based on IATA requirements (Cook et al., 1996; NVSL, 2006).

The DGR outline the procedures for the shipment of infectious substances, which can include diagnostic specimens. Infectious substances are defined in the DGR as substances that are known or are reasonably
expected to contain pathogens. Pathogens are defined as micro-organisms (including bacteria, viruses, rickettsiae, parasites, fungi) or recombinant micro-organisms (hybrid or mutant) that are known or reasonably expected to cause disease in humans or animals.

The IATA (IATA, 2006; WHO, 2005) lists the following exemption from the Dangerous Goods Regulations:

- 3.6.2.2.3.1 Substances which do not contain infectious substances or substances which are unlikely to cause disease in humans or animals are not subject to these Regulations unless they meet the criteria for inclusion in another class.

- 3.6.2.2.3.2 Substances containing microorganisms which are non-pathogenic to humans or animals are not subject to these Regulations unless they meet the criteria for inclusion in another class.

- 3.6.2.2.3.3 Substances in a form that any present pathogens have been neutralised or inactivated such that they no longer pose a health risk are not subject to these Regulations unless they meet the criteria for inclusion in another class.

- 3.6.2.2.3.4 Environmental samples (including food and water samples), which are not considered to pose a significant risk of infection, are not subject to these Regulations unless they meet the criteria for inclusion in another class.

- 3.6.2.2.3.5 Dried blood spots, collected by applying a drop of blood on to absorbent material, or faecal occult blood screening tests and blood or blood components that have been collected for the purposes of transfusion or for the preparation of blood products to be used for transfusion or transplantation and any tissues or organs intended for use in transplantation.

- 3.6.2.2.3.6 Patient specimens for which there is minimal likelihood that pathogens are present are not subject to these Regulations if the specimen is transported in a packaging which will prevent any leakage and which is marked with the words “Exempt human specimen” or “Exempt animal specimen”, as appropriate. The packaging should meet the following conditions:

  (a) The packaging should consist of three components:
      (1) a seal-proof primary receptacle(s);
      (2) a seal-proof secondary packaging; and
      (3) an outer packaging of adequate strength for its capacity, mass and intended use, and with at least one surface having minimum dimensions of 100 mm × 100 mm;

  (b) For liquids, absorbent material in sufficient quantity to absorb the entire contents must be placed between the primary receptacle(s) and the secondary packaging so that, during transport, any release or leak of a liquid substance will not reach the outer packaging and will not compromise the integrity of the cushioning material;

  (c) When multiple fragile primary receptacles are placed in a single secondary packaging, they should be either individually wrapped or separated to prevent contact between them.

*Note: In determining whether a patient specimen has a minimal likelihood that pathogens are present, an element of professional judgment is required to determine if a substance is exempt under this paragraph. That judgment should be based on the known medical history, symptoms and individual circumstances of the source, human or animal, and endemic local conditions. Examples of specimens which may be transported under this paragraph include the blood or urine tests to monitor cholesterol levels, blood glucose levels, hormone levels, or prostate specific antibodies (PSA); tests required to monitor organ function such as heart, liver or kidney function for humans or animals with non-infectious diseases, or therapeutic drug monitoring; tests conducted for insurance or employment purposes and are intended to determine the presence of drugs or alcohol; pregnancy test; biopsies to detect cancer; and antibody detection in humans or animals."

There are also exceptions for some biological products and the shipper of these products is referred to the IATA Regulations for these requirements as not all biological products are exempt. The following is the DGR definition of Biological Products (IATA, 2006; WHO, 2005):

"Biological products are those products derived from living organisms which are manufactured and distributed in accordance with the requirements of appropriate national authorities, which may have special licensing requirements, and are used either for prevention, treatment, or diagnosis of disease in humans or animals, or for development, experimental or investigational purposes related thereto. They include, but are not limited to, finished or unfinished products such as vaccines."
The DGR state that infectious substances (including diagnostic specimens likely to contain animal or human pathogens) are designated as Category A and B and assigned to UN 2814, UN 2900 or UN 3373.

Category A is defined as an: “Infectious substance, which is transported in a form that when exposure to it occurs, is capable of causing permanent disability, life threatening or fatal disease in otherwise healthy humans or animals, indicative examples of substances that meet these criteria are given in Table 1 and 2”. Infectious substances meeting this definition that affect humans, including zoonotic agents, are designated UN 2814 and given the shipping name of “Infectious substance, affecting humans” those affecting animals only are designated UN 2900 and given the shipping name of “Infectious substance, affecting animals”.

Infectious substances shipped for diagnostic purposes that do not meet the criteria for assignment to UN 2814 or UN 2900 are assigned to Category B and must be assigned to UN 3373 and designated as “DIAGNOSTIC SPECIMENS or CLINICAL SPECIMENS or BIOLOGICAL SUBSTANCES CATEGORY B”.

The IATA DGR contains an indicative list of pathogens that must be assigned to UN 2814 or UN 2900 (Tables 1 and 2). The pathogens on these lists cannot be assigned to UN 3373 (IATA, 2006; WHO, 2005).

Table 1. Infectious substances affecting humans that must be designated UN 2814

<table>
<thead>
<tr>
<th>Substances in the list</th>
<th>Shipping name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus anthracis</td>
<td>Japanese encephalitis virus</td>
</tr>
<tr>
<td>Brucella abortus</td>
<td>Junin virus</td>
</tr>
<tr>
<td>Brucella melitensis</td>
<td>Kyasenur Forest disease virus</td>
</tr>
<tr>
<td>Brucella suis</td>
<td>Lassa virus</td>
</tr>
<tr>
<td>Burkholderia mallei</td>
<td>Machupá virus</td>
</tr>
<tr>
<td>Burkholderia pseudomallei</td>
<td>Marburg virus</td>
</tr>
<tr>
<td>Chlamydia psittaci</td>
<td>Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>Clostridium botulinum</td>
<td>Monkeypox virus</td>
</tr>
<tr>
<td>Coccidioides immitis</td>
<td>Nipah virus</td>
</tr>
<tr>
<td>Coxiella burnetii</td>
<td>Omok hemorrhagic fever virus</td>
</tr>
<tr>
<td>Crimean-Congo hemorrhagic fever virus</td>
<td>Poliovirus (cultures only)</td>
</tr>
<tr>
<td>Dengue virus</td>
<td>Rabies virus (cultures only)</td>
</tr>
<tr>
<td>Eastern equine encephalitis virus</td>
<td>Rickettsia prowazekii (cultures only)</td>
</tr>
<tr>
<td>Escherichia coli, verotoxigenic</td>
<td>Rickettsia rickettsii (cultures only)</td>
</tr>
<tr>
<td>Ebola virus</td>
<td>Rift Valley fever virus (cultures only)</td>
</tr>
<tr>
<td>Flexal virus</td>
<td>Russian spring-summer encephalitis virus (cultures only)</td>
</tr>
<tr>
<td>Francisella tularensis</td>
<td>Sabia virus</td>
</tr>
<tr>
<td>Guaranito virus</td>
<td>Shigella dysenteriae type 1</td>
</tr>
<tr>
<td>Hantaan virus</td>
<td>Tick-borne encephalitis virus</td>
</tr>
<tr>
<td>Hantavirus causing haemorrhagic fever with renal syndrome</td>
<td>Variola virus</td>
</tr>
<tr>
<td>Hendra virus</td>
<td>Venezuelan equine encephalitis virus (cultures only)</td>
</tr>
<tr>
<td>Hepatitis B virus</td>
<td>West Nile virus (cultures only)</td>
</tr>
<tr>
<td>Herpes B virus</td>
<td>Yellow fever virus (cultures only)</td>
</tr>
<tr>
<td>Human immunodeficiency virus</td>
<td>Yersinia pestis (cultures only)</td>
</tr>
<tr>
<td>Highly pathogenic avian influenza virus</td>
<td>(cultures only)</td>
</tr>
</tbody>
</table>
Table 2. Indicative examples of animal pathogens forbidden as diagnostic specimens that must be shipped as infectious substances affecting animals (UN 2900)

<table>
<thead>
<tr>
<th>African swine fever virus (cultures only)</th>
<th>Peste des petits ruminants virus (cultures only)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avian paramyxovirus Type 1 – Velogenic Newcastle disease virus (cultures only)</td>
<td>Rinderpest virus (cultures only)</td>
</tr>
<tr>
<td>Classical swine fever virus (cultures only)</td>
<td>Sheep-pox virus (cultures only)</td>
</tr>
<tr>
<td>Foot and mouth disease virus (cultures only)</td>
<td>Goatpox virus (cultures only)</td>
</tr>
<tr>
<td>Lumpy skin disease virus (cultures only)</td>
<td>Swine vesicular disease virus (cultures only)</td>
</tr>
<tr>
<td><em>Mycoplasma mycoides</em> – Contagious bovine pleuropneumonia (cultures only)</td>
<td>Vesicular stomatitis virus (cultures only)</td>
</tr>
</tbody>
</table>

New or emerging pathogens must also be assigned to UN 2814 or UN 2900.

The following is the IATA definition of amplification in culture:

“Cultures are the result of a process by which pathogens are intentionally propagated. This definition does not include patient specimens.”

“Patient specimens are those collected directly from humans or animals, including, but not limited to, excreta, secretia, blood and its components, tissue and tissue fluid swabs, and body parts being transported for purposes such as research, diagnosis, investigational activities, disease treatment and prevention.”

*Note:* Cultures of organisms that do not fit into the definition of Category A infectious substance can be transported as Biological Substances, Category B.

The following flow chart summarises the classification of DIAGNOSTIC SPECIMENS or CLINICAL SPECIMENS or BIOLOGICAL SUBSTANCES CATEGORY B.

Live animals must not be used to transport infectious substances.

Animal carcasses affected by pathogens of category A or which would be assigned to Category A in cultures only, must be assigned to UN 2814 or UN 2900 as appropriate. Other animal carcasses affected by pathogens included in Category B must be transported in accordance with provisions determined by the Competent Authority.
Chapter 1.1.1. – Collection and shipment of diagnostic specimens

The packaging of infectious substances and specimens from suspected serious animal diseases, UN 2814 or UN 2900, are outlined in packing instruction 620; a Shippers Declaration of Dangerous Goods must be completed and submitted with these samples. There is also a requirement that the shipper receive training on the IATA-approved shipping procedures for UN 2814 and UN 2900 shipments. Due to the complexity of these guidelines the shipper is referred to the regulations for further information on all UN 2814 or 2900 shipments (IATA, 2006; WHO, 2005).

The other group, UN 3373, covers ‘Diagnostic Specimens or Clinical Specimens or Biological Substances Category B’. This category has a lower risk and packages containing these specimens should be labelled as ‘Diagnostic Specimens or Clinical Specimens or Biological Substances Category B’; a Declaration of Dangerous Goods is not needed. IATA packing instruction 650 provides the guidelines for packaging infectious substances assigned to UN 3373 and the following is a summary of these packing instructions. However, the complete procedure, as outlined in the most recent IATA Dangerous Good Regulations, must be followed (IATA, 2006; WHO, 2005).

i) Infectious substances assigned to UN 3373 ‘Diagnostic Specimens’ must be packed in good quality packaging, which must be strong enough to withstand the shocks and loadings normally encountered during transport. Packaging must be constructed and closed so as to prevent any loss of contents, which might be caused under normal conditions of transport.

ii) The packaging must consist of three components:
   • a primary receptacle;
   • a secondary packaging; and
   • a rigid outer packaging.

iii) For liquid substances:
   • the primary receptacle(s) must be leak-proof and must not contain more than 1 litre; the secondary packaging must also be leak-proof;
   • adequate adsorbent material must be packed around the primary receptacle(s) to absorb all the fluid in the primary receptacle(s);
   • if multiple primary receptacles are used they should be individually wrapped or separated so as to prevent contact;
   • the primary receptacle or the secondary packaging must be capable of withstanding without leakage an internal pressure of 95 kPa in the range of –40°C to 55°C (–40°F to 130°F);
   • the outer packaging must not contain more than 4 litres. This quantity excludes ice, dry ice, or liquid nitrogen when used to keep specimens cold.

iv) For solid substances:
   • the primary receptacle(s) must be sift-proof and must not exceed the outer packaging weight limit; the secondary packaging must be sift-proof;
   • adequate adsorbent material must be packed around the primary receptacle(s) to absorb all the fluid in the primary receptacle(s);
   • except for packages containing body parts, organs or whole bodies, the outer packaging must not contain more than 4 kg. This quantity excludes ice, dry ice or liquid nitrogen when used to keep specimens cold;
   • if there is any doubt as to whether or not residual liquid may be present in the primary receptacle during transport then packaging suitable for liquids, including absorbent materials, must be used.

v) An itemised list of contents must be enclosed between the secondary packaging and the outer packaging.

vi) If shipped at ambient temperatures or higher, the primary receptacle must have a positive means of ensuring that it is leak proof, such as a leak proof seal, heat seal or skirted stopper. If screw caps are used they should be sealed with parafilm or tape.

vii) Prefrozen packs or dry ice can be packed around the secondary receptacle. If dry ice is used, there must be an internal support to secure the secondary receptacle in the original position after the dry ice has been dissipated. The outer packaging must permit the release of carbon dioxide. There are additional requirements if liquid nitrogen is used and these are described in the DGR.

viii) Packages containing diagnostic or clinical specimens are not required to have the net quantity marked on the outside of the package. However, where dry ice is used as a refrigerant, the net quantity of dry ice must be shown.
ix) The primary and secondary receptacles must be put into a shipping container with adequate cushioning material.

x) The packaging must be able to withstand a 1.2 metre drop test. (There are additional strength requirements for packaging used for UN 2900 and UN 2814 specimens.)

xi) At least one surface of the outer packaging must have a minimum dimension of 100 mm × 100 mm.

xii) For transport, the label 3373 must be displayed on the external surface of the outer packaging on a background of a contrasting colour and must be clearly visible and legible. The mark must be in the form of a square set an angle of 45° (diamond-shaped) with each side having a length of at least 50 mm, the width of the line must be at least 2 mm, and the letters and numbers must be at least 6 mm high. The proper shipping name “Diagnostic specimen”, “Clinical specimen” or “Biological substance category B” in letters at least 6 mm high must be marked on the outer package adjacent to the diamond-shaped mark.

4. Shipping forms

All shipping forms, including the import licence and submission form must be put in an envelope attached to the outside of the shipping container. The forms and labels must be completed as outlined in the DGR and also put on the outside of the container.

E. PRESERVATION OF SAMPLES FOR PROLONGED STORAGE

Establishing a collection of samples for future studies can be very useful. This can include cultures for comparison with future isolates, tissue or serum samples that can be used for the validation of new tests and a collection of fixed tissues, or paraffin blocks, for future histological examination. Possibly the most useful collection is the storage of serum samples. These samples may be useful if a retrospective investigation is carried out to compare the present disease status with that of earlier times.

- Serum banks

Serum samples can provide information about the animals from which the sera were taken. The samples can be tested for a variety of constituents, such as immunoglobulins, trace elements, toxins, hormones and enzymes. If a sufficient number of serum samples has been collected at random from a population, comparisons can be made on the affect of sex, age, breed and geographical location. Results from this comparison can identify high risk groups, vaccination priorities can be established, and patterns and rates of disease determined (Moorhouse & Hugh-Jones, 1981).

A serum bank is a catalogued collection of sera that are stored so as to preserve their immunological and other biochemical properties. Both the catalogue and the storage conditions are essential for a successful serum bank. Each individual sample should be fully documented and identified. The database should contain all relevant information about the origin of the sample and test results obtained. Additional data that may be of interest, such as weather conditions and the animal’s productivity may also be included. Accurate records are essential and must be obtained when the blood samples are collected. The first essential is the complete identification of the animal. The amount of detail recorded should be appropriate to the abilities of the operator, accuracy being more important than quantity of information. Although pooling of sera reduces documentation and storage space, it should be avoided as it greatly reduces the usefulness of the material. Care should be taken to collect the blood as aseptically as possible and sterility should be maintained during separation of the serum and all other manipulations. The serum bank catalogue should be well organised and maintained on a computer database with appropriate backup. A suggested methodology has been described in detail (Moorhouse & Hugh-Jones, 1981).

Sera may be stored for periodic use or kept in long-term storage for historical purposes and these two functions should be separated. Storage conditions should minimise loss of immunological and other biochemical properties of the sera. There are three methods: deep freezing, dry storage on paper disks at ambient temperature and lyophilisation (freeze-drying). For long-term storage of sera by deep freezing, a core temperature below –60°C should be maintained. The lower the temperature the better, but lower temperatures are more expensive to maintain. Liquid phase N2 is at –196°C, vapour phase N2 is at –100°C and an ultra-low deep freezer will maintain –90°C. Some serum banks have been maintained at –20°C, but the serum may deteriorate and not be suitable for detection of some properties, especially if stored for long periods at this temperature. Deep-freezers should have a system to provide a warning if the temperature rises due to mechanical break down or power failure. A stand-by generator is essential together with alternative cold storage space in case the contents of a freezer must be transferred. Paper disk storage is a simple and inexpensive method, but it allows only a small quantity of serum to be stored and the eluted serum is only suitable for a limited number of tests. The disks should be kept in a cool, dry atmosphere. They can probably provide satisfactory results for up to about 5 years. Lyophilisation is generally regarded as the best method for long-term storage of sera. If freeze-drying conditions are optimised the loss of
serum characteristics are minimised. Lyophilisation requires expensive equipment and is a time-consuming process. Lyophilised vials should be stored at 4°C.

REFERENCES


VETERINARY SERVICES (OF THE UNITED STATES DEPARTMENT OF AGRICULTURE) (2005). Regulations for Classifying Infectious Substances and Diagnostic Specimens, USDA Veterinary Services Notice NO. 06-02


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CHAPTER 1.1.2.

TRANSPORT OF SPECIMENS OF ANIMAL ORIGIN

Chapter under study

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  *  *
CHAPTER 1.1.3.

BIOSAFETY AND BIOSECURITY IN THE VETERINARY MICROBIOLOGY LABORATORY AND ANIMAL FACILITIES

INTRODUCTION

Laboratory work of the type described in this Terrestrial Manual should be carried out with a minimum of risk to the health of the staff (biosafety) and the environment (biocontainment). This requires careful consideration of the risks involved in a particular procedure, followed by appropriate measures to minimise the risk of human disease and of possible release into the environment. This is a complex subject that can only be considered in outline in an introductory chapter. This chapter is concerned almost exclusively with risks from infectious agents, but physical and chemical injuries in microbiology laboratories must also be prevented. Risks from infection are reduced by good laboratory techniques and secure facilities, which aid in the containment of pathogens. It is important to understand that containment of pathogens can be used for two purposes. One is to prevent disease in humans in the laboratory; the other is to prevent the release of the pathogen into the environment and causing disease in animals or humans. Often the same methods of containment are used for both preventing laboratory-acquired infection in humans and for preventing escape of pathogens that could cause an outbreak of animal diseases. Although the methods, techniques and facilities required may be the same, the list of pathogens and categorisation into levels of risk will differ depending on whether it is human or animal diseases control that is the primary objective.

Existing national and international reference laboratories have considerable experience in the operation of safe working practices and provision of appropriate facilities. When new laboratories are being established, it would be prudent to seek advice from the relevant regulatory authorities and the competent authorities at established institutes. It is important to comply with legislative requirements.

A. ASSESSMENT OF RISK FROM PATHOGENS

It is necessary first to assess the risk from a pathogen, so that it can be assigned to a Risk Group. A further risk assessment can be conducted, based on the proposed work, to determine the appropriate containment level. To assess the risk to humans and animals from a particular pathogen it is necessary to know whether infection with that organism can cause clinical disease and/or mortality in humans and animals, and whether it could then spread to cause disease in the general human and/or animal population. There are additional requirements related to the containment of animal pathogens and the prevention of the spread of infection to animals. To assess these risks it is necessary to know the epidemiological background of the organism and also such attributes of the organism as infectivity for humans and animals, stability in the environment, ability to infect by different routes of exposure, and susceptibility to specific treatments or prophylaxis (Acha & Szyfres, 2001; Advisory Committee on Dangerous Pathogens, 1995; Bell et al., 1988; Beran & Steele, 1994). It is relatively easy to obtain this information when working with a known pathogen, but the problem is more complex in a diagnostic laboratory receiving clinical material that may be infected with a variety of unknown pathogens, some of which could be extremely hazardous to human health or pose a significant threat to animal populations. Some of the considerations to take into account when evaluating risk are:

1. Known occurrence of human and animal infection with the organism or related organisms with similar characteristics, any history of laboratory-acquired infection, infective dose and disease severity; production of toxins or allergens.
2. The volume of culture to be handled and the concentration of the organism likely to be present. (Procedures such as antigen or vaccine production that require large quantities of organisms usually carry a higher risk than attempted isolation procedures.)

3. The origin of the sample, for example samples from wildlife species may contain human or animal pathogens not normally encountered.

4. The history of the isolate being handled. Pathogens on primary isolation or of low passage level are often more dangerous than pathogens of high passage level. In some cases, pathogenicity may be enhanced by passage or subculture using different media.

5. The possibility of aerosol formation should be especially taken into consideration when handling fluid samples or, for example, during grinding, homogenisation and centrifugation.

6. The threat that the organism may pose to food-producing or companion animals or to wildlife, irrespective of the threat to laboratory personnel. Additional precautions for handling and storage are required for animal disease agents from foreign countries.

7. The physical state of the employees. For example, in the case of pregnancy, immunodeficiency or allergy, special precautions may be required. Sometimes certain individuals have to be excluded from particular types of work that would be especially hazardous to them.

8. A higher level of risk may arise when agents such as Brucella or Mycobacterium are inoculated into animals. To evaluate the impact of animal inoculation, a risk assessment should be conducted and the following factors should be considered:
   i) Host species versus inoculated species;
   ii) Strain/treatment and concentration of the inoculum;
   iii) Route of inoculation;
   iv) Animal housing;
   v) Types of sampling during the experiment.

9. Some pathogens need to be transmitted by specific vectors or require intermediate hosts to complete their life cycles before they can infect animals and cause disease. In countries where such vectors or intermediate hosts do not occur, or where climatic or environmental factors mitigate against their survival, the pathogen poses a lower risk to animal health than in countries where such vectors or intermediate hosts occur naturally or could survive.

B. GROUPING OF MICROORGANISMS BY HUMAN AND ANIMAL HEALTH RISK

The considerations outlined above have been used by several national authorities to designate microorganisms into four Risk Groups (Advisory Committee on Dangerous Pathogens, 1995; Barbeito et al., 1995) representing increasing risks to human health. Such categorisation of pathogens makes no allowance for people who are particularly susceptible, for example due to pre-existing disease, a compromised immune system or pregnancy. The four Groups may be summarised thus:

- **Group 1** – Organisms that are unlikely to cause human or animal disease and are disease-producing organisms in animals that are enzootic but not subject to official control.

- **Group 2** – Organisms that may cause human or animal disease but are unlikely to be spread in the community or animal population and for which effective prophylaxis and treatment are available; examples of Group 2 animal pathogens:
  i) They do not depend on vectors or intermediate hosts for transmission.
  ii) There is very limited or no transmission between different animal species.
  iii) Geographical spread if released from the laboratory is limited.
  iv) Direct animal to animal transmission is relatively limited.
  v) Mode of transmission is primarily through ingestion, inoculation or mucus membrane route.
  vi) The need to confine diseased or infected nondiseased animals is minimal.
  vii) The disease is of limited economic and/or clinical significance.
  viii) Short-term survival in the environment and effective treatment or prevention is available.
  ix) May be either exotic or enzootic but are subject to official control and have a low risk of spread from the laboratory.

- **Group 3** – Organisms that can cause severe human or animal disease and may spread in the community and/or animal population but for which there is usually effective prophylaxis and treatment; examples of Group 3 animal pathogens:
  i) They may depend on vectors or intermediate hosts for transmission.
  ii) Transmission between different animal species may readily occur.
  iii) Geographical spread if released from the laboratory is moderate.
  iv) Direct animal to animal transmission occurs relatively easily.
v) The statutory confinement of diseased, infected and in-contact animals is necessary.
vi) The disease is of severe economic and/or clinical significance.

vii) Prophylactic and/or therapeutic treatments are not readily available or of limited benefit.

viii) Mode of transmission may be through the airborne route or direct contact.

ix) Are either exotic or enzootic but are subject to official control and that have a moderate risk of spread from the laboratory.

• Group 4 – Organisms that cause severe human or animal disease, may represent a high risk of spread in the community or animal population and for which there is usually no effective prophylaxis or treatment.

i) They may depend on vectors or intermediate hosts for transmission.

ii) Transmission between different animal species may occur very readily.

iii) Geographical spread if released from the laboratory is widespread.

iv) Direct animal to animal transmission occurs very easily.

v) Can be transmitted through casual contact or indirectly.

vi) The statutory confinement of diseased, infected and in-contact animals is necessary.

vii) The statutory control of animal movements over a wide area is necessary.

viii) The disease is of extremely severe economic and/or clinical significance.

ix) No satisfactory prophylactic and/or therapeutic treatments are available.

x) Have a high risk of spread from the laboratory into the environment and the national animal population.

Infectious organisms that might be encountered in laboratory work have been assigned to Risk Groups 1–4 by authorities in several countries (Advisory Committee on Dangerous Pathogens, 1995; Barbeito et al., 1995). Some examples of pathogens that may cause disease in humans, and also may be found in a veterinary laboratory, are listed in Table 1. Also, some very serious Group 4 agents, including Hendra and Nipah, have been isolated from diagnostic specimens in veterinary laboratories.

Table 1. Examples of some of the microorganisms in Risk Groups 2 and 3 that are capable of causing human disease and that may be present in a veterinary laboratory

<table>
<thead>
<tr>
<th>Group 2</th>
<th>Viruses:</th>
<th>Influenza viruses types A, B, C other than notifiable avian influenza (NAI); Newcastle disease virus; Orf (parapox virus)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria:</td>
<td>Alcaligenes spp.; Arizona spp.; Campylobacter spp.; Chlamydophila psittaci (nonavian); Clostridium tetani; Clostridium botulinum; Corynebacterium spp.; Erysipelothrix rhusiopathiae; Escherichia coli; Haemophilus spp.; Leptospira spp.; Listeria monocytogenes; Moraxella spp.; Mycobacterium avium; Pasteurella spp.; Proteus spp.; Pseudomonas spp.; Salmonella spp.; Staphylococcus spp.; Yersinia enterocolitica; Yersinia pseudotuberculosis</td>
</tr>
<tr>
<td></td>
<td>Fungi:</td>
<td>Aspergillus fumigatus; Microsporum spp.; Trichophyton spp.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 3</th>
<th>Viruses:</th>
<th>Rabies virus; Equine encephalomyelitis virus (Eastern, Western and Venezuelan); Japanese B encephalitis virus; Louping ill virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria:</td>
<td>Bacillus anthracis; Burkholderia mallei (Pseudomonas mallei); Brucella spp.; Chlamydia psittaci (avian strains only); Coxiella burnetii; Mycobacterium bovis</td>
</tr>
</tbody>
</table>

C. REQUIREMENTS FOR WORK WITH INFECTIOUS AGENTS

1. Known pathogens

Having decided the risk level of certain work, it is then possible to decide the appropriate ‘containment level’ that is needed to minimise the risk of human disease and the risk of spread of disease to animals and the environment. The containment level is defined by a combination of the physical facilities and working practices employed. Organisms of the four Risk Groups indicated above may be placed into containment levels appropriate for safe working, see below. Laboratories usually appoint a Biological Safety Officer, responsible for ensuring that microorganisms are handled at the appropriate containment level. They should have sufficient expertise and be of sufficient seniority to oversee and advise on all safety matters. In large organisations with a network of laboratories, it is appropriate to appoint a central Safety Officer to advise on and coordinate safety matters of a corporate nature, which are implemented by local laboratory Safety Officers at each site. The working methods for a particular procedure or work station should be written out and readily available. Staff must be fully trained and fully aware of any health risks associated with their work and in procedures for reporting incidents or accidents. Staff should also be given a medical card indicating pathogens to which they might be exposed. In some cases, staff can be specially vaccinated to give additional protection, e.g. when working with the rabies virus; this should also be recorded on the medical card. Such information is useful for a medical practitioner in the event of illness.
occurring. Regular medical examinations of employees are recommended and, as appropriate, monitoring tests of employees working with the organisms that cause certain serious human diseases, such as brucellosis and tuberculosis.

Much information is available on containment of pathogens, and sophisticated apparatus and buildings may be constructed for containment of the more hazardous organisms as required by the guidelines, standards and regulations of each country. The requirements depend on the containment required, from the most basic to the highest level.

**Essential requirements for all laboratory work.** The essential requirements for any work with infectious agents, however innocuous they may seem, are as follows:

1. The laboratory should be easy to clean, with surfaces that are impervious to water and resistant to chemicals. There shall be a wash-hand basin and emergency shower, including an eye bath, in each laboratory suite as appropriate for the chemicals and other hazards present. Procedures shall be established for frequent cleaning and disinfection during and at the end of the work period;

2. Personnel access to the work area should be restricted; appropriate security measures such as controlled electronic access may be necessary with higher risk agents.

3. Personal protective equipment such as long-sleeved lab coats or gowns, closed-toe footwear, disposable gloves, masks, safety glasses, face shields, and oro-nasal respirators, as appropriate, shall be worn in the laboratory and removed when leaving the laboratory.

4. The laboratory door should be closed when work is in progress and ventilation should be provided by extracting air from the room. (Where biosafety cabinets are used, care shall be taken to balance ventilation systems);

5. Food (including chewing gum, candy, throat lozenges and cough drops) and/or drink shall not be stored or consumed in laboratories;

6. Smoking and/or application of cosmetics shall not take place in the laboratory;

7. Pipetting shall not be done by mouth;

8. Care shall be taken to minimise the production of aerosols;

9. Emergency response plans should be developed to deal with the biohazard of spills. Some of the items addressed in the plans should include having effective disinfectant available for cleaning spills, removal of and decontamination of contaminated protective clothing, washing of hands, and cleaning and disinfection of bench tops;

10. Used laboratory glassware and other contaminated material shall be stored safely. Materials for disposal shall be transported without spillage in strong containers. Waste material should be autoclaved, incinerated or otherwise decontaminated before disposal. Reusable material shall be decontaminated by appropriate means;

11. No infectious material shall be discarded down laboratory sinks or any other drain;

12. Any accidents or incidents shall be recorded and reported to the Safety Officer.

**Containment level for Group 2 pathogens,** in addition to the points given above, a Class I, II or III microbiological safety cabinet should be used when there is potential for generating aerosols or when handling large quantities of culture or where there is a real need to protect the biological product (see Section D). Appropriate signs are required at all entry doors to indicate the hazard present and the name and telephone number of the person(s) responsible. Emergency protocols should be posted within the laboratory to advise personnel of procedures to follow in case of a pathogen spill or the need to evacuate the laboratory in the event of a fire or other emergency.

**Containment level for Group 3 pathogens,** it is advisable that the laboratory be in an isolated location; access should be limited to appropriately trained level 3 staff. Emergency protocols should be posted within the laboratory to advise personnel of procedures to follow in case of a pathogen spill or the need to evacuate the laboratory in the event of a fire. OIE containment level for Group 3 pathogens surpasses biosafety level-3 (BSL-3) guidelines as outlined by the United States Department of Health and Human Services (DHHS) joint publication with CDC and NIH (2009) and the United States Department of Agriculture (USDA, 2002).

In addition to the previous requirements, the laboratory shall be under negative pressure and the pressure differentials should be monitored; a procedure should be developed to provide an alarm if there is a problem and personnel to respond to the alarm. A ventilation system is required that removes air from the laboratory through a high efficiency particulate air (HEPA) filter. HEPA filters shall be verified regularly (usually annually); this would include HEPA filters in biosafety cabinets and on room and equipment exhausts. The laboratory should be
sealable for fumigation and contain an airlock entry. There is a requirement to treat effluent depending on the pathogen. Biological safety cabinets of Class I, II or III shall be used whenever the process to be undertaken is likely to generate an aerosol (DHHS/CDC/NIH, 2000). It may be necessary for staff to shower on exit from the laboratory and they must wear dedicated laboratory clothing that is left in the laboratory before leaving the building.

**Note.** Because of the link between bovine spongiform encephalopathy (BSE) and new variant Creutzfeldt-Jakob disease in humans, BSE and related agents are now categorised with the human transmissible spongiform encephalopathies in Risk Group 3. Consequently, veterinarians and laboratory workers conducting necropsies on BSE-suspect animals or handling tissues derived from such animals must conduct the work under appropriately strict containment conditions, sometimes with derogations allowed by the nature of the work and the results of local risk assessment. It is important that appropriate protective clothing be worn and that a strict code of practice be followed to prevent exposure to the agent. Laboratories conducting work on BSE must comply with national biocontainment and biosafety regulations (Advisory Committee on Dangerous Pathogens, 1998).

**Containment level for Group 4 pathogens,** the most stringent precautions are required, including access to the building through air locks, and the building being maintained under negative air pressure. Inlet air to the laboratory shall be filtered through a single HEPA filter and extracted air through double HEPA filters in series. All work with infective materials shall be conducted in a Class III cabinet or in a Class II cabinet in conjunction with the use of one-piece positive-pressure suits. All sewage from the laboratory, laboratory effluent and autoclave drain effluent shall be treated by appropriate means to ensure that all infectious material is destroyed before entering the sewerage outside the laboratory. Staff shall shower and change their clothing before leaving the building. Other precautions as described for Group 3 would also apply. The use of one-piece positive-pressure suits is now an internationally accepted way of providing additional protection at level 4.

OIE guidelines for the containment level for Group 4 pathogens are generally equal to the USDA's biosafety level 3 Ag guidelines (USDA, 2002). The primary difference between OIE level 4 and BSL-3 Ag is that the BSL-3 Ag guidelines specify that the laboratory will be airtight and shall pass a pressure decay test to confirm that it does not surpass the prescribed maximum leak rate.

2. **Diagnostic specimens**

Veterinary diagnostic centres readily receive specimens that are submitted because they are suspect for a variety of diseases. The infectious nature of the specimens is usually unknown, but they have the potential to contain biological agents that may cause disease in animals and humans. Practices and procedures need to be in place that will minimise the risk of occupational exposure of employees to such pathogens. Unless suspected of containing a pathogen requiring a higher containment level, it is advisable that initial processing of all unknown specimens should be carried out as though the material contained a Group 2 pathogen. The most important aspects are to prevent percutaneous, mucous membrane exposure, particularly inhalation and ingestion. Biological safety cabinets should be used for all manipulations that may generate aerosols. Class I or II are appropriate depending on the need for protection of the samples from contamination. Additionally, there should be no mouth pipetting, personal protective clothing shall be worn with, in some cases, eye and respiratory protection, depending on the anticipated level of exposure. Although initial diagnostic procedures may be carried out at level 2, once a Group 3 or 4 organism has been isolated (or suspected) further work must be carried out at the higher containment level.

**D. MICROBIOLOGICAL SAFETY CABINETS**

These are used at the different containment levels, as described in Section C above. They are of three types:

Class I: An open-fronted cabinet designed specifically to provide operator and environmental protection and not to give protection to the work being handled.

Class II: An open-fronted safety cabinet, sometimes referred to as a laminar flow recirculating cabinet. They are designed to give operator, product and environment protection.

Class III: These cabinets are closed, with glove ports at the front, and provide the highest degree of containment by complete separation of work and worker. Some cabinets have a removable glove port and are known as Class III/I cabinets, i.e. they can be used in either mode.

Descriptions of safety cabinets and safe working practices have been published (Collins, 1990; International Atomic Energy Agency [IAEA], 1994; DHHS/CDC/NIH, 2000).
E. STORAGE OF PATHOGENS

Storage of live pathogens requires appropriate containment and security to avoid risks due to breakage or unauthorised use of material. Storage facilities should be appropriately labelled to indicate the nature of the pathogens (e.g. their Group) and the contact information for the person(s) responsible for them. A complete inventory of the pathogens in storage should be kept up to date and available. Special care must be taken when opening glass vials of freeze-dried pathogens, as these can sometimes shatter. Care must be taken when working with liquid nitrogen or rooms where asphyxiating gasses may be produced.

Many of the considerations given above relate not only to human safety but also to prevention of the spread of infection to animals. In a veterinary laboratory an important responsibility is to minimise any risk of escape of pathogens to animals, either wild or domestic, in the outside community. Close communication must be maintained with the veterinary authorities. There may be national requirements for special licences to work with certain microorganisms.

F. PHYSICAL AND CHEMICAL HAZARDS

Laboratory work involves many manipulations that are potentially dangerous, such as handling glassware and work with needles or other sharp instruments. There shall be appropriate procedures and equipment for the safe and proper disposal of needles and other ‘sharps’.

Laboratory staff should be protected from the risk of receiving a burn from hot solids or liquids. Autoclaves shall be fitted with safety devices to prevent accidental opening of doors when under pressure, and be regularly serviced and tested. Heat-protective gloves, apron and face shields with brow and chin guards shall be provided. Extreme cold can also be a risk, for example when working with liquid nitrogen; splashes on exposed skin can be very damaging. Gloves should be worn that provide insulation from cold and that are also waterproof, to prevent penetration of the liquid nitrogen. Face shields with brow and chin guards and boots should also be worn when working with liquid nitrogen. Nitrogen evaporating from liquid nitrogen storage in poorly ventilated rooms can lead to depletion of oxygen with fatal consequences.

Irradiation is a serious health risk that may be present due to the use of X-ray machines, or use of gamma-emitters or other sources. Equipment shall be regularly serviced and tested. All use of radioactive material must be meticulously recorded. All staff must wear a personal radiation-monitoring device and have annual health checks. Local and national regulations must be followed (IAEA, 1994).

A wide range of chemicals are used in veterinary laboratories, many of which may be toxic or mutagenic, and some may be carcinogenic. It should be remembered that it is the dose that makes the poison. Vapours are especially hazardous, and some chemicals can be absorbed by penetration of intact skin. Steam sterilisation may make toxic chemicals volatile and endanger personnel who unload the autoclave/pressure steam steriliser. Procedures sufficient to protect pregnant laboratory workers should be followed at all times. A list of hazardous chemicals shall be maintained, and a file record kept of chemicals to which individual staff members could be exposed. This is now a legal requirement in some countries. Chemicals shall be correctly stored in appropriate containers and at the correct temperature. Those that are flammable shall be kept in a fireproof chemical store. A record must be maintained of the purchase and use of hazardous chemicals: how much, when used, by whom and for what purpose. Disposal of some chemicals is subject to official regulation.

Further information on physical and chemical safety precautions can be found in the literature (Office of Biosafety, Laboratory Centre for Disease Control, Health and Welfare Canada, 1996; Rayburn, 1990).

G. LABORATORY ANIMAL FACILITIES

Work with pathogens in laboratory animals poses special risks. Animal rooms have to be constructed to appropriate standards and containment levels, just as laboratories. Containment in animal houses is very important because of the large amount of infectious agents that they may generate. Similar considerations also apply regarding the training of staff, protective clothing and the recording of working procedures. Special care must be taken to avoid injury to staff, e.g. through animals biting and kicking or self inoculation accidents. Any such incidents must be recorded and wounds appropriately treated. There shall be provision for autoclaving steam sterilisation, incineration or rendering of carcases and for the thorough cleansing and disinfection of animal rooms. The animal rooms should not only provide a suitable environment for the animals themselves but should be constructed and ventilated in such a way as to ensure comfort for the attending personnel. This is a large subject that can only be referred to briefly here (Barbeito et al., 1995; Canadian Food Inspection Agency, 1996). Also, an excellent book on health and safety in laboratory animal facilities is available (Wood & Smith, 1999).


**H. EMERGENCY PROVISIONS**

First-aid equipment should be readily available, but stored in a location that is unlikely to be contaminated by work conducted in the laboratory (for example, in the air-lock or ante-room). This equipment shall be appropriate to the work and properly maintained. It shall be kept ready to hand for immediate emergency use by trained first aid personnel. Bandages and dressings should be available. Some staff shall receive training in safety and first aid from recognised authorities and shall possess a valid certificate as evidence of competence. Personnel working in Containment Level 4 facilities shall have advanced first aid competence. Their names and locations should be known to everyone and posted on notice boards. All staff should be aware of the importance of safety. There must be suitable procedures and equipment for dealing with spillages and decontamination. A record must be kept of all incidents and in some countries there may be a legal obligation to report incidents to the enforcing authority.

There must be written procedures for dealing with emergency failure of all safety and containment systems, for example in biosafety cabinets or biocontainment rooms, which can lead to loss of containment.

Many laboratories have a staff safety committee to increase safety awareness and to discuss safety issues with management. Personnel are responsible for their own safety and those around them. Managers are equally responsible for safety in their area of command and should not allow consideration of speed or cost of work to come before the safety of personnel or containment of animal disease agents.

There must be an emergency procedure for obtaining medical assistance if required, and for hospitalisation in appropriate infectious disease facilities when needed. Fire alarms shall be fitted, and tested regularly. The institute or laboratory must designate a warden to control and communicate in emergency situations and conduct periodic drills to make staff aware of what to do and where to assemble in the event of an emergency. The warden is responsible for checking that everyone is in a safe location. Procedures for natural disasters, such as hurricanes and earthquakes, should be in place where they present a risk. All these procedures should be written down and periodically reviewed.

**I. TRANSPORT OF INFECTIOUS MATERIAL**

Great care must be taken when preparing and packing diagnostic specimens, infectious materials and pathogens for transport, to ensure that there is no breakage of containers or leakage of contents that could put at risk personnel in the transport system or animals that may come in contact with contamination. Applicable local, national and international regulations for the transportation of dangerous goods (diagnostic or clinical sample and infectious materials) and importation of animal pathogens must be followed. These are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens.

When categorising animal pathogens into specific Groups, the following criteria should be taken into account:

a) **Group 1 animal pathogens**
   
   Disease-producing organisms that are enzootic but not subject to official control.

b) **Group 2 animal pathogens**
   
   Disease-producing organisms that are either exotic or enzootic but subject to official control and that have a low risk of spread from the laboratory.

c) **Group 3 animal pathogens**
   
   Disease-producing organisms that are either exotic or enzootic but subject to official control and that have a moderate risk of spread from the laboratory.

d) **Group 4 animal pathogens**
   
   Disease-producing organisms that are either exotic or enzootic but subject to official control and that have a high risk of spread from the laboratory into the environment and the national animal population.

**J. CONTAINMENT GROUPS**

1. The principal purpose of containment is to prevent the escape of the pathogen from the laboratory into the national animal population. Some animal pathogens can infect humans. In these instances the risk to human
health may demand additional containment than would otherwise be considered necessary from purely animal health considerations. The risk of human to animal transmission of disease must also be considered and controlled. In addition, other animals being used for experimental work on the pathogen should be held in the appropriate containment level.

2. The level of physical containment and biosafety procedures and practices should be not less than the Group into which the pathogen has been placed and the detailed requirements should be appropriate to the type of organism (i.e. bacterium, virus, fungus or parasite). The lowest containment level will be required for pathogens in Group 1 and the highest level for those in Group 4. Guidance on the containment requirements for Groups 2, 3 and 4 is provided in Section K.

3. Arthropods may be pathogens or vectors for pathogens. If they are a vector for a pathogen being used in the laboratory, the appropriate containment level for the pathogen will be necessary in addition to the containment facilities for the arthropod.

K. GUIDANCE ON THE LABORATORY/ANIMAL FACILITY REQUIREMENTS FOR THE DIFFERENT CONTAINMENT GROUPS

<table>
<thead>
<tr>
<th>Requirements of the laboratory/animal facility</th>
<th>Containment Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) Laboratory/animal facility setting and structure</td>
<td>2</td>
</tr>
<tr>
<td>1. It is advisable that the laboratory/animal facility be in an isolated location</td>
<td>Yes</td>
</tr>
<tr>
<td>2. Not next to known fire hazard</td>
<td>Yes</td>
</tr>
<tr>
<td>3. Workplace separated from other activities</td>
<td>Yes</td>
</tr>
<tr>
<td>4. Personnel access limited</td>
<td>Yes</td>
</tr>
<tr>
<td>5. Protected against entry/exit of rodents and insects</td>
<td>Yes</td>
</tr>
<tr>
<td>6. Liquid effluent must be sterilised and monitored</td>
<td>Yes</td>
</tr>
<tr>
<td>7. Liquid effluent from steam sterilisers shall be sterilised and monitored</td>
<td>Yes</td>
</tr>
<tr>
<td>8. Isolated by airlock. Continuous internal airflow</td>
<td>Yes</td>
</tr>
<tr>
<td>9. The laboratory/animal facility shall be under negative pressure and the pressure differentials should be monitored</td>
<td>Yes</td>
</tr>
<tr>
<td>10. Input air to be filtered using HEPA or equivalent such as gas tight damper; exhaust air to be single HEPA filtration for laboratories and double HEPA filtration for animal facilities</td>
<td>Single on extract</td>
</tr>
<tr>
<td>11. HEPA filters shall be verified regularly (usually annually)</td>
<td>Yes</td>
</tr>
<tr>
<td>12. Mechanical air supply system with fail-safe system and an alarm provided if there is a problem</td>
<td>Yes</td>
</tr>
<tr>
<td>13. Laboratory/animal facility sealable to permit fumigation</td>
<td>Yes</td>
</tr>
<tr>
<td>14. Incinerator, pressure steam steriliser or renderer for disposal of carcasses and waste</td>
<td>Available</td>
</tr>
<tr>
<td>15. The laboratory/animal facility should be easy to clean, with surfaces that are impervious to water and resistant to chemicals. There shall be a wash-hand basin and emergency shower, including an eye bath, in each laboratory suite as appropriate for the chemicals and other hazards present. Procedures shall be established for frequent cleaning and disinfection during and at the end of the work period</td>
<td>Yes</td>
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### Requirements of the laboratory/animal facility

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<th>Requirement</th>
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<td>3</td>
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<td>4</td>
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<tr>
<td>B) Additional Laboratory facility requirements</td>
<td></td>
</tr>
<tr>
<td>16. Class I or II biological safety cabinet available</td>
<td>Yes</td>
</tr>
<tr>
<td>17. Class III biological safety cabinet available</td>
<td>Yes</td>
</tr>
<tr>
<td>18. HEPA filters shall be verified regularly (usually annually)</td>
<td>Yes</td>
</tr>
<tr>
<td>19. Direct access to autoclave/pressure steam steriliser</td>
<td>Yes with double doors</td>
</tr>
<tr>
<td>20. Specified pathogens stored in laboratory</td>
<td>Yes</td>
</tr>
<tr>
<td>21. Double-ended dunk tank required</td>
<td>Preferable</td>
</tr>
<tr>
<td>22. Personal protective clothing and equipment not worn outside laboratory</td>
<td>Yes</td>
</tr>
<tr>
<td>23. Full body shower and change of clothing required before exiting laboratory</td>
<td>It may be necessary for staff to shower on exit the laboratory and they must wear dedicated laboratory clothing that is left in the laboratory before leaving the building</td>
</tr>
<tr>
<td>24. Safety Officer responsible for containment</td>
<td>Yes</td>
</tr>
<tr>
<td>25. Staff receive special training and demonstrate competence in the requirements needed</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
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<td></td>
<td>Yes</td>
</tr>
<tr>
<td>C) Laboratory discipline</td>
<td></td>
</tr>
<tr>
<td>26. Warning notices for containment area to indicate the hazard present and the name and telephone number of the person(s) responsible</td>
<td>Yes</td>
</tr>
<tr>
<td>27. Emergency protocols should be posted within the laboratory to advise personnel of procedures to follow in case of a pathogen spill or the need to evacuate the laboratory in the event of a fire or other emergency</td>
<td>Yes</td>
</tr>
<tr>
<td>28. Laboratory must be lockable</td>
<td>Yes</td>
</tr>
<tr>
<td>29. Authorised entry of personnel</td>
<td>Yes</td>
</tr>
<tr>
<td>30. Protective clothing, including gloves, masks, eye shields, and oro-nasal respirators, as appropriate, shall be worn in the laboratory and removed when leaving the laboratory</td>
<td>Yes</td>
</tr>
<tr>
<td>31. The laboratory door should be closed when work is in progress and ventilation should be provided by extracting air from the room. (Where biosafety cabinets are used, care shall be taken to balance ventilation systems.)</td>
<td>Yes</td>
</tr>
<tr>
<td>32. Food and/or drink shall not be stored or consumed in laboratories</td>
<td>Yes</td>
</tr>
<tr>
<td>33. Smoking and/or application of cosmetics shall not take place in the laboratory</td>
<td>Yes</td>
</tr>
<tr>
<td>34. Pipetting shall not be done by mouth</td>
<td>Yes</td>
</tr>
<tr>
<td>35. Care shall be taken to minimise the production of aerosols</td>
<td>Yes</td>
</tr>
<tr>
<td>36. No infectious material shall be discarded down laboratory sinks or any other drain</td>
<td>Yes</td>
</tr>
<tr>
<td>37. Used laboratory glassware and other materials shall be stored safely before disinfection. Materials for disposal shall be transported without spillage in strong containers. Waste material should be autoclaved, incinerated or otherwise made safe before disposal. Reusable material shall be decontaminated by appropriate means</td>
<td>Yes</td>
</tr>
</tbody>
</table>

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**Containment Group**

- **2**: Controlled environment
- **3**: Reduced containment
- **4**: Maximum containment
Chapter 1.1.3. — Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities

Requirements of the laboratory/animal facility

<table>
<thead>
<tr>
<th>Requirements of the laboratory/animal facility</th>
<th>Containment Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>38. Any accidents or incidents shall be recorded and reported to the Safety Officer</td>
<td>Yes</td>
</tr>
<tr>
<td>39. On entering all clothing removed and clean clothes put on</td>
<td>Yes</td>
</tr>
<tr>
<td>40. On exiting all laboratory clothes removed, individual shall wash and transfer to clean side</td>
<td>Yes</td>
</tr>
<tr>
<td>41. Individual shall shower prior to transfer to clean side</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>D) Handling of specimens</strong></td>
<td></td>
</tr>
<tr>
<td>42. Packaging requirements to be advised prior to submission</td>
<td>Yes</td>
</tr>
<tr>
<td>43. Incoming packages opened by trained staff in appropriately contained reception area</td>
<td>Yes</td>
</tr>
<tr>
<td>44. Movement of pathogens from an approved laboratory to another requires a licence</td>
<td>Yes</td>
</tr>
<tr>
<td>45. Standard Operating Procedures covering all areas must be available</td>
<td>Yes</td>
</tr>
</tbody>
</table>

1. **Additional requirements for work in animal facilities**

1. The animal facility should be easy to clean, with surfaces that are impervious to water and resistant to chemicals used in the area.

2. Personnel access to the work area should be restricted; appropriate security measures such as controlled electronic access may be necessary with higher risk agents.

3. Personal protective equipment such as coveralls, boots, disposable gloves, masks, safety glasses, face shields, and oro-nasal respirators, as appropriate, shall be worn in the animal facility and removed when leaving the animal facility.

4. The animal facility door should be closed when work is in progress and ventilation should be provided by extracting air from the room. (Where biosafety cabinets are used, care shall be taken to balance ventilation systems.)

5. Food (including chewing gum, candy, throat lozenges and cough drops) and/or drink shall not be stored or consumed in animal facilities.

6. Smoking and/or application of cosmetics shall not take place in the animal facility.

10. Used laboratory glassware and other materials shall be stored safely before disinfection. Materials for disposal shall be transported without spillage in strong containers. Waste material should be autoclaved, incinerated or otherwise made safe before disposal. Reusable material shall be decontaminated by appropriate means.

11. No infectious material shall be discarded down animal facility drains without appropriate waste treatment in place.

12. Any accidents or incidents shall be recorded and reported to the Safety Officer.

**L. CONCLUSION**

High standards of laboratory safety and containment that will ensure healthy working conditions for laboratory staff and protection of the environment must be of the greatest priority. They can only be achieved by careful study of the principles involved followed by practical application to premises, facilities, operating procedures and hygiene. Training of all laboratory personnel must be a high priority and no personnel should be allowed to work until appropriate training and competence has been demonstrated and documented. There is a large published literature on all aspects of the subject, and further reading is recommended (Beran & Steele, 1994; European Committee for Standardisation, 2000; Richmond, 1996–2002; Sewell, 1995; World Health Organization, 2004).
REFERENCES


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CHAPTER 1.1.4.

QUALITY MANAGEMENT IN VETERINARY TESTING LABORATORIES

SUMMARY

Valid laboratory results are essential for diagnosis, surveillance and trade. Such results are achieved by the use of good management practices, valid test and calibration methods, proper technique, quality control and quality assurance, all working together within a quality management system. Laboratory quality management includes technical, managerial and operational elements of testing and the interpretation of test results. A quality management system enables the laboratory to demonstrate both competency and an ability to generate consistent technically valid results that meet the needs of its customers. The need for mutual recognition of test results for international trade and the acceptance of international standards such as ISO/IEC\textsuperscript{1} 17025:2005 General Requirements for the Competence of Testing and Calibration Laboratories (ISO/IEC, 2005) requires good laboratory quality management systems. The OIE has published a detailed standard on this subject (OIE, 2008). This chapter is not intended to reiterate the requirements of these two documents, nor has it been endorsed by accreditation bodies. Rather, it outlines the important issues and considerations a laboratory should address in the design and maintenance of its quality management system, whether or not it has been formally accredited.

KEY CONSIDERATIONS FOR THE DESIGN AND MAINTENANCE OF A LABORATORY QUALITY MANAGEMENT SYSTEM

In order to ensure that the quality management system is appropriate and effective, the design must be carefully thought out and, where accreditation is sought, must address all criteria of the appropriate quality standard. The major categories of consideration and the key issues and activities within each of these categories are outlined in the following eight sections of this chapter.

1. The work, responsibilities, and goals of the laboratory

Many factors affect the necessary elements and requirements of a quality management system. These factors include:

i) The type of testing done;

ii) The purpose and requirements of the test results;

iii) The impact of a questionable or erroneous result;

iv) The tolerance level of risk and liability;

v) Customer needs (e.g. sensitivity and specificity of the test method, cost, turnaround time, strain/genotype characterisation);

vi) The role of the laboratory in legal work or in regulatory programmes;

vii) The role of the laboratory in assisting with, confirming, and/or overseeing the work of other laboratories (e.g. as a reference laboratory);

viii) The business goals of the laboratory, including the need for any third party recognition and/or accreditation.

\textsuperscript{1} ISO/IEC - International Organization for Standardization/International Electrochemical Commission.
2. Standards, guides, and references

The laboratory should choose reputable and accepted standards and guides to assist in designing the quality management system. The OIE standard on this subject is a useful guideline (OIE, 2008). For laboratories seeking accreditation of testing, the use of ISO/IEC 17025 (ISO/IEC, 2005) or the OIE Standard (2008) will be essential. Further information on standards may be obtained from the national standards body of each country, from the International Laboratory Accreditation Cooperation (ILAC), and from accreditation bodies, e.g. the National Association of Testing Authorities (NATA), Australia, the United Kingdom Accreditation Service (UKAS), the American Association for Laboratory Accreditation (A2LA), etc. Technical and international organisations such as AOAC International (The Scientific Association Dedicated to Analytical Excellence; formerly the Association of Official Analytical Chemists) and the International Organization for Standardization (ISO) publish useful references, guides and standards that supplement the general requirements of ISO/IEC 17025. The ISO International Standard 9001 (ISO, 2008), is a certification standard for quality management systems and while it may be a useful supplement to a quality system, its requirements do not necessarily ensure or imply technical competence (in the areas listed Section 3 below). ISO 9001 is assessed by a certification body, which is accredited to undertake such assessments by the national accreditation body. When a laboratory meets the requirements of ISO 9001, the term registration or certification is used to indicate conformity, not accreditation.

3. Accreditation

If the laboratory decides to proceed with formal recognition of its quality management system and testing, then third party verification of its conformity with the selected standard(s) will be necessary. ILAC has published specific requirements and guides for laboratories and accreditation bodies. Under the ILAC system, ISO/IEC 17025 is to be used for laboratory accreditation of testing and/or calibration activities. Definitions regarding laboratory accreditation may be found in ISO/IEC International Standard 17000: Conformity Assessment – Vocabulary and Principles (ISO/IEC, 2004). Accreditation is tied to competence, which is significantly more than having and following documented procedures. Having competence also means that the laboratory:

i) Has technically valid and validated test methods, procedures and specifications that are documented in accordance with the requirements of the applicable standard or guidelines;

ii) Has appropriately qualified and trained personnel with a depth of technical knowledge commensurate with appropriate levels of authority;

iii) Has appropriate equipment with planned maintenance/calibration schedules;

iv) Has adequate facilities and environmental control;

v) Has procedures and specifications that ensure accurate and reliable results;

vi) Implements continual improvements in testing and quality management;

vii) Can assess the need for and implement appropriate corrective or preventive actions;

viii) Accurately assesses and controls uncertainty in testing;

ix) Demonstrates proficiency in the test methods used (e.g. by participation in proficiency tests on a regular basis);

x) Has demonstrated competence to generate technically valid results.

4. Selection of an accreditation body

To facilitate the acceptance of the laboratory’s test results for trade, the accreditation standard used must be recognised by the international community and the accreditation body recognised as competent to accredit laboratories. Programmes for the recognition of accreditation bodies are, in the ILAC scheme, based on the requirements of ISO/IEC International Standard 17011: General Requirements for Accreditation Bodies Accrediting Conformity Assessment Bodies (ISO/IEC, 2004a). Information on recognised accreditation bodies may be obtained from the organisations that recognise them, such as the Asia-Pacific Laboratory Accreditation Cooperation (APLAC), the Interamerican Accreditation Cooperation (IAAC), and the European Co-operation for Accreditation (EA).

5. Determination of the scope of the quality management system and/or of the laboratory’s accreditation

The quality management system should cover all areas of activity affecting all testing that is done at the laboratory. While accredited laboratories are obliged to meet the requirements of the standard as detailed below, these principles are relevant to all testing laboratories.
Laboratories accredited to ISO/IEC 17025 have a specific list of those tests that are accredited, called the schedule of accreditation or the scope. If new testing methods are introduced these must be assessed and accredited before they can be added to the scope. The quality management system should ideally cover all areas of activity affecting all testing that is done at the laboratory. However, it is up to the laboratory to decide which tests are to be accredited and included in the scope. If an accredited laboratory also offers unaccredited tests, these must be clearly indicated as such on any reports that claim or make reference to accreditation. Factors that might affect the laboratory’s choice of tests for scope of accreditation include:

i) The impact of initial accreditation on resources within a given deadline;
ii) A contractual requirement for accredited testing (e.g. for international trade, research projects);
iii) The importance of the test and the impact of an incorrect result;
iv) The cost of maintaining an accredited test;
v) Availability of personnel, facilities and equipment;
vi) Availability of reference standards (e.g. standardised reagents, internal quality control samples, reference cultures) and proficiency testing schemes;
vii) The quality assurance necessary for materials, reagents and media;
viii) The validation, technical complexity and reliability of the test method;
ix) The potential for subcontracting of accredited tests;

6. Quality Assurance, Quality Control and Proficiency testing

Quality Assurance (QA) is the systematic and planned process of ensuring that the service offered meets the stated requirements in all areas. The requirements may be internal or defined in an accreditation/certification standard. QA is process orientated and ensures the right things are being done in the right way.

Quality Control (QC) is the systematic and planned monitoring of output to ensure the minimum levels of quality have been met. For a testing laboratory, this is to ensure test processes are working correctly and results are within the expected parameters and limits. QC is test orientated and ensures the results are as expected.

Proficiency Testing (PT), sometimes referred to as External Quality Assurance or EQA, is the determination of a laboratory’s performance by testing specimens of undisclosed content. Ideally, PT schemes should be run by an external independent provider. Participation in proficiency testing enables the laboratory to assess and demonstrate the reliability results by comparison with those from other participating laboratories.

All laboratories should, where possible, participate in external proficiency testing schemes appropriate to their testing. Participation in such schemes is a requirement for accredited laboratories. This provides an independent assessment of the testing methods used and the level of staff competence. If such schemes are not available, valid alternatives may be used, such as ring trials organised by reference laboratories, inter-laboratory testing, use of certified reference materials or internal quality control samples, replicate testing using the same or different methods, retesting of retained items, and correlation of results for different characteristics of a specimen.

Providers and operators of proficiency testing programmes should be accredited to ISO/IEC 17043:2010 – Conformity assessment – General requirements for proficiency testing (ISO/IEC, 2010). This replaces the ISO/IEC Guide 43–1:1997 Proficiency testing by interlaboratory comparisons on which the previous OIE Guidelines were based (OIE, 2008, Guide 4).

Proficiency testing material from accredited providers has been well characterised and any spare material, once the proficiency testing has been completed, can be useful to demonstrate staff competence or for test validation.

7. Test methods

ISO/IEC 17025 requires the use of appropriate test methods and has requirements for their selection, development, and validation. The OIE Quality Standard and Guidelines for Veterinary Laboratories: Infectious Diseases (OIE, 2008) also provides requirements for selection and validation.

This Terrestrial Manual provides recommendations on the selection of test methods for trade, diagnostic and surveillance purposes in the chapters on specific diseases. In addition, a list of prescribed tests for international trade is provided. As stated in the introduction to this list, the prescribed tests that are listed are those that are required by the OIE Terrestrial Animal Health Code. These tests are considered to be adequately validated to give reliable results to qualify animals for international movement. Also listed are alternative tests that may be
suitable for use within a local setting, but that may have limited validation. The fact that a test is recommended does not necessarily mean that a laboratory is competent to perform it. The laboratory quality system should incorporate provision of evidence of competency.

In the veterinary profession, other standard methods (published in international, regional, or national standards) or fully validated methods (having undergone a full collaborative study and that are published or issued by an authoritative technical body such as the AOAC International) may be preferable to use, but may not be available. Many veterinary laboratories develop or modify methods, and most laboratories have test systems that use non-standard methods, or a combination of standard and non-standard methods. In veterinary laboratories, even with the use of standard methods, some in-house evaluation, optimisation, and/or validation generally must be done to ensure valid results.

Customers and laboratory staff must have a clear understanding of the performance characteristics of the test, and customers should be informed if the method is non-standard. Many veterinary testing laboratories will therefore need to demonstrate competence in the development, adaptation, and validation of test methods.

This *Terrestrial Manual* provides more detailed and specific guidance on test selection, optimisation, standardisation, and validation in Chapter 1.1.5 *Principles and methods of validation of diagnostic assays for infectious diseases*. The following are key test method issues for those involved in the quality management of the laboratory.

**a) Selection of the test method**

Valid results begin with the selection of a test method that meets the needs of the laboratory’s customers in addressing their specific requirements. Some issues relate directly to the laboratory, others to the customer. Considerations for the selection of a test method include:

- International acceptance;
- Scientific acceptance;
- Appropriate or current technology;
- Suitable performance characteristics (e.g. analytical and diagnostic sensitivity and specificity, repeatability, reproducibility, isolation rate, limits of detection, precision, trueness, and uncertainty);
- Suitability of the test in the species and population of interest;
- Sample type (e.g. serum, tissue, milk) and its expected quality/state on arrival at the laboratory;
- Test target (e.g. antibody, antigen, live pathogen, nucleic acid sequence);
- Test turnaround time;
- Resources and time available for development, adaptation, evaluation;
- Intended use (e.g. export, import, surveillance, screening, diagnostic, confirmatory);
- Safety factors;
- Customer expectations;
- Throughput of test samples required;
- Cost of test, per sample;
- Availability of reference standards, reference materials and proficiency testing schemes.

**b) Optimisation and standardisation of the test method**

Once the method has been selected, it must be set up at the laboratory. Additional optimisation is necessary, whether the method was developed in-house or imported from an outside source. Optimisation establishes critical specifications and performance standards for the test process as used in a specific laboratory. Optimisation should determine:

- Critical specifications for equipment and instruments;
- Critical specifications for reagents (e.g. chemicals, biologicals), reference standards, reference materials, and internal controls;
- Robustness – critical control points and acceptable ranges, attributes or behaviour at critical control points, using statistically acceptable procedures;
- Quality control activities necessary to monitor critical control points;
v) The type, number, range, frequency, and arrangement of test run controls;
vi) Criteria for non-subjective acceptance or rejection of a batch of test results;
vii) Criteria for the interpretation and reporting of test results;
viii) A documented test method and reporting procedure for use by laboratory staff;
ix) Evidence of technical competence for those who perform the test processes and interpret results.

c) Validation of the test method
Validation evaluates the test for its fitness for a given use by establishing test performance characteristics, such as sensitivity, specificity, and isolation rate; and diagnostic parameters such as positive/negative cut-off, and titre of interest or significance. Validation should be done using an optimised, documented, and fixed procedure. The extent and depth of the validation process will depend on logistical and risk factors. It may involve any number of activities and amount of data, with subsequent data analysis using appropriate statistical methods. Validation activities might include:
i) Field and/or epidemiological studies;
ii) Repeat testing to establish the effect of variables such as operator, reagents, equipment;
iii) Comparison with other, preferably standard, methods and with reference standards (if available);
iv) Collaborative studies with other laboratories using the same documented method. Ideally organised by a reference laboratory and including testing a panel samples of undisclosed composition or titre with expert evaluation of results and feedback to the participants;
v) Reproduction of data from an accepted standard method, or from a reputable publication;
vi) Experimental infection or disease outbreak studies;
vii) Analysis of internal quality control data.

Validation is always a balance between cost, risk, and technical possibilities. There may be cases where quantities such as accuracy and precision can only be given in a simplified way. Criteria and procedures for the correlation of test results for diagnosis of disease status or for regulatory action must be developed. The criteria and procedures developed should account for screening methods, retesting and confirmatory testing.

Test validation is covered in chapter 1.1.5.

d) Uncertainty of the test method
Measurement of Uncertainty (MU) is “a parameter associated with the result of a measurement that characterises the dispersion of values that could reasonably be attributed to the measure” (Eurachem, 2000). Uncertainty of measurement does not imply doubt about a result but rather increases confidence in its validity. It is not the equivalent to error, as it may be applied to all test results derived from a particular procedure.

Laboratories must estimate the MU for each test method resulting in a measurement included in their scope of accreditation and for any methods used to calibrate equipment (ISO/IEC, 2005).

Tests can be broadly divided into two groups: quantitative (biochemical assays, enzyme-linked immunosorbent assays ELISA, titrations, real-time polymerase chain reactions PCR, pathogen enumeration, etc.); and qualitative (bacterial culture, parasite identification, virus isolation, endpoint PCR, immunofluorescence, etc.).

The determination of MU is well established in quantitative measurement sciences (ANSI, 1997). It may be given as a numeric expression of reliability and is commonly shown as a stated range. Standard deviation (SD) and confidence interval (CI) are examples of the expression of MU, for example the Optical Density result of an ELISA expressed as ± n SD, where n is usually 1, 2 or 3. The Confidence Interval (usually 95%) gives an estimated range in which the result is likely to fall, calculated from a given set of test data.

The application of the principles of MU to qualitative testing is less well defined. The determination and expression of MU has not been standardised for veterinary (or medical, food, or environmental) testing laboratories, but sound guidance exists and as accreditation becomes more important, applications are being developed. The ISO/IEC 17025 standard recognises that some test methods may preclude metrologically and statistically valid calculation of uncertainty of measurement. In such cases the laboratory must attempt to identify and estimate all the components of uncertainty based on knowledge of the performance of the method and making use of previous experience, validation data, internal control results etc.
Many technical organisations and accreditation bodies (e.g. AOAC International, ISO, NATA, A2LA, SCC, UKAS, Eurachem, CITAC) teach courses and/or provide guidance on MU for laboratories seeking accreditation.

The ISO/IEC 17025 requirement for “quality control procedures for monitoring the validity of tests” implies that the laboratory must use quality control procedures that cover all major sources of uncertainty. There is no requirement to cover each component separately. Laboratories may establish acceptable specifications, criteria, ranges etc. at critical control points for each component of the test process. The laboratory can then implement appropriate quality control measures at these critical points, or seek to reduce or eliminate the uncertainty effect of each component. Components of tests with sources of uncertainty include:

i) Sampling;
ii) Contamination;
iii) Sample transport and storage conditions;
iv) Sample processing;
v) Reagent quality, preparation and storage;
vi) Type of reference material;
vii) Volumetric and weight manipulations;
viii) Environmental conditions;
ix) Equipment effects;
x) Analyst or operator bias;
xi) Biological variability;
xii) Unknown or random effects.

Systematic errors or bias determined by validation must be corrected by changes in the method, adjusted for mathematically, or have the bias noted as part of the report statement.

If an adjustment is made to a test or procedure to reduce uncertainty or correct bias then a new source of uncertainty is introduced (the uncertainty of the correction). This must be assessed as part of the MU estimate.

Additional information on the analysis of uncertainty may be found in the Eurachem Guides to Quantifying Uncertainty in Measurement (Eurachem, 2000) and Use of uncertainty information in compliance assessment (Eurachem, 2007).

e) Implementation and use of the test method

Training should be a planned and structured activity with steps to ensure adequate supervision is maintained while analysts are being trained. Analysts should be able to demonstrate proficiency in using the test method prior to producing reported results, and on an ongoing basis.

The laboratory must be able to demonstrate traceability for all accredited tests and the principle should apply to all tests whether accredited or not. This covers all activities relating to test selection, development, optimisation, standardisation, validation, implementation, reporting, personnel, quality control and quality assurance. Traceability is achieved by using appropriate documented project management, record keeping, data management and archiving systems.

8. Strategic planning

Laboratories should have evidence of continual improvement, which is an obligatory requirement for accredited laboratories. The laboratory must be knowledgeable of and stay current with the quality and technical management standards and with methods used to demonstrate laboratory competence and establish and maintain technical validity. Evidence of this may be provided by:

i) Attendance at conferences, organisation of in-house or external meetings on diagnostics and quality management;
ii) Participation in local and international organisations;
iii) Participation in writing national and international standards (e.g. on ILAC and ISO committees);
iv) Current awareness of publications, writing and reviewing publications about diagnostic methods;
v) Training programmes, including visits to other laboratories;
v) Conducting research;
vii) Participation in cooperative programmes (e.g. Inter-American Institute for Cooperation in Agriculture);
viii) Exchange of procedures, methods, reagents, samples, personnel, and ideas;
ix) Planned, continual professional development and technical training;
x) Management reviews;
xii) Analysis of customer feedback;
xii) Root cause analysis of anomalies and implementation of corrective, preventive and improvement actions.

REFERENCES


* * *

2 NCSL: The National Conference of Standards Laboratories.
3 CITAC: The Cooperation of International Traceability in Analytical Chemistry.
CHAPTER 1.1.5.

PRINCIPLES AND METHODS OF VALIDATION OF DIAGNOSTIC ASSAYS FOR INFECTIOUS DISEASES

INTRODUCTION

Validation is a process that determines the fitness of an assay, which has been properly developed, optimised and standardised, for an intended purpose. Validation includes estimates of the analytical and diagnostic performance characteristics of a test. In the context of this chapter, an assay that has completed the first three stages of the validation pathway (see Figure 1), including performance characterisation, can be designated as “validated for the original intended purpose(s)”\(^1\). To maintain a validated assay status, however, it is necessary to carefully monitor the assay’s daily performance, often by tracking the behaviour of internal controls over time. This ensures that the assay, as originally validated, consistently maintains its performance characteristics. Should it no longer produce results consistent with the original validation data, the assay may be rendered unfit for its intended purpose. Thus, a validated assay is continuously assessed to assure it maintains its fitness for purpose through assessment of results of internal controls in each run of the assay.

Assays applied to individuals or populations have many purposes, such as aiding in: documenting freedom from disease in a country or region, preventing spread of disease through trade, eradicating an infection from a region or country, confirming diagnosis of clinical cases, estimating infection prevalence to facilitate risk analysis, identifying infected animals toward implementation of control measures, and classifying animals for herd health or immune status post-vaccination. A single assay may be validated for one or several intended purposes by optimising its performance characteristics for each purpose, e.g. setting diagnostic sensitivity (DSe) high, with associated lower diagnostic specificity (DSp) for a screening assay, or conversely, setting DSp high with associated lower DSe for a confirmatory assay.

The ever-changing repertoire of new and unique diagnostic reagents coupled with many novel assay platforms and protocols has precipitated discussions about how to properly validate these assays. It is no longer sufficient to offer simple examples from serological assays, such as the enzyme-linked immunosorbent assay, to guide assay developers in validating the more complex assays, such as nucleic acid detection tests. In order to bring coherence to the validation process for all types of assays, this chapter focuses on the criteria that must be fulfilled during assay development and validation of all assay types. The inclusion of assay development as part of the assay validation process may seem counterintuitive, but in reality, three of the validation criteria that must be assessed in order to achieve a validated assay, comprise steps in the assay development process. Accordingly the assay development process seamlessly segues into an assay validation pathway, both of which contain validation criteria that must be fulfilled. This chapter also provides guidance for evaluation of each criterion through provision of best scientific practices contained in the chapter’s appendices. The best practices are tailored for each of several fundamentally different types of assays (e.g. detection of nucleic acids, antibodies, or antigens).

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\(^1\) Validation does not necessarily imply that test performance meets any minimum value or that the test has comparable performance to any comparative test, unless this has been specifically considered in the design of the test evaluation study.
DIRECT AND INDIRECT METHODS THAT REQUIRE VALIDATION

The diagnosis of infectious diseases is performed by direct and/or indirect detection of infectious agents. By direct methods, the particles of the agents and/or their components, such as nucleic acids, structural or non-structural proteins, enzymes, etc., are detected. The indirect methods demonstrate antibodies, or cell-mediated immune responses, induced by exposure to infectious agents or their components. The most common indirect methods of infectious agent detection are antibody assays such as classical virus neutralisation, antibody enzyme-linked immunosorbent assay (ELISA), haemagglutination inhibition, complement fixation, and the recently appearing novel methods, such as biosensors, bioluminometry, fluorescence polarisation, and chemoluminescence.

The most common direct detection methods are isolation or in-vitro cultivation of viable organisms, electron microscopy, immunofluorescence, immunohistochemistry, antigen-ELISA, Western immunoblotting, and nucleic acid detection systems (NAD). The NAD systems include nucleic-acid hybridisation (NAH), macro- and microarrays and the various techniques of nucleic acid amplification, such as the polymerase chain reaction (PCR), or the isothermal amplification methods, such as nucleic acid sequence-based amplification (NASBA), and invader or loop-mediated isothermal amplification (LAMP). NAD assays are rapidly becoming commonplace and in many cases replacing virus isolation and bacteria cultivation, particularly for the detection of agents that are difficult or impossible to culture. NAD tools are also used as a secondary means for highly specific identification of strains, groups, or lineages of organisms following isolation or culture of viruses, bacteria and parasites. Molecular diagnostics, such as PCR, do not require: a) the presence of replicating organisms, b) expensive viral isolation infrastructure, c) up to several weeks to achieve a diagnosis, or d) special expertise, which is often unavailable in many laboratories – all practical advantages. These methods have become relatively inexpensive, safe and user-friendly (Ballagi-Pordány & Belák, 1996; Belák, 2005; 2007; Belák & Thorén, 2001; Burns et al., 2005; Bustin, 2005; Huggett et al., 2005; Lauerman, 2004; Louie et al., 2000). Various real-time PCR methods, nucleic acid extraction robots, and automated workstations for NAD, antibody, antigen, and agent detection have resulted in a large repertoire of high throughput, robust, very rapid and reliable assays. Although NAD systems often have the advantage of a greater diagnostic sensitivity and analytical specificity than infectious agent recovery or antigen-capture ELISA procedures, that advantage usually carries with it a greater challenge for validation of such assays.

PRELIMINARY CONSIDERATIONS IN ASSAY DEVELOPMENT AND VALIDATION

The first consideration is to define the purpose of the assay, because this guides all subsequent steps in the validation process. By considering the variables that affect an assay’s performance, the criteria that must be addressed in assay validation become clearer. The variables can be grouped into three categories: (a) the sample – individual or pooled, matrix composition, and host/organism interactions affecting the target analyte quantitatively or qualitatively; (b) the assay system – physical, chemical, biological and operator-related factors affecting the capacity of the assay to detect a specific analyte in the sample; and (c) the test result interpretation – the capacity of a test result, derived from the assay system, to predict accurately the status of the individual or population relative to the analyte in question.

The matrix in which the targeted analyte may reside (serum, faeces, tissue, etc.) may contain endogenous or exogenous inhibitors that prevent enzyme-dependent tests such as PCRs or ELISAs from working. Other factors that affect the concentration and composition of analyte (mainly antibody) in the sample may be mainly attributable to the host and are either inherent (e.g. age, sex, breed, nutritional status, pregnancy, immunological responsiveness) or acquired (e.g. passively acquired antibody, active immunity elicited by vaccination or infection). Non-host factors, such as contamination or deterioration of the sample, also affect the ability of the assay to detect the specific targeted analyte in the sample.

Factors that interfere with the analytical performance of the assay system include instrumentation, operator error, reagent choice (both chemical and biological) and calibration, accuracy and acceptance limits of assay controls, reaction vessels and platforms, water quality, pH and ionicity of buffers and diluents, incubation temperatures and durations, and error introduced by detection of closely related analytes. It is also important that biological reagents are free of extraneous agents.

Factors that may negatively impact diagnostic performance of the assay are primarily associated with choice of reference sample panels from known infected/exposed or known uninfected animals selected for evaluating the diagnostic sensitivity (DSe) and diagnostic specificity (DSP) of the assay. This is particularly difficult because the degree to which the reference animals represent all of the host and environmental variables in the population targeted by the assay has a major impact on the confidence of test-result interpretation. For example, experienced serologists are aware that an assay, validated by using serum samples from northern European cattle, may not give valid results for the distinctive populations of cattle in Africa. Diagnostic performance of the
assay is further complicated when sample panels of known infection status are not available, often because they are impossible to obtain. In this situation, DSe and DSp may be estimated, in certain circumstances, by use of latent class models (Enoe et al., 2000; Greiner & Gardner, 2000; and Appendix 1.1.4.5).

THE CRITERIA OF ASSAY DEVELOPMENT AND VALIDATION

Assay performance is affected by many factors that span from the earliest stages of assay development through the final stage of performance assessment when the test is applied to targeted populations of animals. An assay, therefore, cannot be considered validated unless a specific set of essential validation criteria (see accompanying box) have been tested and affirmed or fulfilled, either quantitatively or qualitatively. Lack of attention to any one of these criteria will likely reduce the level of confidence that an assay is fulfilling the purpose(s) for which it is intended. The first four of these criteria typically are addressed during development of the assay (the Development Pathway), and the remaining eight are evaluated during the first three stages of assay validation (the Validation Pathway) as described below.

A. ASSAY DEVELOPMENT PATHWAY

1. Definition of the intended purpose(s) for an assay

The OIE Standard for Management and Technical Requirements for Laboratories Conducting Tests for Infectious Diseases (2008) states that test methods and related procedures must be appropriate for specific diagnostic applications in order for the test results to be of any relevance. In other words, the assay must be “fit for purpose”. The capacity of a positive or negative test result to predict accurately the infection or exposure status of the animal or population of animals is the ultimate consideration of assay validation. This capacity is dependent on development of a carefully optimised (Section A.2.d), and standardised (Section A.2.g) assay that, through accrual of validation data, provides less biased and more precise estimates of DSe and DSp. These estimates, along with evidence-based data on prevalence of infection in the population being tested, are the basis for providing a high degree of confidence in the predictive values of positive and negative test results. In order to insure that test results provide useful diagnostic inferences about animal population infection/exposure status, the validation process encompasses initial development and assay performance documentation, as well as on-going assessment of quality control and quality assurance programmes. Figure 1 shows the assay validation process, from assay design through the development and validation pathways to implementation, deployment, and maintenance of the assay.

As outlined in the background information in Certification of diagnostic assays on the OIE website (www.oie.int), the first step is selection of an assay type that likely can be validated for a particular use.

a) Fitness for purpose

The most common purposes are to:

1) Demonstrate freedom from infection in a defined population (country/zone/compartment/herd) (prevalence apparently zero):
   1a) ‘Free’ with and/or without vaccination,
   1b) Re-establishment of freedom after outbreaks

2) Certify freedom from infection or presence of the agent in individual animals or products for trade/movement purposes.

3) Eradication of disease or elimination of infection from defined populations.

2 For detail on terms, see glossary in the OIE Quality Standard and Guidelines for Veterinary Laboratories: Infectious Diseases (World Organisation for Animal Health [OIE], 2008).
3 This is a specific interpretation of the more generally stated requirements of the ISO/IEC 17025:2005 international quality standard for testing laboratories (ISO/IEC, 2005). The OIE Standard further states that in order for a test method to be considered appropriate, it must be properly validated and that this validation must respect the principles outlined in the validation chapters of the Terrestrial and Aquatic Manuals.
4) Confirmatory diagnosis of suspect or clinical cases (includes confirmation of positive screening test).

5) Estimate prevalence of infection or exposure to facilitate risk analysis (surveys, herd health status, disease control measures).

6) Determine immune status of individual animals or populations (post-vaccination).

These purposes are broadly inclusive of many narrower and more specific applications of assays (see Appendices for each assay type for details). Such specific applications and their unique purposes need to be clearly defined within the context of a fully validated assay.

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<table>
<thead>
<tr>
<th>Assay Development Pathway</th>
<th>Assay Validation Pathway</th>
<th>Validation Status Retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Essential prerequisites</td>
<td>Reagents and controls</td>
<td>Replacement of depleted reagents</td>
</tr>
<tr>
<td>Study design and protocol</td>
<td>Optimisation, Robustness, Calibration to Standards</td>
<td>Assay-modifications and re-validation</td>
</tr>
<tr>
<td>Analytical specificity</td>
<td>Repeatability and preliminary Reproducibility</td>
<td>Comparability assessments</td>
</tr>
<tr>
<td>Analytical sensitivity</td>
<td>Candidate test compared with standard test method</td>
<td>Monitoring and maintenance of validation criteria</td>
</tr>
<tr>
<td>Provisional recognition</td>
<td>Samples from reference animals</td>
<td>Monitoring precision and accuracy</td>
</tr>
<tr>
<td>STAGE 1</td>
<td>Samples from experimental animals (where used)</td>
<td>Daily in-house QC</td>
</tr>
<tr>
<td>Diagnostic specificity</td>
<td>Reproducibility</td>
<td>Proficiency testing</td>
</tr>
<tr>
<td>Diagnostic sensitivity</td>
<td>Ruggedness</td>
<td></td>
</tr>
<tr>
<td>Cut-off determination</td>
<td>STAGE 2</td>
<td></td>
</tr>
<tr>
<td>STAGE 2</td>
<td>STAGE 3</td>
<td></td>
</tr>
<tr>
<td>Analytical characteristics</td>
<td>Reproducibility</td>
<td></td>
</tr>
<tr>
<td>Diagnostic characteristics</td>
<td>Reproducibility</td>
<td></td>
</tr>
<tr>
<td>STAGE 4</td>
<td>Implementation</td>
<td></td>
</tr>
<tr>
<td>Reproducibility</td>
<td>Reference standards selected</td>
<td></td>
</tr>
<tr>
<td>Ruggedness</td>
<td>International recognition (OIE)</td>
<td></td>
</tr>
<tr>
<td>Interpretation of test results</td>
<td>Deployment to other labs</td>
<td></td>
</tr>
<tr>
<td>Definition of evaluation panel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Select collaborating labs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Definition of evaluation panel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Provisional recognition</td>
<td>STAGE 1</td>
<td></td>
</tr>
<tr>
<td>STAGE 1</td>
<td>Repeatability and preliminary Reproducibility</td>
<td></td>
</tr>
<tr>
<td>Analytical specificity</td>
<td>Candidate test compared with standard test method</td>
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</tr>
<tr>
<td>Analytical sensitivity</td>
<td>Samples from reference animals</td>
<td></td>
</tr>
<tr>
<td>Provisional recognition</td>
<td>Samples from experimental animals (where used)</td>
<td></td>
</tr>
<tr>
<td>STAGE 2</td>
<td>STAGE 3</td>
<td></td>
</tr>
<tr>
<td>Diagnostic specificity</td>
<td>Reproducibility</td>
<td></td>
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Figure 1. The assay development and validation pathways with assay validation criteria highlighted in bold typescript within shadowed boxes.

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b) **Fitness for use**

While this chapter deals with validation and fitness for purpose from a scientific perspective, it should also be noted that other practical factors might impact the relevance of an assay with respect to its intended application. These factors include not only the diagnostic suitability of the assay, but also its acceptability by scientific and regulatory communities, acceptability to the client, and feasibility given available laboratory resources. An inability to meet operational requirements of an assay also may make it unfit for its intended use. Such requirements may include performance costs, equipment availability, level of technical sophistication and interpretation skills, kit/reagent availability, shelf life, transport requirements, safety, biosecurity, sample throughput, turn-around times for test results, aspects of quality control and quality assurance, and whether the assay can practically be deployed to other laboratories. Test kits used in the
field are highly desirable from an ease-of-use viewpoint, but because they are performed outside the confines of a controlled laboratory environment, they require added precautions to maintain fitness for purpose (Crowther et al., 2006).

2. Assay development – the experimental studies

a) Essential prerequisites: factors that impact assay validation

i) Quality assurance

Whether developing assays in the laboratory or performing analyses of clinical material, the objective is to produce data of high quality. This requires that key requirements have to be fulfilled within the laboratory (see Chapter 1.1.4 Quality management in veterinary testing laboratories) The establishment of quality assurance (QA) and quality control (QC) systems is essential, i.e. a set of quality protocols, including the use of assay control samples that ensure that the system is working properly and confirms data reproducibility and quality. QA and QC systems, together with trained and competent personnel, have already been established in many laboratories world-wide.

ii) Equipment selection

Equipment that is not maintained and calibrated can be a major impediment to achieving a quality assay. Apparatus (freezers, heating blocks, incubators, refrigerators, optical colorimeters, thermocyclers, plate washers, pipettes, etc.) must be calibrated according to the laboratory’s quality assurance protocols. Examples of this need include robotics used for automation of entire assays, or parts thereof, for routine diagnostic processing. It is not sufficient to assume that robotic extraction of nucleic acid, for example, is equivalent to previously used manual extraction methods or that an automated ELISA plate washer provides uniform washing among wells of the plate. The instrument must be calibrated and the protocol validated to confirm performance efficiency and to ensure that cross-contamination does not occur in NAD systems or that washing is adequate for all wells in a plate. (See Appendices on best practices for more details.)

iii) Selection and integrity of samples

Selection, collection, preparation and management of samples are critical variables in designing, developing, and validating an assay. Other variables such as transport, chain of custody, tracking of samples, and the laboratory information management system are also critical sources of variation/error that become especially important when the assay is implemented for routine testing. Integrity of experimental outcomes during assay development and validation is only as good as the quality of the samples used in experimentation or routine diagnostic testing. Anticipating the factors that can negatively impact sample quality must precede launching an assay validation effort. Reference samples used in assay development and validation should be in the same matrix used in the assay (e.g. serum, tissue, whole blood) and representative of the species to be tested by the resulting assay. The reference materials should appropriately represent the range of analyte concentration to be detected by the assay. Details on proper sample collection, preparation, management, and transport are available in the Chapter 1.1.1 Collection and shipment of diagnostic specimens.

b) Test method design and proof of concept

Considerable thought and planning needs to go into designing all steps of a new assay destined for validation, or an existing assay that is being modified. Assistance is offered in Appendices to this chapter, which cover best practices for development and validation of assays for detection of various analytes (e.g. antibody, antigen, and nucleic acid detection).

i) Analyte reference samples

Development of all assays is dependent on analyte reference samples that reflect the target analyte and the matrix in which the analyte is found in the population for which the assay is intended. The reference samples may be sera, fluids or tissues that contain the analyte of interest or a genomic construct consistent with the target analyte. These reference materials are used in experiments conducted throughout the development process and carried over into the validation of the assay.

Operating range of the assay

During development of the assay, the lower and upper detection limits are established. To formally establish this range, a high positive reference sample is selected. (Ideally, this sample will be the same one from among

Analyte reference samples, containing the analyte of interest in varying concentrations, are useful in developing and evaluating the candidate assay’s validation criteria.

Operating range of an assay: an interval of analyte concentrations (amounts) over which the method provides suitable accuracy and precision.
the three samples described under “Optimisation” below). This high positive sample is serially diluted to extinction in an analyte-negative matrix of the same constitution as the sample matrix of samples from animals in the population targeted by the assay. The results are plotted as a response-curve, with the response (e.g. OD, Ct, etc.) a function of analyte concentration (amount). The curve, establishes the range of the assay, which is the interval between the upper and lower concentration (amounts) of analyte in the sample for which a suitable level of precision\(^4\) and accuracy has been demonstrated. For most diagnostic assays, the response is the result of interaction of the analyte with an antibody or other binding reagent. These are known as ligand binding assays (LBAs). The typical calibration curve for LBAs is sigmoidal in shape, with a lower asymptote (asymptote) near the background response (non-specific binding) and an upper asymptote near the maximum response. Typically, LBA data are transformed to approximate a linear relationship between response and concentration. This transformation simplifies interpolation of data using linear regression analysis, but with the disadvantage of introduced bias. As linearisation is imperfect leading to compromised estimates of accuracy and precision, numerous data-fitting algorithms have been applied to experimental calibration curve data from LBAs (Findlay & Dillard, 2007). The currently accepted reference model for calibration of LBAs is the 4-parameter logistic model, which usually optimises accuracy and precision over the maximum usable calibration range (Findlay & Dillard, 2007). Such transformations are now more practical for the general user because of many user-friendly statistical software programs available on the internet.

d) Optimisation

It is useful to select at least three well-defined reference samples, representing the analyte ranging from high positive to negative (e.g., negative, low- and high-positive). These samples ideally should represent known infected and uninfected animals from the population that eventually will become the target of the assay once it is validated. Obtaining such reference samples, however, is not always possible, particularly for nucleic acid and antigen detection assays. The alternative of preparing reference samples spiked with cultured agents or positive sera is inferior because the matrix of field samples may be very different from spiked-sample matrix. But, when no other alternative exists, spiking a sample with a known amount of the analyte derived from culture, or diluting a high positive serum in negative serum of the same species may be all that is available. In either case, it is imperative that the matrix, into which analyte is placed or diluted, is identical to, or resembles as closely as possible the samples that ultimately will be tested in the assay. Ideally, reference samples have been well characterised by one or preferably at least two alternate methodologies. These samples can be used in experiments to determine if the assay is able to distinguish between varying quantities of analyte, and for optimising the reagent concentrations and perfecting the protocol. In principle, for all assay types, it is highly desirable to prepare and store a sufficient amount of each reference sample in aliquots for use in every run of the candidate assay as it is evaluated through the entire development and validation process. Switching reference samples during the validation process introduces an intractable variable that can severely undermine interpretation of experimental data and, therefore, the integrity of the development and validation process.

The labour-intensive process of optimising an assay is fundamental and critical to achieving a quality assay. Scientific judgment and use of best scientific practices provided in accompanying Appendices to this chapter are the best assets to guide optimisation of all elements of an assay. The approach outlined provides a solid foundation for development of an assay that fulfils the criteria of ‘robustness’ and ‘ruggedness’ when used over extended periods of time within a laboratory or when implemented in other laboratories. Often, prototype assays are developed using reagents and equipment at hand in the laboratory. However, if the assay is intended for routine diagnostic use in multiple laboratories, optimisation becomes extremely critical. Every chemical and buffer formulation must be fully described. All reagents must be defined with respect to purity and grade (including water). Acceptable working ranges must be established for parameters such as pH, molarity, etc. Likewise for biologicals, standards for quality, purity, concentration and reactivity must be defined. Shelf lives and storage conditions must also be considered for both chemicals and biologicals. Acceptable ranges for reaction times and temperatures need also be established. Essential equipment

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\(^4\) Precision may be evaluated in several ways by testing the same replicated sample: 1) within a plate or plates in a run of the assay, 2) between plates run concurrently within a run of the assay, 3a) between assay runs at different times in the same day or on different days under similar conditions, 3b) between assay runs on different days with different operators, 4) between laboratories. In this chapter, precision categories 1–3 are estimates of repeatability, and precision category 4 is synonymous with reproducibility. Levels 3a and 3b are also known as intermediate precision.
critical to assay performance must be described in detail, including operational specifications and calibration. Process (quality) control is often an add-on at the end of assay development but it should be an integral part of optimisation from the very beginning. In addition to the above, downstream aspects such as data capture, manipulation and interpretation may also require standardisation and optimisation. Finally, all of these parameters, once optimised, must be fully described in the test method protocol.

In some assay types, a correct assay result is fully dependent on getting a particular step in the testing process correct, requiring special attention during optimisation. A case in point is nucleic acid extraction from the sample. Both commercial (robotic, spin columns, and magnet-based extractions, etc.) and standard chemistry-based methods are used for DNA or RNA extraction. It is crucial to determine the most reproducible and efficient extraction method through optimisation experiments. Extraction needs to be optimised for every type of tissue that may be targeted by the assay. If the method of extraction is changed, at a minimum, comparable efficiency of extraction should be demonstrated (see Section B.6 below and associated Appendix 1.1.4.6 for additional information on establishing comparability when reagents are changed).

A variety of analyte reference samples and other process controls that are routinely included in any assay system are identified in the following sections. These provide critical assay monitoring functions that require special attention during assay optimisation. In addition, proper preparation and storage of all biological reagents and reference materials must be heeded to ensure stability (see chapter 1.1.1).

During experimentation to optimise the assay, take note of assay parameters that have a narrow range in which they perform optimally, as these are the critical points that may affect an assay’s robustness (see Section A.2.f).

e) Inhibitory factors in sample matrix

Generally, for antibody detection in serum, assays are rather resistant to inhibitory factors, with the exception of certain assays, e.g. toxic factors in viral neutralisation assays, or when endogenous substances found in certain sample types inhibit enzymatic reactions in ELISAs. For nucleic acid detection, sample matrices including blood, serum, body tissues, and swab samples allow for easy extraction of target nucleic acids, while faeces, autolysed tissues and semen samples can be more difficult to handle because of the presence of factors which can inhibit downstream assays such as PCR.

f) Robustness

Robustness refers to an assay’s capacity to remain unaffected by minor variations in test situations that may occur over the course of testing in a single laboratory. It is assessed by deliberate variations in method parameters (International Conference on Harmonisation, 2005). Assessment of robustness should begin during assay development and optimisation stages. The deliberate variations in method parameters may be addressed in experiments after optimal conditions for an assay are established. However, when multi-factorial titrations of reagents are used for optimising the assay, indications of a compromised robustness may surface. If slight differences in conditions or reagent concentrations cause unacceptable variability, the assay most likely will not be robust. Early knowledge of this situation elicits a critical decision point for determining whether to continue with validation of the assay would be worthwhile, because if an assay is not robust within one laboratory under rather ideal conditions, it is unlikely to exhibit ruggedness (reduced reproducibility) when transferred to other laboratories (ruggedness is addressed in Section 4).

The factors most likely to affect assay robustness are quantitative (continuous) such as pH and temperature; qualitative (categorical) such as batch of reagents or brand of microtitre plates; and mixture-related such as aqueous or organic matrix factors (Dejaegher & Vander Heyden, 2006). For ligand-binding assays (LBAs), lack of robustness is not only due to less-than-optimal concentration/amount of the bio-reagent specified in the method, but may also be due to the intrinsic characteristics of the biological reagent (e.g. monoclonal antibody affinity or polyclonal antibody avidity and/or valency). Robustness, therefore, particularly of LBA-based assays, may be affected by systematic and/or random errors (Thompson et al., 2002).

Robustness testing is demonstrated on a method-by-method basis. All critical reagents are identified and subjected to a factorial assessment which compares all possible combinations of reagents. For example, in antibody detection by ELISA, factors may include concentration of antigen bound to the solid phase, conjugate dilution and several test sera representing the operating range of assay. The response of the assay with respect to these small changes shall not result in unacceptable variability. Alternatively, robustness can be demonstrated through the application of factorial design experiments (Dejaegher & Vander Heyden, 2006; Youden & Steiner, 1987).
Robustness is further verified during Stage 1 of assay validation. When the optimised test is first run under routine laboratory conditions, this practical measure of robustness is referred to as repeatability (see Section 2.a) and it is continually monitored as part of process control procedures for the duration of the life of the assay (see Section 6.a).

g) Calibration of the assay to standard reagents
   i) International and national analyte reference standards
      Ideally, international reference standards, containing a known concentration of analyte, are the reagents to which all assays are standardised. Such standards are prepared and distributed by international reference laboratories. National reference standards are calibrated by comparison with an international standard reagent whenever possible; they are prepared and distributed by a national reference laboratory. In the absence of an international reference standard, a national reference standard becomes the standard of comparison for the candidate assay. These standard reagents are highly characterised through extensive analysis, and preferably the methods for their characterisation, preparation, and storage have been published in peer-reviewed publications.

   ii) In-house standard reagent
      An in-house reference standard generally has the highest metrological quality available at a given location in a given organisation, and is calibrated against an International or National standard. In the absence of either of these calibrators and to the extent possible, the in-house standard is highly characterised in the same manner as international and national analyte standards. This local in-house standard therefore becomes the best available standard, and is retained in sufficient aliquotted volumes for periodic use as the standard to which working standards are calibrated.

   iii) Working standard reagent
      One or more working standard reagent(s), commonly known as analyte or process controls, are calibrated to an international, national, or in-house standard reagent, and are prepared in large quantities, aliquotted and stored for routine use in each diagnostic run of the assay.

h) ‘Normalising’ test results to a working standard(s)
   Due to the inherent variation in raw test results that are often observed between test runs of the same assay or among laboratories using the same or similar assays, it is virtually impossible to directly compare (semi-) quantitative data. To markedly improve the comparability of test results both within and between laboratories, one or more working standard reagent(s) are included in each run of an assay. Raw test values for each test sample can then be converted to units of activity relative to the working standard(s) by a process called ‘normalisation’ [not to be confused with transformation of data to achieve a ‘normal’ (Gaussian) distribution]. The ‘normalised’ values may be expressed in many ways, such as a per cent of a positive control (e.g. in an ELISA), or as a concentration or titre of an analyte derived from a standard curve, or as a number of targeted genomic copies also derived from a standard curve of Ct (cycle threshold) values for real time PCR. It is good practice to include working standards [or at least a reasonably well characterised sample(s) in all runs of the assay during assay development and validation because this allows ‘normalisation’ of data which provides a valid means for direct comparison of results between runs of an assay. Automated assay systems may calculate and report ‘normalised’ data by, for example, a standard curve, or by reporting the cycle number at which the cycle threshold is exceeded as in real-time PCR. For more information, see Appendices 1.1.4.1 and 1.1.4.3.

B. ASSAY VALIDATION PATHWAY

1. Definition of a validated assay
   Validation is a process that determines the fitness of an assay that has been properly developed, optimised and standardised for an intended purpose. Validation includes estimates of the analytical and diagnostic performance characteristics of a test. In the context of this document, an assay that has completed the first three stages of the validation pathway (Figure 1), including performance characterisation, can be designated as “validated for the original intended purpose(s)"

   To retain the status of a validated assay, however, it is necessary to assure that the assay as originally validated consistently maintains the performance characteristics as defined during validation of the assay (see Section 6 below). This can be determined in a quality assurance program characterised by carefully monitoring the assay’s daily performance, primarily through precision and accuracy estimates for internal controls, and by scheduled external proficiency testing. Should the assay cease to produce results consistent with the original validation data,
the assay would be rendered unfit for its intended purpose. Thus, a validated assay must be continuously assessed to assure it maintains its fitness for purpose.

2. Stage 1 – Analytical performance characteristics

Ideally, the design for studies outlined in the following sections should be done with assistance of a statistician. It is possible to design experiments that efficiently provide information on likely within- and among-laboratory sources of variation in assay precision (see footnote 4 in Section A.2.c, above) which will define the performance characteristics of the assay. Stage 1 studies for repeatability, reproducibility and assessment of analytical sensitivity (limit of detection) should be performed in a blinded fashion with random selection of samples. The choice of organisms, strains or serotypes to assess analytical specificity should reflect current knowledge and therefore inform the best possible experimental design for targeting specific analytes.

a) Repeatability

Repeatability is estimated by evaluating variation in results of replicates from a minimum of three (preferably five) samples representing analyte activity within the operating range of the assay. Each of these samples is then aliquoted into individual vessels as three identical replicates of the original sample containing the original analyte and matrix concentration. Each replicate is then run through all steps of the assay, including creating the working dilution, as though it were a test sample derived from the population targeted by the assay. It is not acceptable to prepare a final working dilution of a sample in a single tube from which diluted aliquots are pipetted into reaction vessels, or to create replicates from one extraction of nucleic acid rather than to extract each replicate before dilution into the reaction vessels. Such ‘samples’ do not constitute valid replicates for repeatability studies. Between-run variation is determined by using the same samples in multiple runs (approximately 20) involving two or more operators, done on at least five separate days. The variation in replicate results can be expressed as standard deviations, confidence intervals, or other possible options (see Appendix 1.1.4.4 on measures of uncertainty for assessments of repeatability).

b) Analytical specificity (ASp)

Analytical specificity distinguishes between the target analyte and other components in the assay in at least three distinctive ways. These are described as the selectivity, exclusivity, and inclusivity of the assay.

- Selectivity refers to the extent to which a method can accurately quantify the targeted analyte in the presence of: 1) interferents such as matrix components (e.g. inhibitors of enzymes in the reaction mix); 2) degradants (e.g. toxic factors); 3) non-specific binding of reactants to a solid phase, (e.g. conjugate of an ELISA adsorbed to well of microtiter plate); 4) antibodies to vaccination which maybe confused with antibodies to active infection. Such interferents may cause falsely reduced or elevated responses in the assay that negatively affect its analytical specificity. Vessman et al. (2001) is a useful overview of selectivity as defined for analytical chemistry from which a modification described herein was deduced for application to diagnostic tests.

- Exclusivity is the capacity of the assay to detect an analyte or genomic sequence that is unique to a targeted organism, and excludes all other known organisms that are potentially cross-reactive. This would also define a confirmatory assay.

- Inclusivity is the capacity of an assay to detect several strains or serovars of a species, several species of a genus, or a similar grouping of closely related organisms or antibodies thereto. It characterises the scope of action for a screening assay.

After eliminating interferents to the extent possible, the next step is to test a panel of samples appropriate for evaluating either inclusivity, exclusivity, or both, depending on the intended purpose of the assay.

ASp applies to both direct and indirect methods of analyte detection. If exclusivity is required, field samples should be obtained from animals infected with genetically-related, non-pathogenic organisms, but this may prove difficult or even impossible. In such cases, cultivated organisms can be used in direct detection methods, or serum from animals exposed experimentally by natural routes for indirect detection methods. Acceptable cross-reactivity is largely dependent on the intended purpose of the test and the prevalence of the cross-reactive organisms/analytes in the target population samples – which must be determined for each case (see Appendix 1.1.4.5 for more detail). For PCR, it is useful to perform computer simulation studies as an adjunct to laboratory assessment of ASp; however, such studies are not sufficient by themselves to evaluate ASp.
A factor unique to viral antibody detection is the possible antibody response of animals to carrier proteins found in vaccines – another type of interferent that may negatively affect selectivity. If such proteins are also present in the solid phase antigen on ELISA plates, they may bind antibody developed to vaccine carrier proteins and give false-positive results (lack of exclusivity in the assay). Use of vaccine preparations as antigens in ELISAs is, therefore, not recommended. See Appendix 1.1.4.1 for specific practices to determine ASp.

c) **Analytical sensitivity (ASe)**

Analytical sensitivity is synonymous with the lower limit of detection (LOD) of an analyte in an assay. For direct-detection assays, this may be expressed as the number of genome copies, infectious dose, colony-forming units, plaque forming units, etc., of the agent that can be detected and distinguished from the result of a matrix background. Most commonly, this is expressed as a number of copies, complement-fixing units or plaque-forming units giving at least 50% positive results among the replicates of a sample for a specified volume or weight (see Appendix 1.1.4.5 for detail on LOD determination). For indirect detection assays, it is the least amount of antibody detected, usually, the penultimate dilution of sample in which the analyte is indistinguishable from the activity of a sample matrix control.

If the intended purpose is to detect low levels of analyte or subclinical infections and it is difficult to obtain the appropriate reference materials, for example samples from early stages of the infection process, it may be useful to determine comparative analytical sensitivity by running a panel of samples on the candidate assay and on another independent assay. This would provide a relative comparison of analytical sensitivity between the assays, but care must be taken in choosing the independent assay used in the comparison to ensure that the analytes being detected (if different) demonstrate the same type of pathogenic profile in terms of time of appearance after exposure to the infectious agent, and relative abundance in the test samples chosen.

Where a new, more sensitive test is developed, it may be necessary to test serial samples taken from infected animals early after infection, on through to the development clinical or fulminating disease, and to run these in parallel with previously used tests to demonstrate the increased sensitivity. This would also provide a temporal comparison of the earliest point of detection relative to the pathogenesis of the disease.

d) **Standard test method for comparison with the candidate assay test method**

There are situations where it is not possible or desirable to continue to Stage 2 of the Validation Pathway because appropriate samples from the target population are scarce and animals are difficult to access (such as for exotic diseases). However, a small but select panel of highly characterised test samples representing the range of analyte concentration should be run in parallel on the candidate assay method and by the standard method if it exists.

e) **Analytical accuracy of adjunct tests or procedures**

Some test methods or procedures may be qualified for use as analytical tools in the diagnostic laboratory. These usually are secondary adjunct tests or procedures that are applied to an analyte that has been detected in a primary assay. The purpose of such analytical tools is to further characterise the analyte detected in the primary assay. Examples of such adjunct tests include virus neutralisation to type an isolated virus, or molecular sequencing to confirm a real-time PCR test result. Pathogenicity indices, haemagglutination inhibition, drug resistance determinations, etc. are other examples where adjunct tests or procedures are performed, either independent of, or as part of a primary assay.

Such adjunct tests must be validated for analytical performance characteristics (Sections A.2 through B.2.c., above), but differ from routine diagnostic tests because they do not require validation for diagnostic performance characteristics (Sections B.3 through B.5, below). The analytical accuracy of these tools may be determined by comparison with a reference reagent standard, or by characteristics inherent in the tool itself (such as endpoint titration). In all of these examples, the targeted analyte is further characterised quantitatively or qualitatively by the analytical tool.

f) **Preliminary evaluation of reproducibility**

Preliminary reproducibility estimates of the candidate assay may be useful at this time in the validation process, where only a small panel of highly characterised samples is available. This panel could be used for a limited evaluation of reproducibility to enhance provisional acceptance status for the assay. The candidate
test method is then duplicated in laboratories at one or more different institutes, and the panel of samples is evaluated using the candidate assay in each of these laboratories, using the same protocol, same reagents as specified in the protocol, and comparable equipment. This is a scaled-down version of Stage 3 of assay validation.

g) **Provisional assay recognition**

Experience has shown that the greatest obstacle for continuing through Stage 2 of the Validation Pathway is the number of defined samples required to calculate DSe and DSp (see requirements for Stage 2, Diagnostic Performance, below). The formula is well known and tables are available for determining the number of samples required to estimate various levels of DSe and DSp, depending on the amount of allowable error and the level of confidence in the estimates (Table 1 and Jacobson, 1998). The formula assumes that the myriad of host/organism factors that may affect the test outcome are all accounted for. Since that assumption may be questionable, the estimated sample sizes are at best minimal. For a disease that is not endemic or widespread, it may be impossible, initially, to obtain the number of samples required, but over time, accrual of additional data will allow adjustment of the threshold or if no adjustment is needed, enhance confidence in the estimates.

Historical precedent would suggest that assays were generally the product of laboratory experiments with an emphasis on analytical sensitivity and analytical specificity, and evaluation of panels of field samples was nominal. Such bench validation for bovine spongiform encephalopathy (BSE) is a classical example where positive field samples were not available. Nevertheless, during extended periods of usage in diagnostic settings, such tests have undergone adjustments based on empirical evidence to reduce false-positive and false-negative results. For some of the BSE rapid tests, adjustments had to be made in the cut-off to reduce false-positive results apparent in early implementation. Bench validation provided a level of confidence in diagnostic performance that was adequate for conditional diagnostic use as determined by national authorities. But, it must never become a replacement for a full field validation. Therefore bench validation of diagnostic assays can only offer provisional recognition with anticipation that full field validation will follow.

A provisional recognition of an assay by state or national authorities recognises that the assay has not been evaluated for diagnostic performance characteristics. As such, the laboratory should develop and follow a protocol for adding and evaluating samples, as they become available, to fulfill this requirement. Ideally, this process should be limited to a specific timeframe in which such an accrual would be directed toward fulfilling Stages 2 and 3 of the validation pathway. This concept should be limited to emergency situations where rapid introduction of new tests is deemed essential by authorities. There may be other situations where bilateral trade agreements, based on tests (e.g. standard test methods) that have not been fully validated within a given country, are mutually accepted. In exceptional cases for rare diseases where no other assay option exists, provisional recognition may be allowed, but reporting of results should include a statement of the provisional nature of the validation of the assay. In all cases, sound evidence must exist for preliminary estimates of DSp and DSe based on a small select panel of well-characterised samples containing the targeted analyte.

### 3. Stage 2 – Diagnostic performance of the assay

**Diagnostic Sensitivity** and **Diagnostic Specificity**. Estimates of DSe and DSp are the primary performance indicators established during validation of an assay. These estimates are the basis for calculation of other parameters from which inferences are made about test results (e.g. predictive values of positive and negative test results). Therefore, it is imperative that estimates of DSe and DSp are as accurate as possible. Ideally, they are derived from testing a panel of samples from reference animals, of known history and infection status relative to the disease/infection in question and relevant to the country or region in which the test is to be used. Receiver operating characteristic curve analysis is a useful adjunct to estimation of DSe and DSp because it assesses the global accuracy of a quantitative diagnostic test across possible assay values (Greiner & Gardner, 2000; Greiner et al., 2000; Zweig & Campbell, 1993). This approach is described in-depth in Appendix 1.1.4.5.

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5 Provisional recognition does not imply certification by the OIE. It does, however, recognise an informed decision of authorities at local, state, national or international levels of their conditional approval of a partially validated assay, usually for a time-limited use in emergency situations or as the basis for bi-lateral agreements between countries that choose to accept results from such an assay for trade purposes.
A sampling design must be chosen that will allow estimation of DSe and DSp. The designated number of known positive and known negative samples will depend on the likely values of DSe and DSp of the candidate assay and the desired confidence level for the estimates (Table 1 and Jacobson, 1998). An abbreviated Table 1 provides two panels of the theoretical number of samples required, when either a 5% or 2% error is allowed in the estimates of DSe or DSp. Comparison of a 5% vs 2% error shows a considerable reduction in the number of samples required. A rather large number of samples is required to achieve a very high confidence for DSe and DSp when a minimal amount of error in the estimate is desired. Logistical and financial limitations may require that less than an optimal number of samples will be evaluated. However, by reducing the DSe and DSp confidence levels to less than 90% usually would not be recommended. Sample size also may be limited by the fact that reference populations and gold standards (perfect reference standards) may be lacking (see Appendix 1.1.4.5 for further detail). It may, therefore, be necessary to use a sub-optimal number of samples initially. It is, however, highly desirable to enhance confidence and reduce allowable error in the DSe and DSp estimates by adding more samples as they become available.

Table 1. Theoretical number of samples from animals of known infection status required for establishing diagnostic sensitivity (DSe) and specificity (DSp) estimates with known confidence

<table>
<thead>
<tr>
<th>Estimated DSe or DSp</th>
<th>2% error allowed in estimate of DSe and DSp</th>
<th>5% error allowed in estimate of DSe and DSp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>75%</td>
<td>80%</td>
</tr>
<tr>
<td>90%</td>
<td>257</td>
<td>369</td>
</tr>
<tr>
<td>92%</td>
<td>210</td>
<td>302</td>
</tr>
<tr>
<td>94%</td>
<td>161</td>
<td>232</td>
</tr>
<tr>
<td>95%</td>
<td>136</td>
<td>196</td>
</tr>
<tr>
<td>96%</td>
<td>110</td>
<td>158</td>
</tr>
<tr>
<td>97%</td>
<td>83</td>
<td>119</td>
</tr>
<tr>
<td>98%</td>
<td>56</td>
<td>80</td>
</tr>
<tr>
<td>99%</td>
<td>28</td>
<td>41</td>
</tr>
</tbody>
</table>

Per cent error allowed in the estimate of DSe or DSp = 2% in the left panel and 5% in the right panel. For the number of samples required for 1%, 3%, and 4% allowable error in the estimate of DSe and DSp, multiply the number of samples in the left panel of the table by a factor of 4.0, 0.44, and 0.25, respectively.

The following are examples of reference populations and methodologies that may aid in determining performance characteristics of the test being validated.

a) Reference animal populations

Ideally, selection of reference animals requires that important host variables in the target population are represented in animals chosen for being infected with or exposed to the target agent, or that have never been infected or exposed. The variables to be noted include but are not limited to species, age, sex, breed, stage of infection, vaccination history, and relevant herd disease history.

i) Negative reference samples: True negative samples, from animals that have had no possible infection or exposure to the agent, may be difficult to locate. It is often possible to obtain these samples from countries that have eradicated or have never had the disease in question. Such samples are useful as long as the targeted population for the assay is similar to the sample-source population.

ii) Positive reference samples: It is generally problematic to find sufficient numbers of true positive reference animals, as determined by isolation of the organism. It may be necessary to resort to samples from animals that have been tested by another test such as a nucleic acid detection system.

iii) Samples from animals of unknown status: See Appendix 1.1.4.5, and Section 3.a.iii of this chapter for a discussion of latent class models.
b) Reference animal infection status

i) So-called “Gold Standard” model

The term “gold standard” is commonly used to describe any standard of comparison, but it should be limited to methods or combination of methods that unequivocally classify animals as infected/exposed or uninfected. Some isolation methods themselves have problems of repeatability and analytical sensitivity, so are not truly gold standards particularly for purportedly negative samples. When the so-called reference standard is imperfect, which is the common scenario for most ante-mortem tests, estimates of DSe and DSp for the candidate assay may be compromised because the error in estimates obtained by comparison to the relative standard is carried over into the estimates for the candidate assay. Indeed, when using imperfect reference assays, the DSe and DSp performance estimates of the candidate assay will be flawed and often overestimated.

NAD assays may be more sensitive and specific than existing gold standard methods, which render the established gold standard as not suitable for use as a comparison. If the NAD is more sensitive than the gold standard, an apparent lower relative specificity will be misleading. This problem may be partially resolved by assessing sample derivation, clinical history and sequencing of any PCR products to confirm analyte identity.

ii) Latent-class models

Latent-class models (Branscum et al., 2005; Enoe et al., 2000; Georgiadis et al., 2003; Hui & Walter, 1980) do not rely on the assumption of a perfect reference test but rather estimate the accuracy of the candidate test and the reference standard with the joint test results. Because these statistical models are complex and require critical assumptions, statistical assistance should be sought to help guide the analysis and describe the sampling from the target population(s), the characteristics of other tests included in the analysis, the appropriate choice of model and the estimation methods based on peer-reviewed literature (for details see Appendix 1.1.4.5 on statistical considerations).

c) Sera obtained sequentially from experimentally infected or vaccinated animals are useful for determining the kinetics of antibody responses or the presence/absence of antigen or organisms in samples from such animals. However, multiple serially acquired pre- and post-exposure results from individual animals are not acceptable for establishing estimates of DSe and DSp because the statistical requirement of independent observations is violated. Only single time-point sampling of individual experimental animals is acceptable. Also, for indirect methods of analyte detection, exposure to organisms under experimental conditions, or vaccination may elicit antibody responses that may not be quantitatively and qualitatively typical of natural infection in the target population (Jacobson, 1998). The strain of organism, dose, and route of administration to experimental animals are examples of variables that may introduce error when extrapolating DSe and DSp estimates to the target population. For these reasons, validation of an assay should not be based solely on samples from experimental animals.

d) Threshold (cut-off) determination

To obtain DSe and DSp estimates of the candidate assay, the test results first must be reduced to categorical (positive, negative, or intermediate) status. This is accomplished by insertion of one or two cut-off points (threshold or decision limits) on the continuous scale of test results. The selection of the cut-off(s) should reflect the purpose of the assay and its application, and must support the required DSe and DSp of the assay. Options and descriptive methods for determining the best way to express DSe and DSp are available (Branscum et al., 2005; Georgiadis et al., 2003; Greiner et al., 1994; 2000; Jacobson, 1998; Zweig & Campbell, 1993; and Appendix 1.1.4.5 on statistical considerations). If considerable overlap occurs in the distributions of test values from known infected and uninfected animals, it is difficult to select a single cut-off that will accurately classify these animals according to their infection status. Rather than a single cut-off, two cut-offs can be selected that define a high DSe (e.g. inclusion of 99% of the values from infected animals), and a high DSp e.g. 99% of the values from uninfected animals). The values that fall between these percentiles may be classified as intermediate (see box), and would require testing by a confirmatory assay, retesting for detection of seroconversion, or sequencing for identity.

The main difficulty in establishing cut-offs based on diagnostic performance characteristics is the lack of availability of the required number of well-characterised samples. Alternatives are discussed in Section B.2.g. on provisional acceptance of an assay for enhancement of data to enhance estimates of DSe and DSp.

Threshold and cut-off are considered to be synonymous. A cut-off is the test value selected for distinguishing between negative and positive results on a continuous scale of test values. Intermediate, inconclusive, suspicious, or equivocal are terms used synonymously for a zone of test values between the positive and negative cut-offs.
e) Calculation of DSe and DSp based on test results of reference sera

A typical method for determining DSe and DSp estimates is to test the reference samples in the new assay, and cross tabulate the categorical test results in a 2 x 2 Table. In a hypothetical example, assume the test developer decided that estimated DSe and DSp for the new assay should be 97% and 99%, respectively, with a desired confidence of 95% for both estimates. The amount of allowable error in the estimates was set at 2%. Table 1 indicates that 279 samples from known infected animals are required for the DSe assessment, and 95 known negative samples are needed for establishing the DSp estimate. The samples were then run in the new assay. Table 2 is a hypothetical set of results and the calculated DSe and DSp estimates based on the samples tested.

Table 2. Diagnostic sensitivity and specificity estimates calculated from hypothetical set of results for samples tested from known infected and non-infected populations

<table>
<thead>
<tr>
<th>Test results</th>
<th>Number of reference samples required*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Known positive (279)</td>
</tr>
<tr>
<td></td>
<td>270</td>
</tr>
<tr>
<td>Negative</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>88</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diagnostic sensitivity*</th>
<th>Diagnostic specificity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP/(TP + FN)</td>
<td>TN/(TN + FP)</td>
</tr>
<tr>
<td>96.7% (94.0 – 98.5%)**</td>
<td>92.0% (84.3 – 96.7%)**</td>
</tr>
</tbody>
</table>

* Based on Table 1 for an assay with the following parameters:
1) Prior to testing, estimated DSe of 97% and DSp of 99%
2) 95% = required confidence in DSe and DSp estimates
3) 2% = Allowable error in the estimates of DSe and DSp
TP and FP = True Positive & False Positive, respectively
TN and FN = True Negative and False Negative, respectively
** 95% exact binomial confidence limits for DSe and DSp calculated values (see Appendix 1.1.4.5 for information on confidence limits)

In this example, the DSe estimates are as anticipated, but the DSp is much reduced from the anticipated 99%. As a consequence, the width of the confidence interval for DSp is greater than expected. Re-inspection of Table 1 indicates that 707 samples are necessary to achieve an error margin of ± 2% at a DSp of 92% but such an increase in sample size might not be feasible.

4. Stage 3 – Reproducibility and augmented repeatability estimates

Reproducibility is an important measure of the precision of an assay when used in several laboratories located in distinct or different regions or countries using the identical assay (protocol, reagents and controls). Each of at least three laboratories test the same panel of samples (blinded) containing a minimum of 20 samples, with identical aliquots going to each laboratory (see Appendix 1.1.4.7 on panels of samples). This exercise also generates preliminary data on non-random effects attributable to deployment of the assay to other laboratories – also known as ruggedness of the assay. In addition, within-laboratory repeatability estimates are augmented by the replicates used in the reproducibility studies. Measurements of precision can be estimated for both the reproducibility and repeatability data (see Appendix 1.1.4.4 on Measurement of Uncertainty for further explanation of the topic and its application).
Chapter 1.1.5. – Principles and methods of validation of diagnostic assays for infectious diseases

5. Stage 4 – Programme implementation

a) Interpretation of test results.

Predictive values of test results. An assay’s test results are most useful when the inferences made from them are accurate. Predictive values for test results need to be based on the true prevalence of exposure/infection in the targeted population. For screening assays used in surveillance of a disease-free population, false-positive results are a significant problem. For instance, an assay may have impeccable credentials (e.g. high precision and accuracy, 99% DSe and 99.9% DSp). But if the prevalence of disease is close to zero, and the assay has one false positive for every 1000 animals tested, false-positive inferences are a problem (see Jacobson, 1998 for PV tables for various estimates of DSe and DSp). Similarly, if the assay for a highly virulent disease has one false negative for every 100 animals, false negative inferences could have devastating consequences. This illustrates the critical importance of choosing diagnostic thresholds that are appropriate for the application at hand. Thresholds should be chosen to minimise the effect of false positives and/or false negatives on the predictive values of the test given its application and the prevalence of exposure/infection in the target population. It may also be prudent to have highly specific confirmatory assays to determine whether screening assay reactors are true or false positives.

For nucleic acid assays, it may be necessary to confirm NAD-positive results by sequence analysis of the amplified product (an example of an assay to assist in resolving errors due to non-specific target or primer binding).

b) International recognition

Traditionally, assays have been recognised internationally by the OIE when they are designated as prescribed or alternate tests for trade purposes. This has often been based on evidence of their usefulness on a national, regional or international basis. For test kits that have completed the certification process, the final step is listing of the test in the OIE Register. Tests listed in the Register are certified as fit for a specific purpose if they have completed Validation Stages 1, 2 and 3. The Register is intended to provide potential test users with an informed and unbiased source of information about the test and its performance characteristics for an intended purpose. The Register is available on the OIE website at: http://www.oie.int/en/our-scientific-expertise/certification-of-diagnostic-tests/background-information/

c) Deployment of the assay

Ultimate evidence of the usefulness of an assay is its successful application(s) in other laboratories and inclusion in national, regional and/or international programmes. Reference laboratories play a critical role in this process. In the natural progression of diagnostic and/or technological improvements, new assays will become the new standard method to which other assays will be compared. As such, they may progressively achieve national, regional and international recognition. As a recognised standard, these assays will also be used to develop reference reagents for quality control, proficiency and harmonisation purposes. These reference reagents may also become international standards.

An assessment of ruggedness should be repeated when the test is transferred from the development laboratory to the field, whether for use in local laboratories or in pen-side applications. Predictable changes, e.g. extremes of temperature and levels of operator experience, should be assessed as additional sources of variation in assay results that may affect estimates of ruggedness (which is mostly derived from reproducibility estimates).

6. Monitoring assay performance after initial validation

a) Monitoring the assay

A validated assay in routine use needs to be consistently monitored for repeatability through process controls to evaluate possible temporal changes in test precision and accuracy. These changes can be monitored graphically by plotting control values in control charts. Deviations from the expected performance should be investigated so corrective action can be taken if necessary. Such monitoring provides critical evidence that the assay retains its ‘validated’ designation during the implementation phase of the assay. Subsequent on-going evaluation of the assay’s performance is also essential and is usually done through assessments of precision, accuracy, and
outlier tendencies using control charts. Reproducibility is assessed through external quality control programmes such as proficiency testing.

b) Diagnostic modifications – considerations for changes in the assay

Over time, modification of the assay likely will be necessary to address changes in the analytes targeted (i.e. modification of the assay to adjust diagnostic performance) or technical modifications may be needed to improve assay efficiency or cost-effectiveness.

If the assay is to be applied in another geographical region and/or population, revalidation of the assay under the new conditions is recommended. Lineages or sub-lineages of a virus, derived from animals in different geographic locations, are known to have different target sequences or primer sites, requiring revalidation of the assay. This is especially true for NAD systems as it is very common for point mutations occur in many infectious agents (i.e. RNA viruses). Mutations, which may occur within the primer or probe sites can affect the efficiency of the assay and even invalidate the established performance characteristics. It is also advisable to regularly confirm the target sequence at the selected genomic regions for national or regional isolates of the infectious agents. This is especially true for the primer and probe sites, to ensure that they remain stable and the estimates of DSe for the assay are not compromised.

A similar situation may occur with incursion of new viral lineages into countries or regions where that viral lineage did not previously exist. In these circumstances, existing NAD assays which did not target these novel lineages may need to be modified to include primers or probes targeting these new analytes. The same would be true for typing sera used in virus neutralisation assays.

i) Technical modifications and comparability assessments

Technical modifications to a validated assay such as changes in instrumentation, extraction protocols, and conversion of an assay to a semi-automated or fully automated system using robotics will typically not necessitate full revalidation of the assay. Rather, a methods comparison study is done to determine if the relatively minor modifications of the assay affect the test results. Comparability can be established by running the modified procedure and original procedure side-by-side, with the same panel of samples in both, over several runs. The panel chosen for this comparison should represent the entire operating range of both assays. If the results from the modified procedure and originally validated method are determined to be comparable in an experiment based on a pre-specified criterion, the modified assay remains valid for its intended purpose. See Appendix 1.1.4.6 for description of experiments that are appropriate for comparability testing and Appendix 1.1.4.7 on reference sample panels.

A comparability assessment may or may not be adequate if the test is applied to a different sample matrix, e.g. validated on blood and used on another tissue in the sample species. Revalidation may be necessary if the test is designed for use in a new species.

ii) Replacement of depleted reagents

When a reagent such as a control sample is nearing depletion, it is essential to prepare and repeatedly test a replacement before such a control is depleted. The prospective control sample should be included in multiple runs of the assay in parallel with the original control to establish their proportional relationship. Whenever possible, it is important to change only one reagent at a time to avoid the compound problem of evaluating more than one variable.

c) Enhancing confidence in validation criteria

Because many host variables have an impact on the diagnostic performance of assays, it is highly desirable over time to increase the number of reference samples from animals of known infection status. This improves the precision of the overall estimates of DSe and DSp, and may allow calculations of DSe estimates by factors such as age, stage of disease, and load of organisms. New data should be included annually in relevant test dossiers.

REFERENCES


Chapter 1.1.5. – Principles and methods of validation of diagnostic assays for infectious diseases


* * *

APPENDICES TO THIS CHAPTER – ALL UNDER STUDY:

Appendix 1.1.4.1. Development and optimisation of antibody detection assays

Appendix 1.1.4.2. Development and optimisation of antigen detection assays

Appendix 1.1.4.3. Development and optimisation of nucleic acid detection (NAD) assays

Appendix 1.1.4.4. Measurement uncertainty

Appendix 1.1.4.5. Statistical approaches to validation

Appendix 1.1.4.6. Comparability of assays after minor changes in a validated test method

Appendix 1.1.4.7. Selection and use of reference samples and panels
CHAPTER 1.1.6.

PRINCIPLES OF VETERINARY VACCINE PRODUCTION

SUMMARY

A reliable supply of pure, safe, potent, and effective vaccines is essential for maintenance of animal health and the successful operation of animal health programmes. Immunisation of animals with high quality vaccines is the primary means of control for many animal diseases. In other cases, vaccines are used in conjunction with national disease control or eradication programmes.

The requirements and procedures described here are intended to be general in nature and to be consistent with published standards that are generally available for guidance in the production of veterinary vaccines. The approach to ensuring the purity, safety, potency, and efficacy of veterinary vaccines may vary from country to country depending on local needs. However, proper standards and production controls are essential to ensure the availability of consistent, high quality products for use in animal health programmes.

As the pathogenesis and epidemiology of each disease varies, the role and efficacy of vaccination as a means of control also varies from one disease to another. Some vaccines may be highly efficacious, inducing an immunity that not only prevents clinical signs of the disease, but may also prevent infection and reduce multiplication and shedding of the disease-causing agent. Other vaccines may prevent clinical disease, but not prevent infection and/or the development of the carrier state. In other cases, immunisation may be completely ineffective or only able to reduce the severity of the disease. Thus the decision whether to recommend vaccination as part of an animal disease control strategy requires a thorough knowledge of the characteristics of the disease agent and its epidemiology, as well as the characteristics and capabilities of the various available vaccines. There is also growing public interest in the beneficial implications for animal welfare of the use of veterinary vaccines as a means of disease control. In any case, if vaccines are used, successful performance requires that they be produced in a manner that ensures a uniform and consistent product of high quality.

NOMENCLATURE

The nomenclature for veterinary biological products varies from country to country. For example, in the United States of America (USA) the term ‘vaccine’ is used for products containing live or inactivated viruses or protozoa, live bacteria, or nucleic acids. Products containing killed bacteria and other microorganisms are identified as bacterins, bacterial extracts, conventional or recombinant subunits, bacterintoxoids, or toxoids, depending on the type of antigen they contain. For example, products containing antigenic or immunising components of microorganisms may be called ‘subunits’ or ‘bacterial extracts’, and those produced from the inactivation of toxins are called ‘toxoids’. In the European Union (EU), Immunological Veterinary Medicinal Products are defined as ‘products administered to animals in order to produce active or passive immunity or to diagnose the state of immunity’, see Directive 2001/82/EC, as amended by Directive 2004/28/EC. For this chapter, however, the term ‘vaccine’ will include all products designed to stimulate active immunisation of animals against disease, without regard to the type of microorganism or microbial toxin from which they may be derived or that they contain. This use is more consistent with international nomenclature. ‘Vaccine’ will not be used in this discussion in reference to biological products recommended for passive immunisation, immunomodulation, treatment of allergies, or diagnosis.

VACCINE TYPES OR FORMS

Vaccines may be prepared as live or inactivated (killed) products. Some live vaccines are prepared from low virulence, mild, field isolates of a disease-causing agent that have been found to be safe and effective when
administered by an unnatural route or under other conditions where exposure to the microorganism will immunise rather than cause disease. Other live vaccines are prepared from isolates of disease-causing agents that have been modified by passage through laboratory animals, culture media, or avian embryos to select a variant of reduced virulence. The development of recombinant DNA (rDNA) procedures has provided some unique opportunities for vaccine production. Modified live vaccines may now be specifically produced by deletion of virulence-related genes from a microorganism. Others are produced by the insertion of genes that code for specific immunising antigens from a disease-causing microorganism into a nonvirulent vector microorganism. Nucleic-acid-mediated vaccines containing plasmid DNA are being developed. The DNA is usually in plasmid form and codes for immunising antigens from disease-causing microorganisms.

Killed products may contain: 1) Cultures of microorganisms that have been inactivated by chemical or other means; 2) Inactivated toxins; or 3) Subunits (antigenic parts of microorganisms) that have been extracted from cultures or that have been produced through rDNA procedures.

Both live and inactivated vaccines may be formulated with adjuvants designed to enhance their efficacy. Frequently used adjuvants are typically water-in-oil emulsions (either single or double), made with mineral or vegetable oil and an emulsifying agent. Other adjuvants, such as aluminium hydroxide gel or saponin, are also used. In addition to these traditional adjuvants, vaccines are being developed that include additional ingredients that induce immunomodulatory effects in the host animal and serve to enhance the efficacy of the product. These ingredients may include immunogenic components of microorganisms such as killed bacteria, which stimulate the immune response to other fractions contained in the vaccine, or cytokines, which may be used to regulate specific aspects of the immune system and are included in rDNA constructs used in products manufactured through biotechnology.

**QUALITY ASSURANCE**

The consistent production of pure, safe, potent, and efficacious vaccines requires quality assurance procedures to ensure the uniformity and consistency of the production process. As production processes for vaccines provide a great opportunity for variability, care must be taken to control variability to the greatest extent possible, preferably using validated procedures, and to protect the product from contamination through all stages of production.

Vaccine purity, safety, potency, and efficacy must be ensured by consistency in the production process. Consistent product quality (batch-to-batch uniformity) must be built in at each stage. Final product testing is used as a check to verify that the controls on the production procedures have remained intact and that the released product meets the specification previously agreed with the licensing authority.

Regulatory authorities in different countries have developed various approaches to ensuring the quality of vaccines. Although alike in their ultimate goal, these systems may vary in the emphasis given to control of the production process (process standards) in comparison with control through testing of the final product (performance standards). The control procedures selected should be those that best fit the conditions under which vaccines are being produced and, where possible, comply with good manufacturing practice.

The control standards and procedures established for a product define the risk or possibility of producing and releasing a product that is worthless, contaminated, dangerous, or harmful. The acceptable degree of risk may depend on the benefits to be gained by having the product available to prevent disease losses. Thus standards may justifiably vary from country to country or product to product, depending on local animal health conditions. However, control authorities should strive to establish control standards and procedures that ensure a finished product of the highest purity, safety, potency, and efficacy possible.

The optimal quality assurance system should address both production procedures and final product testing in proper balance. An absolutely fail-safe system that would result in no risk of releasing an unsatisfactory product would probably be too expensive with regard to cost of production as well as control. Thus regulatory officials and manufacturers of vaccines must select control procedures that are capable of ensuring an acceptable low level of risk in relation to hazard. Such procedures, however, must not be burdensome to the extent that they inhibit the development and availability of the products needed to provide proper preventative medical care at a cost that is acceptable to the consumer.

**PRODUCTION FACILITIES**

Facilities used for the production of vaccines should be designed to protect the purity of the product throughout the production process and to safeguard the health of the personnel. They must be constructed so that: 1) they can be readily and thoroughly cleaned; 2) they provide adequate separation of preparation rooms; 3) they have adequate ventilation; 4) they have ample clean hot and cold water and efficient drainage and plumbing; and 5) they have dressing rooms and other facilities for personnel that are accessible without passing through
biological product preparation areas. Facilities must be adequate to provide for all applicable production functions, such as: storage of master seeds, ingredients, and other production materials; preparation of growth media and cell cultures; preparation of glassware and production equipment; inoculation, incubation, and harvest of cultures; storage of in-process materials; inactivation, centrifugation, addition of adjuvant, and formulation of product; filling, desiccation, sealing of containers, labelling and storage of final product; quality control testing of in-process materials and final product; and research and development.

Separate areas are generally required for different activities. All rooms and air-handling systems must be constructed so as to prevent cross-contamination from other products and to prevent contamination by people or equipment. Virulent or dangerous microorganisms must be prepared and stored in rooms separate from the remainder of the establishment. In particular, challenge organisms must be completely separated from vaccine strains. All equipment that comes into contact with product must be sterilised using validated procedures.

Production facilities have to be designed in such a way that contamination of the external environment is prevented. Any material used during production has to be made safe before leaving the facility. If highly contagious microorganisms are propagated, the exhaust air must be treated to prevent escape of infectious agents. Personnel must follow safety procedures such as showering, and avoid contact with susceptible animals after leaving the production facilities.

Although the quality and design of production facilities may vary significantly, they must always meet standards considered to be appropriate for the vaccines that are to be produced. For example, the requirements for facilities for the production of chicken embryo vaccines administered by oral, intranasal or intraocular routes in chickens may not need to be quite as demanding as those for the production of cell culture vaccines administered subcutaneously or intramuscularly.

FACILITIES PLAN

For each vaccine made in a facility, there should be a detailed production plan that describes where each step in the production process will occur. This plan should be documented in a detailed standard operating procedure (SOP) or by providing a building blueprint and accompanying blueprint legend. Each room in the establishment should be uniquely identified, and all functions performed and all microorganisms involved should be specified for each room. Disinfection procedures, monitoring of equipment and other procedures used in the operation of the facilities to prevent contamination or errors during production should also be documented. This plan should be updated as new products or microorganisms are added to the facility, or other changes or improvements in procedures are developed.

DOCUMENTATION OF THE MANUFACTURING PROCESS

A detailed Outline of Production, a series of SOPs, or other documents should also be prepared to describe the protocol for the manufacture and testing of each product produced in an establishment. Criteria and standards for source materials should be clearly and accurately documented. Documentation should also address such things as: the source, isolation, and passage (subculturing) history of each strain of microorganism; the source and sequence of nucleic acid elements, or peptides included in products derived from biotechnology, including plasmids or other vectors used in the construction of genetically modified microorganisms for use as master seeds; methods for identifying the microorganisms and determining their virulence and purity; the medium or cell culture system used for seed and production cultures, including the methods used to demonstrate that media are free from contamination; the source of ingredients of animal origin; methods of media sterilisation; storage conditions of cell lines and seed cultures; size and types of containers used for growth of cultures; methods for preparing seed cultures and inoculating production cultures; time and conditions for incubation; observations during growth; criteria and specifications for satisfactory harvest material; and harvest techniques. There should be documentation on measures implemented by the firm to minimise the risk of transmissible spongiform encephalopathies (TSE) agent contamination in ingredients of animal origin and procedures to insure that fetal bovine serum is free of pestiviruses. It should also include: a description of all tests conducted to assess the purity and quality of the product as it proceeds through the production process; each step in the formulation of the final product; the tests used for assessing the purity, safety, potency, and other requirements of each batch/serial of completed product; the specifications for finishing, including packaging and labelling with complete indications and recommendations for use; and the expiry date established for the product.

Guidelines for the preparation of such documents for veterinary vaccines are published by competent control authorities. This documentation is intended to define the product and to establish its specifications and standards. It should serve along with the blueprints and blueprint legends (or production plan and SOPs) as a uniform and consistent method of producing the product that should be followed in the preparation of each batch/serial.
Chapter 1.1.6. — Principles of veterinary vaccine production

RECORD KEEPING

The producer should establish a detailed record-keeping system capable of tracking the performance of successive steps in the preparation of each biological product. Records kept should indicate the date that each essential step was taken, the name of the person who carried out the task, the identity and quantity of ingredients added or removed at each step, and any loss or gain in quantity in the course of the preparation. Records should be maintained of all tests conducted on each batch/serial. All records relevant to a batch/serial of product should be retained for at least 2 years after the expiry date on the label, or in line with the requirements of the competent control authority. In addition, a record should be maintained of all labels used on all products, with each label identified as to its name, product number, product licence number, package size, and label identification number. All labels printed should be accounted for. Records must be kept concerning sterilisation and pasteurisation procedures. These are usually made by means of automatic recording devices. The manufacturer must also keep complete records for all animals at the establishment, including health prior to being used for any tests, results of tests performed, treatment administered, maintenance, necropsy, and disposal.

MASTER SEED

The objective of testing the master seed is to ensure vaccine safety, quality and efficacy. Safety should be tested in an early stage. A master seed should be established for each microorganism used in the production of a product to serve as the source of seed for inoculation of all production cultures. Working seeds and production seeds may be prepared from the master seed by subculturing; generally the final production cultures should not be more than five (sometimes ten) passages from the master seed. The number of passages should be determined by data and designated in each case. Using a master seed and limiting the number of passages of seed microorganism in this manner assists in maintaining uniformity and consistency in production. Records of the source of the master seed should be maintained. For genetically modified microorganisms, the source of the gene(s) for the immunogenic antigens and the vector microorganism should be identified. Furthermore, the gene sequences introduced into the seed microorganism genome during construction of the modified seed should be provided. The master seed should consist of a single uniform batch/serial of seed that has been mixed and filled into containers as one batch/serial. Master seed should be frozen or desiccated and stored at low temperatures such as –40°C or –70°C, or under other conditions found to be optimal for maintaining viability. Each master seed should be tested to ensure its identity, safety and efficacy. Genetically modified seeds should also be tested to ensure stability and safety of the inserted gene sequences. Purity should also be established by testing to ensure freedom from extraneous bacteria, fungi, mycoplasma, and viruses.

MASTER CELL STOCKS

When cell cultures are used to prepare a product, a master cell stock (MCS) should be established for each type of cell to be used. Records of the source of the master cell stock should be maintained. For each product, the highest and lowest passage levels of cells that may be used for production should be established and specified in the Outline of Production or SOP. Some control authorities do not permit more than 20–40 subcultivations. Each MCS should be characterised to ensure its identity, and its genetic stability should be demonstrated when subcultured from the lowest to the highest passage used for production. The karyotype of the MCS should be shown to be stable with a low level of polyploidy. Freedom from oncogenicity or tumorogenicity should be demonstrated by in-vivo studies in appropriate species using the highest cell passage that may be used for production. Purity of MCSs should be established by testing to ensure freedom from extraneous bacteria, fungi, mycoplasma, and viruses.

- Primary cells

Primary cells are defined as a pool of original cells derived from normal tissue up to and including the tenth subculture used in the production of biologicals. In the case of products for use in poultry, these cells are usually obtained from specific pathogen free embryonating chicken eggs that have originated in an unvaccinated flock subjected to intensive microbiological monitoring. Other primary cells are derived from normal tissue of healthy animals and are tested for contamination with a wide variety of microorganisms as appropriate, including bacteria, fungi, mycoplasmas, and cytopathic and/or haemadsorbing-inducing agents or other extraneous viruses. The use of primary cells has an inherently higher risk of introducing extraneous agents compared with the use of cell lines and should be avoided where alternative methods of producing effective vaccines exist. Indeed, some control authorities only allow the use of primary cells in exceptional cases.

- Embryonating eggs

Embryonating eggs are also commonly used in the production of biologicals. In almost all cases they should be derived from specific pathogen free chicken flocks that have been intensively monitored for infectious agents and
have not been vaccinated. The route of inoculation of the egg and the choice of egg material to be harvested are dependent on the particular organism that is being propagated.

**INGREDIENTS**

The specifications and source of all product ingredients should be defined in the Outline of Production, SOP, or other appropriate documents. The Outline of Production must be approved by the National licensing agency. All ingredients of animal origin that are not subject to a validated sterilisation procedure should also be tested to ensure freedom from extraneous bacteria, fungi, mycoplasma, and viruses. Their country of origin should be known. Measures should be implemented by the firm to avoid the risk of TSE agent contamination by ingredients of animal origin. Some control authorities discourage the use of preservatives or (more importantly) antibiotics as a means of controlling adventitious contamination during production and prefer the use of strict aseptic techniques to ensure purity. However, they sometimes allow the use of preservatives in multidose containers to protect the product during use. These control authorities usually limit any addition of antibiotics in the manufacture of the product to cell culture fluids and other media, egg inocula, and material harvested from skin or possibly other tissues. They normally permit the use of no more than three antibiotics in the same product. Some control authorities prohibit the use of penicillin or streptomycin in vaccines administered by aerosol or parenterally. If the antibiotics used are not recommended for use in the target species, they should be shown to have no harmful effects in the vaccinated animals and not result in the contamination of food derived from vaccinated animals.

**SAFETY TESTS**

The intrinsic safety of vaccines should be demonstrated early in the development stage and documented as part of the licensing dossier. Safety studies during development and licensing for all products should include the safety of a single dose, of an overdose and of repeated single doses. Additional data are derived for live vaccines from the increase in virulence tests and by assessing risk to the environment and in-contact animals, as discussed below. Safety should be demonstrated in each species for which the product is indicated. As a general rule, overdose studies are required for all vaccines: ×10 for live and ×2 for inactivated vaccines (if this is not practical, an indication of safety may be obtained from the results of the potency tests). For inactivated virus or bacterial products, where host animals are used for potency testing, safety may be determined by measuring local and systemic responses following vaccination and before challenge in the potency tests. Further evidence concerning the safety of products is derived from field safety trials (discussed below). Vaccines derived through biotechnology should be evaluated as discussed in the classification of biotechnology-derived vaccines and release of live rDNA vaccines below.

**INCREASE IN VIRULENCE TESTS**

With live vaccines, there is concern that the organism might be shed from the host and transmitted to contact animals, causing disease if it retains residual virulence or reverts to virulence. Therefore, all live vaccines should be tested for virulence by means of passage studies. Vaccine organisms are propagated in vivo by inoculating a group of target animals with master seed, in principle; this inoculation uses the natural route of infection for that organism that is most likely to result in infection and reversion and, if possible, that represents a recommended route of administration of the vaccine manufactured from this master seed. The vaccine organism is recovered from tissues or excretions and is used directly to inoculate a further group of animals, and so on. After not less than four passages, i.e. use of a total of five groups of animals (more for poultry products), the isolate must be fully characterised, using the same procedures used to characterise the master seed. Regulatory authorities opinion varies in whether or not it is acceptable to propagate in vitro between passages organisms that otherwise cannot be passaged five times because of their degree of attenuation. The vaccine organism must retain an acceptable level of attenuation after propagation in this way.

**ASSESSING RISK TO THE ENVIRONMENT**

The ability of each live vaccine to shed, to spread to contact target and non-target animals, and to persist in the environment must be evaluated to provide information for assessing the risk of the vaccine to the environment, taking into account human health. In some cases this may be done in conjunction with the increase in virulence tests. These and additional considerations are especially important in the case of products based on biotechnology or recombinant DNA techniques; more information about such products is provided in the sections at the end of this chapter.
EFFICACY TESTS

The efficacy of veterinary vaccines should be demonstrated by statistically valid vaccination–challenge studies in the host animal, using the most sensitive, usually the youngest, animals for which the product is to be recommended. Data should support the efficacy of the vaccine in each animal species by each vaccination regimen that is described in the product label recommendation, including studies on the onset of protection when claims for onset are made in the product labelling and for the duration of immunity. The tests should be performed under controlled conditions starting, wherever possible, with seronegative animals. Where validated potency tests are available, target species vaccination–challenge studies may not be required if predictive serological test results are available. The application of procedures to replace, reduce, and refine animal tests (the ‘three Rs rule’) should be encouraged whenever possible.

Efficacy studies should be conducted with final product vaccine that has been produced at the highest passage level from the master seed that is permitted in the Outline of Production, or other documentation of the manufacturing process. This will have specified the minimum amount of antigen per dose that must be in the final product throughout the entire authorised shelf-life. Where a range of antigen level per dose is permitted, the antigen level per dose in the vaccine tested for efficacy must be at or below the minimum permitted amount. The precise challenge method and the criteria for determining protection vary with the immunising agent and should be standardised whenever possible.

Field efficacy studies may be used to confirm the results of laboratory studies or to demonstrate efficacy when meaningful vaccination–challenge studies are not feasible. However, it is generally more difficult to obtain statistically significant data to demonstrate efficacy under field conditions. Protocols for field studies are more complex, and care must be given to establish proper controls to ensure the validity of the data. Even when properly designed, field efficacy studies may be inconclusive because of uncontrollable outside influences. Some problems include: a highly variable level of challenge; a low incidence of disease in nonvaccinated controls; and exposure to other organisms causing a similar disease. Therefore, efficacy data from both laboratory and field studies may be required to establish the efficacy of some products, as well as ‘posteriory’ field trials linked to vaccinovigilance.

INTERFERENCE TESTS

For products with two or more antigenic components, tests must confirm that there is no interference between individual components, that is, one component causing a decrease in the protective immunological response to another component. Interference testing should be conducted for each combination product prior to approval.

A loss of potency may also result when residual inactivating agent in a killed liquid product used as a diluent for a desiccated live fraction reduces the viability of the live organisms because of viricidal or bacteriocidal activity. Each batch/serial of liquid killed vaccine that is to be used as a diluent for live vaccines must, therefore, be tested for viricidal or bacteriocidal activity prior to release.

Consideration must also be given to possible interference between two different vaccines from the same manufacturer recommended to be given to the same animal within a 2-week period.

CONSISTENCY OF PRODUCTION

Prior to marketing approval of any new product, each establishment should produce in its facilities three consecutive production batches/serials of completed product to evaluate the consistency of production. These batches/serials should be prepared according to the procedures described in the Outline of Production and blueprints and legends, SOPs or other documentation of the manufacturing process and should therefore be ‘typical of production’. Some authorities require that the size of each of the three batches/serials should be at least one-third the size of the average batch/serial that will be produced once the product is in production.

The manufacturer should test each of these batches/serials for purity, safety, and potency as provided in the Outline of Production or other documentation of the manufacturing process. Applicable Standard Requirements and test procedures, for example those described in CFR (Code of Federal Regulations) Title 9 part 113, in the Annex to EU Directive 2001/82/EC (as amended), in the European Pharmacopoeia, or as described in this Terrestrial Manual may be used. Satisfactory test results should be demonstrated for all three batches/serials prior to approving the production of the product in the facilities and its release for marketing. Each subsequent batch/serial should be tested in the same manner with satisfactory results prior to release for marketing.
STABILITY TESTS

Stability studies (based on an acceptable potency test) are required to establish the validity of the expiry date that appears on the product package. Some authorities allow the use of accelerated stability tests to determine a provisional expiry date for products, e.g., incubating at 37°C for 1 week for each year of dating. Such estimates must be confirmed by periodic real-time potency tests on at least three different batches/serials through the period of time indicated by the expiry date, and 3–6 months beyond. For products containing viable organisms, testing should be done at release and at the approximate expiry date until a statistically valid record has been established. For non-viable products, each batch/serial presented for licensing is tested at release and at periodic intervals through, or past, the requested expiry date. If at the end of the dating period (shelf life) specified, the product is tested and found still to be above the release quality, consideration can be given to extending the designated shelf life, by request to the control authority. Stability testing also provides the opportunity to test for residual moisture and for other important parameters, such as the stability of adjuvant emulsions.

BATCH/SERIAL RELEASE FOR DISTRIBUTION

Prior to release, the manufacturer must test each batch/serial for purity, safety, and potency, as well as perform any other tests described in the firm’s Outline of Production or other documentation of the manufacturing process for that product. In countries that have national regulatory programmes that include official control authority re-testing (check testing) of final products, samples of each batch/serial should also be submitted for testing in government laboratories by competent authorities. If unsatisfactory results are obtained for tests conducted either by the manufacturer or by competent authorities, the batch/serial should not be released. In such cases, subsequent batches/serials of the product should be given priority for check testing by competent authorities.

1. **Batch/serial purity test**

Purity is determined by testing for a variety of contaminants. Tests to detect contaminants are performed on: master seeds, primary cells, MCSs, ingredients of animal origin if not subjected to sterilisation (e.g., fetal bovine serum, bovine albumin, or trypsin), and each batch/serial of final product prior to release.

Purity test procedures have been published, for example in CFR Title 9 part 113, in the annex to EU Directive 2001/82/EC (as amended), in the European Pharmacopoeia, or in this Terrestrial Manual, for the detection of extraneous viruses, bacteria, mycoplasma and fungi, including for example: *Salmonella*, *Brucella*, chlamydial agents, haemagglutinating viruses, avian lymphoid leukemia, pathogens detected by a chicken inoculation test, chicken embryo inoculation test, lymphocytic choriomeningitis, cytopathic and haemadsorbing agents, and pathogens detected by enzyme-linked immunosorbent assay, polymerase chain reaction, or the fluorescent antibody technique. Procedures used to ensure that fetal or calf serum and other ingredients of bovine origin are free of pestiviruses should be of high concern and well documented. Tests to be used to ensure purity vary with the nature of the product, and should be prescribed in the Outline of Production or other documentation of the manufacturing process. As tests for the detection of TSE agents in ingredients of animal origin have not been developed, vaccine manufacturers should document in their Outlines of Production or SOPs the measures they have implemented to minimise the risk of such contamination in ingredients of animal origin. This relies on three principles: first, verification that sources of all ingredients of animal origin in production facilities are from countries recognised as having the lowest possible risk of bovine spongiform encephalopathy; second, that the tissues or other substances used are themselves recognised as being of low or nil risk of containing TSE agents; third, where relevant, that the processes applied to the material have been validated for inactivation of TSE agents. Methods of production should also document the measures taken to prevent cross contamination of low risk materials by higher risk materials during processing.

2. **Batch/serial safety test**

Batch/serial safety tests are required for the release of each batch/serial and typical tests are described in CFR Title 9 part 113, in the European Pharmacopoeia, in this *Terrestrial Manual* and elsewhere. Standard procedures are given for safety tests in mice, guinea-pigs, cats, dogs, horses, pigs, and sheep and are generally conducted using fewer animals than are used in the safety tests required for licensing. Batches/serials are considered satisfactory if local and systemic reactions to vaccination with the batch/serial to be released are in line with those described in the registration dossier and product literature. Some authorities do not permit batch/serial safety testing in laboratory animals, requiring a test in one of the target species for the product.

3. **Batch/serial potency test**

Batch/serial potency tests, required for each batch/serial prior to release, are designed to correlate with the host animal vaccination–challenge efficacy studies. For inactivated viral or bacterial products, potency tests may be
conducted in laboratory or host animals, or by means of quantitative *in-vitro* methods that have been validated reliably to correlate *in vitro* quantification of important antigen(s) with *in vivo* efficacy. The potency of live vaccines is generally measured by means of bacterial counts or virus titration. Recombinant DNA or biotechnology-based vaccines should also be tested. Live genetically modified organisms can be quantified like any other live vaccine by titration, and expressed products of recombinant technology are quantified by *in vitro* tests, which can be easier to perform compared with tests on naturally grown antigens because of the in-process purification of the desired product.

When testing a live bacterial vaccine for release for marketing, the bacterial count must be sufficiently greater than that shown to be protective in the master seed immunogenicity (efficacy) test to ensure that at any time prior to the expiry date, the count will be at least equal to that used in the immunogenicity test. When testing a live viral vaccine for release, the virus titre must, as a rule, be sufficiently greater than that shown to be protective in the master seed immunogenicity test in order to ensure that at any time prior to the expiry date, the titre will be at least equal to that used in the immunogenicity test. Some control authorities specify higher bacterial or viral content than these. It is evident that the appropriate release titre is primarily dependent on the required potency and secondarily dependent on the rate of decay of the bacteria or viruses in the vaccine, as indicated by the stability test.

Standard Requirements have been developed and published by competent authorities for potency testing several vaccines. These tests can be found in CFR Title 9 part 113, in the European Pharmacopoeia, and in this *Terrestrial Manual*.

**OTHER TESTS**

Depending on the form of vaccine being produced, certain tests may be indicated and should be provided as appropriate in the Outline of Production or other documentation of the manufacturing process. These tests may concern: the level of moisture contained in desiccated products, the level of residual inactivant in killed products, the complete inactivation of killed products, pH, the level of preservatives and permitted antibiotics, physical stability of adjuvants, retention of vacuum in desiccated products, and a general physical examination of the final vaccine. Tests for these purposes may also be found in CFR Title 9 part 113, in EU Directive 2001/82/EC (as amended), in the European Pharmacopoeia, or in this *Terrestrial Manual*.

**SAMPLING**

Samples should be selected from each batch/serial of product. The selector should pick representative final containers from each batch/serial and store these samples at the storage temperature recommended on the label. The producer should keep these reserve samples at the recommended storage temperature for a minimum of 6 months after the expiry date shown on the label, so that they are available to assist in evaluating the cause of any field problems reported from the use of the vaccine. The samples should be stored in a secure storage area and be tamper-evident.

**LABELLING**

Standards for labelling products will vary from country to country; however, the label indications and all claims that are made on the label should be supported by appropriate data that have been reviewed and approved by competent authorities. It is recommended that all labels for veterinary vaccines be water-proof and contain the following information, although for very small containers, the label may instead refer to the carton label or to an enclosed package insert for some of the less prominent information:

1. The true name of the product, prominently lettered and with equal emphasis on each word;
2. The name and address of the producer (and also the importer for imported products);
3. The recommended storage temperature;
4. A statement that the product is ‘for veterinary (or animal) use only’. Full instructions for use, including all required warnings;
5. For food animals, a statement indicating that the animals should not be vaccinated within a specified number of days before slaughter. This will depend on the vaccine (e.g. type of adjuvant) and is not required for all products;
6. The expiry date;
Chapter 1.1.6. – Principles of veterinary vaccine production

7. The batch/serial number by which to identify the product in the producer’s record of preparation;
8. The licence number for the product; in some countries this is replaced by the licence number of the establishment/manufacturer;
9. The recoverable quantity and number of doses;
10. A statement that the entire contents of a multidose container should be used when the container is first opened (or with appropriate holding time for certain products, as supported by data) and that any unused portions should be disposed of in a proper manner;
11. A safety warning to the operator, if appropriate, e.g. accidental self-injection with oil emulsion vaccines.
12. Where it is allowed for an antibiotic to be added to a vaccine during the production process, the statement “Contains (antibiotic name) as a preservative” or an equivalent statement indicating the antibiotic added should appear on the carton or enclosures if used. If cartons are not used, such information should appear on the final container label.

Labels may also include other factual statements that are not false or misleading. Special restrictions concerning the use or handling of the product, when applicable, should also be indicated.

Similar information should also be given in a Product Data Sheet that is provided as a package insert. This will also contain much more detail about method of use and possible adverse reactions.

FIELD TESTS (SAFETY AND EFFICACY)

All veterinary biological products administered to animals should be tested for safety and, if possible, for efficacy in the field, using good clinical practice, before being authorised for general use. Field studies are designed to demonstrate efficacy under working conditions and to detect unexpected reactions, including mortality, that may not have been observed during the development of the product. Under field conditions there are many uncontrollable variables that make it difficult to obtain good efficacy data, but demonstration of safety is more reliable. The tests should be done on the host animal, at a variety of geographical locations, using appropriate numbers of susceptible animals. The test animals should represent all the ages and husbandry practices for which the product is indicated; unvaccinated controls must be included. The product tested should be one or more production batches/serials. A protocol should be developed indicating the observation methods and the recording methods.

INSPECTION OF PRODUCTION FACILITIES

Establishments that are approved to produce veterinary biologicals should be subject to in-depth inspections of the entire premises by national competent authorities to ensure compliance with the Outline of Production and blueprints and legends, SOPs, or other documentation of the manufacturing process. These inspections may include such items as: personnel qualifications; record keeping; general sanitation and laboratory standards; research activities on products being developed; production procedures; operation of sterilisers, pasteurisers, incubators, and refrigerators; filling, desiccating, and finishing procedures; care and control of animals; testing procedures; distribution and marketing; and product destruction. It is desirable to have good manufacturing practice (for manufacturing) and good laboratory practice (for quality assurance testing). (See Chapter 1.1.4 Quality management in veterinary testing laboratories, for guidelines.)

The inspectors should prepare a comprehensive report documenting the findings of the inspection and stating the actions that the establishment must take to improve its production processes. The establishment should receive a copy of the report. When necessary a follow-up inspection should be conducted to determine whether appropriate action has been taken to correct deficiencies. Continued reassessment in this manner is needed to ensure that production facilities continue to be operated in an acceptable manner.

UPDATING THE OUTLINE OF PRODUCTION

Before production procedures are changed, the corresponding Outline of Production or other documentation of the manufacturing process should be changed. Establishments should have internal review procedures to evaluate all changes in production before they are initiated. Changes should also be reviewed and approved by competent authorities prior to their implementation. In cases where a significant production step is altered, revisions may require additional data to support the purity, safety, potency, and/or efficacy of the product. In
countries with regulatory programmes that include check testing the final product at national laboratories, revisions should entail testing of the new product by competent authorities.

**PERFORMANCE MONITORING**

Manufacturers are required to maintain an adverse reaction notification system and an effective mechanism for rapid product recall. These should both be subject to audit by regulatory bodies. In many countries, the manufacturer must notify all adverse reactions immediately to the regulatory authority, along with any remedial action taken. An alternative used in some countries is that if at any time, there are indications that raise questions regarding the purity, safety potency, or efficacy of a product, or if it appears that there may be a problem regarding the preparation, testing or distribution of a product, the manufacturer must immediately notify the regulatory authorities concerning the circumstances and the action taken.

After release of a product, its performance under field conditions should continue to be monitored by competent authorities. Consumer complaints may serve as one source of information; however, such information needs to be investigated to determine whether or not the reported observations are related to the use of the product. Users of veterinary vaccines should be informed of the proper procedures for making their complaints. The manufacturer of the product should be informed of all complaints received by competent authorities. Competent authorities should also ascertain whether the manufacturer has received other similar complaints for this product and, if so, whether the manufacturer has taken appropriate action. Control laboratories may test samples of the batch/serial of product involved, if necessary.

When the investigation is complete, a final report should be prepared and a summary of the findings sent to the complainant and to the manufacturer. When it is determined that a product is causing serious problems, immediate action should be taken to remove the product from the market and to notify animal health authorities.

**ENFORCEMENT**

National programmes established to ensure the purity, safety, potency, and efficacy of veterinary vaccines must have adequate legal authority to ensure compliance with product registration conditions and other programme requirements. The goal should be to obtain voluntary compliance with established regulatory requirements. However, when violations occur, competent authorities must have adequate legal authority to protect animal and human health. Authority for detention, seizure, and condemnation of products found to be worthless, contaminated, dangerous, or harmful may be valuable for this purpose. Under such authority, product may be detained for a period of time, and if during that time compliance cannot be achieved, competent authorities may seek a court order or decree for seizure and condemnation.

The authority to remove or suspend establishment and/or product licenses, obtain injunctions, and stop the sale of product is also needed. Civil penalties or criminal prosecution may also be necessary for serious or deliberate violations.

** LICENSING OF PRODUCTS DERIVED THROUGH BIOTECHNOLOGY**

Recent advances in biotechnology have made possible the development and commercialisation of new biological products with useful antigenic and diagnostic properties. Many such products have now been licensed or approved, and more are being developed. Products of rDNA technology do not differ fundamentally from conventional products. Therefore, existing laws and regulations are fully applicable to these new products.

**CLASSIFICATION OF BIOTECHNOLOGY-DERIVED VACCINES**

Each competent authority with power to regulate organisms and products derived from recombinant techniques should ensure that the public health and the environment are protected from any potentially harmful effect. For the purpose of evaluating licence applications, veterinary vaccines derived through rDNA technology may be divided into three broad categories. The division is based on the products’ biological properties and on the safety concerns they present.

Category I consists of nonviable or killed products that pose no risk to the environment and present no new or unusual safety concerns. Such products include inactivated microorganisms, either whole or as subunits, created by using rDNA techniques.
Category II products contain live microorganisms modified by adding or deleting one or more gene(s). Added genes may code for marker antigens, enzymes, or other biochemical by-products. Deleted genes may code for virulence, oncogenicity, marker antigens, enzymes, or other biochemical by-products. The licence application must include a characterisation of the DNA segments added or deleted, as well as a phenotypic characterisation of the altered organism. The genetic modifications must not result in any increase in virulence, pathogenicity, or survivability of the altered organism in comparison with the wild-type form. It is important that the genetic modification does not cause a deterioration in the safety characteristics of the organism.

Category III products make use of live vectors to carry recombinant-derived foreign genes that code for immunising antigens. Live vectors may carry one or more foreign gene(s) that have been shown to be effective for immunising target host animals. The use of DNA vaccines containing recombinant-derived foreign genes that code for immunising antigens (plasmid DNA vaccines) constitutes a new approach to vaccine development. The proper categorisation of this type of rDNA-derived product will be established as biological properties and safety characteristics are determined. These new vaccines may find application in a wide variety of situations such as conventional products have. Guidelines for the development, production, characterisation, and control of these new products are still preliminary and subject to change as new data and knowledge are developed. Information concerning the current thinking on regulatory guidelines may be found on the Internet at the following addresses:


RELEASE OF LIVE rDNA PRODUCTS

The release of live rDNA microorganisms (Categories II and III) for field testing or general distributions as an approved or licensed product may have a significant effect on the quality of the human and animal environment. Before release is authorised, the manufacturers of the vaccine should conduct a risk assessment to evaluate the impact on the human and animal environment. In the USA, for example, a procedure is adopted that could be used as a model system in other countries. The European Union has adopted a similar system. It is performed as follows:

A risk assessment is carried out that should contain the following information: the purpose and need for the proposed action; the alternatives considered; a list of the government agencies, organisations, and persons consulted; and the affected environment and the potential environmental consequences. The topics discussed should include: the characteristics of the vaccine organism, human health risks, animal health risks for both target and nontarget animals, persistence in the environment, and increase in virulence.

If the risk assessment results in a finding by competent authorities that the proposed release of the recombinant vaccine into the environment for field trials or general distribution would not have a significant impact on the environment, a notice should be published and distributed to the public announcing this and that the risk assessment and findings are available for public review and comment. If no substantive comments are received to refute the findings, competent authorities may authorise the field testing or grant the license or approval for general distribution.

The preparation of a risk assessment and the findings made from the assessment may also include the scheduling of one or more public meetings if a proposed action has ecological or public health significance. Such meetings should be announced through a public notice. Interested persons should be invited to make presentations, along with presentations by the producer of the product, and government personnel. The transcripts of such meetings should become part of the public record.

If, in the course of preparing a risk assessment, competent authorities conclude that the proposed action may have a significant effect on the human environment, an Environmental Impact Statement (EIS) should be prepared. The EIS provides a full and fair discussion of the significant environmental impacts, and informs decision-makers and the public of any reasonable alternatives that would avoid or minimise the adverse impacts. (Environmental documents are considered in CFR Title 40 part 1508.) See also EU Directive 2001/18/EC and http://www.emea.europa.eu/pdfs/vet/iwp/000404en.pdf

FURTHER READING

The following are some suggested texts that contain guidelines on aspects of vaccine production.

Chapter 1.1.6. – Principles of veterinary vaccine production

European Pharmacopoeia 7.0. (2012). European Directorate for the Quality of Medicines and HealthCare (EDQM), Council of Europe, Strasbourg, France.


USDA-APHIS-VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1995). Veterinary Biologics General Licensing Considerations No. 800.200, Efficacy Studies. USDA-APHIS-Veterinary Biologics, 4700 River Road, Riverdale, Maryland 20737, USA.


* * *

APPENDIX 1.1.6.1.

RISK ANALYSIS FOR BIOLOGICALS
FOR VETERINARY USE

GENERAL CONSIDERATIONS

All products, including biologicals for veterinary use, derived from animals have some capacity to transmit animal disease. The level of this capacity depends on the inherent nature of the products, their source, the treatment that they might have undergone, and the purpose for which they are intended. Biologicals for in vivo use in particular will have the highest probability of exposure to animals and as such present the highest risk. Products used for in vitro purposes can introduce disease into animal populations through deliberate or inadvertent use in vivo, contamination of other biologicals, or spread by other means. Even products for diagnosis and research have the potential for close contact with animals. Exotic micro-organisms, some highly pathogenic, which may be held for research and diagnostic purposes in countries free from infection or the diseases they cause, could possibly contaminate other biological products.

Veterinary Authorities of importing countries shall make available specific procedural requirements for approval or licensing of biologicals for veterinary use. They may limit supply to registered institutions or in vitro use or for non-veterinary purposes where such assurance cannot be provided.

* * *
APPENDIX 1.1.6.2.

RISK ANALYSIS FOR VETERINARY VACCINES

INTRODUCTION

Risk analysis for veterinary vaccines has to be founded on the principles of quality assurance, which includes quality control, in the production of veterinary vaccines. These recommendations are focused mainly on the risk related to the contamination of vaccines by infectious agents particularly in regard to the risk of importing exotic diseases. The major risk of introducing a disease into a country is through importation of live animals or animal products and rarely through veterinary vaccines. Veterinary vaccines can however be contaminated by disease agents if master seeds, strains, cell cultures, animals or ingredients of animal origin such as fetal calf serum used in production are contaminated or if cross contamination occurs during the production process.

PRINCIPLES

Exporting countries and importing countries should agree on a system of classification of risks associated with veterinary vaccines taking into account factors such as purification procedures which have been applied.

Exporting countries and importing countries should agree on risk analysis models to address specific issues and products. Such risk analysis models should include a scientific risk assessment and formalised procedures for making risk management recommendations and communicating risk. The regulation of veterinary vaccines should include the use of either qualitative or quantitative models.

Risk analysis should be as objective and transparent as possible. Step risk and scenario tree methods should be used in risk assessment whenever appropriate, as they identify the critical steps in the production and use of the products where risks arise and help to characterise those risks.

The same conclusions about risk analysis may be reached by differing methods. Where methods may differ in countries, the concept of equivalence should apply wherever possible and the methods should be validated to ensure they are of comparable sensitivity.

MANUFACTURING PRACTICES

The manufacture of veterinary vaccines has special characteristics which should be taken into consideration when implementing and assessing the quality assurance system. Due to the large number of animal species and related pathogenic agents, the variety of products manufactured is very wide and the volume of manufacture is often low; hence, work on a group basis is common. Moreover, because of the very nature of this manufacture (cultivation steps, lack of terminal sterilisation, etc.), the products must be particularly well protected against contamination and cross contamination. The environment must also be protected especially when the manufacture involves the use of pathogenic or exotic biological agents and the worker must be particularly well-protected when the manufacture involves the use of biological agents pathogenic to man.

These factors, together with the inherent variability of immunological products, means that the role of the quality assurance system is of the utmost importance. It is important that vaccines should be manufactured in accordance with a recognised codified system that includes specifications regarding equipment, premises, qualification of personnel as well as quality assurance and regular inspections.

A commonly agreed system of facility inspection carried out by qualified and specialised inspectors must be in place to assure confidence.
INFORMATION TO BE SUBMITTED WHEN APPLYING FOR REGISTRATION IN THE IMPORTING COUNTRY

The manufacturer or Veterinary Authority of the exporting country should make available to the importing country the pharmacopoeia it uses. For the importing country it is necessary to have documented both the quality control methods used and the source of each batch of starting materials. The key steps of the manufacturing process of veterinary vaccines should be described in detail to help risk analysis. Risk analysis has to be focused on the quality and safety parts of the application file. Laboratory safety testing should cover target and non-target organisms to obtain sufficient biological data. All test procedures used should correspond with the state of scientific knowledge at the time and should be validated.

The description of the method of preparation of the finished product should include an adequate characterisation of the substances needed to prepare the working seeds, the description of the treatments applied to starting materials to prevent contamination, and a statement of the stages of manufacture at which sampling is carried out for process control tests.

The results of control tests during production and on finished product, as well as the sensitivity of these tests, have to be available for risk analysis. The stepwise procedures of the control tests should also be available.

CATEGORISATION OF VETERINARY VACCINES

To assist in risk analysis, countries should establish a system of categorisation of veterinary vaccines taking into account criteria such as pathogens used as active ingredients, their inherent characteristics and the risk they pose.

In case of live vectored vaccines, the safety of the vector to the targeted and non-targeted species and to human beings must be assessed. Special attention should be paid to potential tissue tropism or host range modification of the recombinant.

VACCINOVIGILANCE

Exporting countries and importing countries should ensure that a reliable system of vaccinovigilance (post licensing monitoring) is established to identify, at the earliest stage, any serious problems encountered from the use of veterinary vaccines. Vaccinovigilance should be ongoing and an integral part of all regulatory programmes for veterinary vaccines, especially live vaccines.

RISK COMMUNICATION

Reliable data in support of applications submitted in importing countries should be provided by the manufacturer or the Veterinary Authority of the exporting country. Relevant data on risk analysis, changes in animal health situations and vaccinovigilance should be shared by Veterinary Authorities on a continuous basis.

* *
* *
CHAPTER 1.1.7.

TESTS FOR STERILITY AND FREEDOM FROM CONTAMINATION OF BIOLOGICAL MATERIALS

INTRODUCTION

Sterility is defined as the absence of living organisms. It is achieved by heating, by filtration, by treatment with ethylene oxide or by ionising irradiation, and by conducting any subsequent processes aseptically. Freedom from contamination is defined as the absence of specified living organisms. This may be achieved by selecting materials from sources shown to be free from the specified organisms and by conducting subsequent procedures aseptically. Adequate assurance of sterility and freedom from contamination can only be achieved by proper control of the primary materials used and their subsequent processing and storage. Tests on the product are necessary to check that this control has been achieved.

A. GENERAL PROCEDURES

1. Primary materials must be collected from sources shown to be free from contamination and handled in such a way as to minimise contamination and the opportunities for any contaminants to multiply.

2. Materials that can be sterilised without their biological activities being affected unduly must be sterilised by a method effective for the materials concerned. The method must reduce the level of contamination to be undetectable, as determined by an appropriate sterility test (see paragraph B.3 below).

3. If a sterilisation process is used, it shall be validated to demonstrate its suitability and adequately controlled to show that it has functioned properly on each occasion.

4. Materials that are not sterilised and those that are to be processed further after sterilisation must be handled aseptically.

5. The environment in which any aseptic handling is carried out must be maintained in a clean state and protected from external sources of contamination, and must be controlled to prevent internal contamination.

B. LIVING VIRAL VACCINES FOR ADMINISTRATION BY INJECTION

1. Materials of animal origin shall be (a) sterilised, or (b) obtained from healthy animals that, in so far as is possible, should be shown to be free from pathogens that can be transmitted from the species of origin to the species to be vaccinated, or any species in contact with them, or (c) the material shall be shown to be free from such pathogens.

2. Seed lots of virus and of any continuous cell line used for virus growth shall be shown to be free from bacteria, fungi, mycoplasmas, extraneous viruses and other pathogens that can be transmitted from the species of origin to the species to be vaccinated or any species in contact with them. For the production of avian vaccines and the quality control procedures for these vaccines, it is recommended that specific pathogen-free embryonated chicken eggs be used.


4. Each batch of vaccine shall pass tests appropriate to prove that the vaccine is free from extraneous viruses. (Such tests include tests in cell cultures susceptible to viruses of the species to be vaccinated, tests in embryonated eggs, and, where necessary, tests in animals.)
5. Some countries require that each batch of vaccine pass a test for freedom from mycoplasma. Suitable test methods have been published (Code of Federal Regulations, 2007; European Pharmacopoeia, 2012; WHO, 1998).

6. Tests for freedom from certain specific bacteria may be required, e.g. tests for *Salmonella*, *Mycobacterium tuberculosis* and *M. paratuberculosis*, *Brucella* spp. and *Leptospira* spp. (Code of Federal Regulations, 2007; European Pharmacopoeia, 2012).

C. LIVING VIRAL VACCINES FOR ADMINISTRATION THROUGH DRINKING WATER, SPRAY, OR SKIN SCARIFICATION

1. Paragraphs B.1, 2, 4, 5, and 6 apply.
2. A limited number of contaminating, nonpathogenic bacteria and fungi may be permitted (see Section J.2.5).

D. INACTIVATED VIRAL VACCINES

1. Paragraphs B.1, 2 and 3 apply.
2. Each batch of vaccine shall pass a test for inactivation of the vaccinal virus. This is done before addition of preservative. The inactivation process and the tests used to detect live virus after inactivation must be validated and shown to be suitable for their intended purpose.
3. Demonstration that the method of inactivation also inactivates representative pathogens may be required unless the vaccine satisfies the conditions of paragraphs B.4 and B.5.

E. LIVING BACTERIAL VACCINES

1. Paragraphs B.1 applies.
2. Seed lots of bacteria shall be shown to be free from other bacteria as well as fungi and mycoplasmas.
3. Each batch of vaccine shall pass a test for purity carried out using solid media and ignoring the growth of the vaccinal bacterium.
4. Some countries require that each batch of bacterial vaccine passes a test for freedom from mycoplasmas. Suitable test methods have been published (WHO, 1998, and, for avian mycoplasmas: European Pharmacopoeia, 2012).

F. INACTIVATED BACTERIAL VACCINES

1. Paragraphs B.1, B.3, and E.2 apply.
2. Each batch of vaccine shall pass a test for inactivation of the vaccinal bacterium. If appropriate, the test for sterility may be used for this purpose.

G. SERA FOR ADMINISTRATION TO ANIMALS

1. Paragraph B.1 applies. Some countries require quarantine, health certification, and specific disease tests be completed for all serum donor animals (Code of Federal Regulations, 2007).
2. Paragraph B.2 or E.2 applies, as appropriate, if a virus or a bacterium is used in serum production.
4. Each batch of serum shall pass tests appropriate to prove that the serum is free from extraneous viruses. (Such tests include tests in cell cultures susceptible to viruses of the species to be treated, tests in embryonated eggs and, where necessary, tests in animals.)

5. Some countries require that each batch of serum passes a test for freedom from mycoplasmas. Suitable test methods have been published (WHO, 1998, and, for avian mycoplasmas: European Pharmacopoeia, 2012).

H. DIAGNOSTIC AGENTS FOR ADMINISTRATION TO ANIMALS

1. Paragraphs B.1 and 3 apply.

2. Paragraphs B.2 and D.2 apply if a virus is used in the production of the diagnostic agent; E.2 and F.2 apply if a bacterium is used.

I. EMBRYOS, OVA, AND SEMEN

Special precautions must be taken with relation to the use of embryos, ova and semen (Hare, 1985).

J. PROTOCOL EXAMPLES

1. General procedures

Materials used in the production of biological products should be sterilised and/or tested to ensure freedom from contaminants before being used. Samples of the finished biological product should also be tested for bacterial, fungal, or mycoplasmal contaminants.

The assays for bacteria, mycoplasma, fungi, and viruses described here are derived from various sources and they are given as examples of methods that can be used with confidence.

2. Detection of bacteria and fungi

These assays describe the materials and methods that are used for the detection of bacteria and fungi by either the membrane filtration method, or the direct inoculation of fluid media method used for materials that are unsuitable for membrane filtration.

2.1. General procedure for detecting viable bacteria and fungi

Standard tests for detecting extraneous bacteria and fungi in raw materials, seed stocks, or final product are: the membrane filtration test or the direct inoculation sterility test.

For the membrane filtration technique, a filter having a nominal pore size not greater than 0.45 µm and a diameter of at least 47 mm should be used. Cellulose nitrate filters should be used if the material is aqueous or oily; cellulose acetate filters should be used if the material is strongly alcoholic, oily or oil-adjuvanted. Immediately before the contents of the container or containers to be tested are filtered, the filter is moistened with 20–25 ml of Diluent A or B.

**Diluent A** – for aqueous products or materials: Dissolve 1 g peptic digest of animal tissue in water to make 1 litre, filter or centrifuge to clarify, adjust the pH to 7.1 ± 0.2, dispense into containers in 100 ml quantities, and sterilise by steam.

**Diluent B** – for oil-adjuvanted products or materials: Add 1 ml polysorbate 80 to 1 litre Diluent A, adjust the pH to 7.1 ± 0.2, dispense into containers in 100 ml quantities, and sterilise by steam.

If the biological being tested has antimicrobial properties, the membrane is washed three times after sample application with approximately 100 ml of the appropriate diluent (A or B). The membrane is then transferred whole to culture media, aseptically cut into equal parts and placed in media, or the media is transferred to the membrane in the filter apparatus. If the test sample contains merthiolate as a preservative, fluid thioglycollate medium (FTM) is used and the membranes are incubated at both 30–35°C and 20–25°C. If the test sample is a killed biological without merthiolate preservative, FTM is used at 30–35°C and soybean casein digest medium (SCDM) at 20–25°C. If the sample tested is a live viral biological, SCDM is used at both incubation temperatures. Recently, it has been suggested that sulfite-polymyxin-sulfadiazine agar be
Chapter 1.1.7. — Tests for sterility and freedom from contamination of biological materials

used to enhance the detection of *Clostridium* spp. when the membrane filtration technique is used (Tellez *et al.*, 2005).

If direct inoculation of culture media is chosen, a sterile pipette or syringe and needle are used to aseptically transfer the biological material directly into liquid media. If the biological being tested has antimicrobial properties, the ratio of the inoculum to the volume of culture medium must be determined before the test is started. To determine the correct medium volume to negate antimicrobial activity, 100 colony-forming units (CFU) of the control microorganisms listed in Table 1 are used. If the test sample contains merthiolate as a preservative, FTM is used in test vessels incubated at both 30–35°C and 20–25°C. Growth should be clearly visible after an appropriate incubation time (see Section J.2.2). If the test sample is a killed biological without merthiolate, or a live bacterial biological, FTM is used at 30–35°C and SCDM at 20–25°C. If the test sample is a live viral biological, SCDM is used at both incubation temperatures. If the inactivated bacterial vaccine is a clostridial biological, or contains a clostridial component, the use of FTM with 0.5% added beef extract (FTMB) in place of FTM is preferred. It may also be desirable to use both FTM and SCDM for all tests.

### Table 1. Some American type culture collection\(^1\) strains with their respective medium and incubation conditions

<table>
<thead>
<tr>
<th>Medium</th>
<th>Test microorganism</th>
<th>Temperature (°C)</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTM</td>
<td><em>Bacillus subtilis</em> ATCC # 6633</td>
<td>30–35</td>
<td>Aerobic</td>
</tr>
<tr>
<td>FTM</td>
<td><em>Candida krusei</em> ATCC # 6258</td>
<td>20–25</td>
<td>Aerobic</td>
</tr>
<tr>
<td>SCDM</td>
<td><em>Bacillus subtilis</em> ATCC # 6633</td>
<td>30–35</td>
<td>Aerobic</td>
</tr>
<tr>
<td>SCDM</td>
<td><em>Candida kursei</em> ATCC # 6258</td>
<td>20–25</td>
<td>Aerobic</td>
</tr>
<tr>
<td>FTMB</td>
<td><em>Clostridium sporogenes</em> ATCC # 11437</td>
<td>30–35</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>FTMB</td>
<td><em>Staphylococcus aureus</em> ATCC #6538</td>
<td>30–35</td>
<td>Aerobic</td>
</tr>
</tbody>
</table>

For both membrane filtration and direct inoculation sterility tests, all media are incubated for no fewer than 14 days. At intervals during incubation, and after 14 days' incubation, the test vessels are examined for evidence of microbial growth. Microbial growth should be confirmed by subculture and Gram stain.

### 2.2. Growth promotion and test interference

The sterility of the media should be confirmed by incubating representative containers at the appropriate temperature for the length of time specified for each test.

The ability of the culture media to support growth in the presence and absence of product, product components, cells, seeds, or other test material should be validated for each product to be tested, and for each new batch or lot of culture media.

To test for ability to support growth in the absence of the test material, media should be inoculated with 10–100 viable control organisms of the suggested American Type Culture Collection (ATCC) strains listed in Table 1 and incubated according to the conditions specified.

To test for ability of the culture media to support growth in the presence of the test material, containers should be inoculated simultaneously with both the test material (see Section J.2.3) and 10–100 viable control organisms. The number of containers used should be at least one-half the number used to test the product or product component. The test media are satisfactory if clear evidence of growth of the control organisms appears in all inoculated media containers within 7 days. In the event that growth is evident, the organism should be identified to confirm that it is the organism originally added to the medium. The sterility test is considered invalid if any of the media show inadequate growth response, or if the organism recovered is not the organism used to inoculate the material.

### 2.3. Number of items to be tested

The number of items in a batch determines the number of containers that should be tested for sterility. If the batch size is not more than 100, then 10% or four containers, whichever is the greater, should be tested. If the batch contains between 100 and 500 containers, then ten containers should be tested. If the batch has more than 500 containers, then 2% or 20 containers, whichever is the lesser, should be tested. An alternative is to test a maximum of 10 containers for all serials other than autogenous products.

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\(^1\) American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, USA.
The amount of sterility test inoculum is dependent on the quantity of biological in each container. If the quantity is less than 1 ml, then the entire contents are used for each medium. If the quantity is from 1 to 4 ml, then half the contents are used in each medium. If the quantity is from 4 to 20 ml, then 2 ml inoculum per medium is used. If the quantity in each container is from 20 to 100 ml, then 10% of the contents are used per medium. If the quantity per container is greater than 100 ml, then 10% or 50 ml, whichever is the greater, is used to inoculate each medium.

2.4. Interpretation of sterility test results

If growth is found in any medium but it can be demonstrated by controls that the media or technique were faulty, then the first test is declared invalid and may be repeated. If microbial growth is found in any of the test vessels of the first test but there is no evidence invalidating it, then a retest may be conducted. The minimum number of biological containers, test vessels, and membrane filters in a retest is double the number used in the first test. If no growth is found in the first test or retest, the biological meets the requirements of the test and is considered satisfactory for sterility. If microbial growth is found in any of the retest vessels, the biological is considered unsatisfactory for sterility. If, however, it can be demonstrated by controls that the media or technique of the retest were faulty, then the retest may be repeated.

2.5. General procedure for testing live viral vaccines produced in eggs and administered through drinking water, spray, or skin scarification for the presence of bacteria and fungi

Each batch of final container biological should have an average contamination of not more than one bacterial or fungal colony per dose for vaccines recommended for poultry, or ten colonies per dose for other animals (see Section J.2.3 above to determine the number of samples to test). From each container sample, each of two Petri dishes are inoculated with vaccine equal to ten doses if the vaccine is recommended for poultry, or one dose if recommended for other animals. To each plate add 20 ml of brain–heart infusion agar containing 0.007 IU (International Units) of penicillinase per ml. One plate should be incubated at 30–35°C for 7 days and the other at 20–25°C for 14 days. Colony counts are made at the end of each incubation period. An average colony count of all the plates representing a batch should be made for each incubation condition. If the average count at either incubation condition exceeds one colony per dose for vaccines recommended for poultry, or ten colonies per dose for vaccines recommended for other animals in the initial test, one retest to rule out faulty technique may be conducted using double the number of unopened final containers. If the average count at either incubation condition of the final test for a batch exceeds one colony per dose for vaccines recommended for poultry, or ten colonies per dose for vaccines recommended for other animals, the batch of vaccine should be considered unsatisfactory.

2.6. General procedure for testing seed lots of bacteria and live bacterial biologicals for purity

Each seed lot of bacteria or batch of live bacterial biological should be tested for purity by inoculation of SCDM, which is incubated at 20–25°C for 14 days, and FTM, which is incubated at 30–35°C for 14 days (see Section J.2.3 above to determine the number of samples to be tested and the amount of test inoculum to be used). A sterile pipette or syringe and needle is used to aseptically transfer the quantity of biological directly into the two types of culture medium. The minimum ratio of inoculum to culture medium is 1/15.

If the inoculum or growth of the bacterial vaccine renders the medium turbid so that the absence of atypical microbial growth cannot be determined by visual examination, subcultures should be made from all turbid tubes on day 3 through to day 11. Subculturing is done by transferring 0.1–1.0 ml to differential broths and agar and incubating for the balance of the 14-day period. Microscopic examination by Gram stain should also be done.

If no atypical growth is found in any of the test vessels when compared with a positive control included in the test, the lot of biological may be considered satisfactory for purity. If atypical growth is found but it can be demonstrated by control that the media or technique were faulty, then the first test may be repeated. If atypical growth is found but there is no evidence invalidating the test, then a retest may be conducted. Twice the number of biological containers and test vessels of the first test are used in the retest. If no atypical growth is found in the retest, the biological is considered to be satisfactory for purity. If atypical growth is found in any of the retest vessels, the biological is considered to be unsatisfactory for purity. If, however, it can be demonstrated by controls that the media or technique of the retest were faulty, then the retest may be repeated.

3. Detection of Mycoplasma contamination

3.1. General procedure for detecting Mycoplasma contamination

Each batch of live viral vaccine, each lot of master seed virus (MSV), each lot of primary and master cell stock (MCS), and all ingredients of animal origin not steam sterilised should be tested for the absence of mycoplasmas. Solid and liquid media that will support the growth of small numbers of test organisms, such
as typical contaminating organisms *Acholeplasma laidlawii*, *Mycoplasma arginini*, *M. fermentans*, *M. hyorhinis*, *M. orale*, and *M. synoviae* should be used. The nutritive properties of the solid medium should be such that no fewer than 100 CFU should occur with each test organism when approximately 100–200 CFUs are inoculated per plate. An appropriate colour change should occur in the liquid media when approximately 20–40 CFUs of each test organism are inoculated. The ability of the culture media to support growth in the presence of product should be validated for each product to be tested, and for each new batch or lot of culture media.

One sample of each lot of vaccine, MSV, etc., should be tested. Inoculate each of four plates of solid medium with 0.25 ml of the sample being tested, and inoculate 100 ml of the liquid medium with 10 ml of the sample. An alternative is to inoculate each of the plates with 0.1 ml and to inoculate 100 ml of liquid medium with 1 ml of the sample being tested. Incubate two plates at 35–37°C aerobically (an atmosphere of air containing 5–10% CO₂ and adequate humidity) and two plates anaerobically (an atmosphere of nitrogen containing 5–10% CO₂ and adequate humidity) for 28 days. On day 3 or day 4 after inoculation, subculture 0.25 ml from the liquid media on to two plates of solid media. Incubate one plate aerobically and the second anaerobically at 35–37°C until day 28 of the test. Repeat the subculture procedure on day 6, 7, or 8 and again on day 13 or 14. An alternative method is to subculture on days 3, 5, 10, and 14 on to a plate of solid medium. All the subculture plates are incubated for 10 days except for the 14-day subculture, which is incubated for 14 days. Observe the liquid media every 2–3 days and, if any colour change occurs, subculture immediately.

3.2. Interpretation of *Mycoplasma* test results

At the end of the incubation period (day 28), examine all the inoculated solid media microscopically for the presence of mycoplasma colonies. The test sample passes the test if the growth of mycoplasma colonies has occurred on the positive controls, and if growth has not occurred on any of the solid media inoculated with the test material. If at any stage of the test, more than one plate is accidentally contaminated with bacteria or fungi, or is broken, the test is invalid and should be repeated. If mycoplasma colonies are found on any agar plate, the test should be repeated once to confirm the mycoplasma contamination. Twice the volume (0.5 ml) of biological material being tested may be used in the retest. If mycoplasma colonies are found on any of the agar plates of the retest, the test sample should be considered unsatisfactory because of mycoplasma contamination. Some mycoplasmas cannot be cultivated, in which case the MSV and MCS have to be tested using an indicator cell line (Vero cells), DNA staining, or polymerase chain reaction (PCR) methods.

4. Detection of *Salmonella* contamination

Each batch of live virus biological made in eggs should be free from contamination with *Salmonella*. This testing must be done before bacteriostatic or bactericidal agents are added. Five samples of each batch should be tested; 5 ml or one-half of the container contents, whichever is the lesser, of the sample should be used to inoculate 100 ml of tryptose broth and tetrathionate broth. The inoculated broths should be incubated for 18–24 hours at 35–37°C. Transfers from these broths should be made on to MacConkey and Salmonella–Shigella agar, incubated for 18–24 hours, and examined. If no growth typical of *Salmonella* is noted, the agar plates should be incubated an additional 18–24 hours and again examined. If colonies typical of *Salmonella* are observed, further subculture on to suitable differential media should be made for positive identification. If *Salmonella* is found, the batch of biological is unsatisfactory.

5. Detection of viruses in biological materials

Biological materials subject to viral contamination that cannot be sterilised before use, such as ingredients of animal origin (for example, serum), primary cells, line cells or viral seed stocks, should be tested before they are used. Assays are described to detect viral contaminants by cytopathic effects (CPE), haemadsorption, haemagglutination, fluorescent antibody techniques and other suitable methods, e.g. PCR and enzyme-linked immunosorbent assay. All biological materials should be specifically tested for pestviruses. Avian materials and vaccines should be inoculated on to primary avian cell cultures, eggs and/or chicks for the detection of avian viruses. In addition to examining for CPE and cellular abnormalities in these inoculated cells/eggs/chicks, tests for haemadsorbing and haemagglutinating viruses should also be included.

Cells shall be tested in the following manner. On day 0, primary or frozen cells to be tested are seeded on 75 cm² (or similar) flasks; 7 days later, at least two 75 cm² flasks are prepared. On day 14, one flask is used to test the cells for cytopathology, haemadsorption, and fluorescent antibody staining (procedures follow). The other flask is passaged a second time, and on day 21 is subjected to three freeze–thaw cycles. An alternative method is to freeze–thaw the cells at 26 days instead of 21 days. After the third freeze–thaw cycle, the cells are centrifuged at 2000 g for 10 minutes, and the supernatant is used to inoculate appropriate virus-sensitive cells, i.e. cells susceptible to viruses that may be present in the species of animal from which the cells were obtained, cells susceptible to viruses that may occur in the animals in which the material is going to be used and cells...
susceptible to pestiviruses. These cells are then passed twice at 7-day intervals, and tested for cytopathology, haemadsorption and by fluorescent antibody staining.

Ingredients of animal origin are tested on both African green monkey kidney (Vero) cells and on a cell line or primary cells derived from the same species as the ingredient under test. Cells are inoculated using 75 cm² flasks with 3.75 ml of test material in 25 ml of media or 15% of the test material, whichever is the lesser. The cells are passaged two or three times at 7-day intervals, and tested for cytopathology, haemadsorption and fluorescent antibody staining. The cells should be observed for cytopathology every 2 to 3 days, and prior to each subculture, throughout the incubation period.

MSV are tested on Vero cells, cell lines or primary cells of the species for which the product is intended, and cell lines or primary cells of the species in which the product is prepared (if different from the intended species).

For each cell type required for testing, 1 ml of the test MSV is thawed or reconstituted and neutralised with the addition of 1 ml monospecific antiserum. The serum must be shown to be free from antibodies against any of the contaminants for which the test is intended. Antisera should also be tested for nonspecific inhibiting affects. At least two cell types are always required, so a minimum of 2 ml of MSV and 2 ml of antiserum are required. The antiserum is allowed to neutralise the MSV at room temperature for 1 hour. Of the MSV/antiserum mixture, 2 ml is then inoculated onto a 75 cm² flask of the appropriate cells. If the MSV is known to be high-titred or is a difficult agent to neutralise, or if the blocking serum is known to be low-titred, the blocking antiserum can be added to the growth medium at a final concentration of 1–5%. The cells should be passaged at least twice over a 14-day period, and the final culture is examined for cytopathology, haemadsorption and by fluorescent antibody staining.

The May–Grünwald–Giemsa staining procedure is usually used to detect cytopathology caused by extraneous viruses. Monolayers are usually prepared on two-chambered tissue culture slides and incubated for 7 days. The plastic wells of the slides are removed leaving the rubber gasket attached to the slide. The slides are rinsed in warm Dulbecco’s phosphate buffered saline (PBS), fixed in alcohol and placed on a staining rack. The slides are stained for 15 minutes at room temperature with May–Grünwald stain diluted 1/5 with absolute methanol. The May–Grünwald stain is removed by inverting the slides. The slides are then stained for 20 minutes with Giemsa stain diluted 1/15 in deionised water. The Giemsa stain is removed by inverting the slide. The slides are then stained for 10–20 seconds. The slides are air-dried, and paraffin oil and a cover-slip are applied. The May–Grünwald–Giemsa stain will differentially stain DNA and RNA nucleoproteins. DNA nucleoproteins stain red-purple, while RNA nucleoproteins stain blue. The monolayers are examined with a conventional microscope for the presence of inclusion bodies, an abnormal number of giant cells, or other cytopathology attributable to a viral contaminant. The inoculated monolayers are compared with the noninoculated monolayers. If specific cytopathology attributable to an extraneous virus is found, the test material should be considered unsatisfactory.

Testing to detect extraneous viruses that produce haemadsorption in infected cells is usually carried out on monolayers of the second passage of test-material-inoculated cell cultures and noninoculated cell cultures. The monolayers are on 75 cm² plastic flasks. Guinea-pig, chicken, and any other blood for use in this assay is collected in an equal volume of Alsever’s solution. The blood may be stored at 4°C for up to 7 days if it is washed several times in Alsever’s solution before storage in an equal volume of Alsever’s. Just prior to use, the stored erythrocytes are again washed by adding 5 ml of blood in Alsever’s solution to 45 ml of calcium- and magnesium-free PBS and centrifuging in a 50 ml centrifuge tube at 500 g for 10 minutes. The supernatant is removed by suction and the erythrocytes are suspended in PBS and recentrifuged. This washing procedure is repeated at least twice until the supernatant is clear. Erythrocytes from each species are combined by adding 0.1 ml of each type of packed blood cells to 100 ml of PBS. The erythrocytes from different species may be kept separate or combined, as desired. To each flask, add 5 ml of the erythrocyte suspension, and incubate the flasks at 4°C for 30 minutes. The monolayers are washed twice with PBS and examined for haemadsorption. If no haemadsorption is apparent, 5 ml of the erythrocyte suspension is added to each flask, the flasks are incubated at 20–25°C for 30 minutes, rinsed as before, and examined for haemadsorption. Separate flasks may be used for each incubation temperature if desired. Monolayers are examined for the presence of haemadsorption both grossly (using an illuminated glovebox) and microscopically. It is important to compare the noninoculated monolayers with the test monolayers to detect nonspecific haemadsorption that may occur with some cell types. The use of calcium- and magnesium-free PBS and fresh erythrocytes should prevent most nonspecific haemadsorption from occurring. If specific haemadsorption attributable to an extraneous agent is found, the test material should be considered to be unsatisfactory.

Tests to detect extraneous viruses by fluorescent antibody usually use monolayers of the second passage of test-material-inoculated cell cultures and noninoculated cell cultures. The monolayers are usually on eight-chamber tissue culture slides. One positive control slide (consisting of eight monolayers) is made for each antiviral conjugate by inoculating each monolayer with approximately 100 TCID₅₀ (50% tissue culture infective dose) of the appropriate virus. Three groups of monolayers are stained with each antiviral conjugate. They are Group 1 – the second passage of test-material-inoculated cell cultures; Group 2 – the second passage of the noninoculated cell cultures; and Group 3 – the second passage of noninoculated cell cultures (for the production of positive control cell cultures). At the time of staining, the plastic walls of the slides are removed, leaving the rubber gasket
attached to the slide. The slides are rinsed in Dulbecco's PBS, fixed for at least 10 minutes in acetone at 4°C, and dried. Approximately 0.1 ml of each conjugate is placed on each well of one slide from Groups 1, 2, and the corresponding positive control slide from Group 3. The slides are incubated in a humidified chamber at 37°C for 30 minutes, rinsed once in Dulbecco's PBS, and placed in a container of Dulbecco's PBS for 10 minutes. The slides are rinsed thoroughly in deionised water and dried. All slides are examined for fluorescence attributable to each specific virus. The three slides from each group with the same conjugate are compared. If the slide prepared from cells inoculated with test material shows any evidence of specific viral fluorescence, the MSV should be considered unsatisfactory.

REFERENCES


EUROPEAN PHARMACOPOEIA 7.0. (2012). European Directorate for the Quality of Medicines and HealthCare (EDQM), Council of Europe, Strasbourg, France.


FURTHER READING

Details of methods and culture media will be found in the following books, and also in commercial catalogues.


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APPENDIX 1.1.9.1.

RISK ANALYSIS FOR BIOLOGICALS FOR VETERINARY USE OTHER THAN VACCINES

INTRODUCTION

For the purpose of this chapter, the term ‘biologica ls’ means ‘biologica ls for veterinary use other than veterinary vaccines’.

CATEGORISATION OF BIOLOGICALS

Categorisation provides a means of facilitating risk analysis for the international trade in biologicals.

The categorisation system should take into account the source, the nature and the stated purpose of the biologicals. By conducting generic risk analyses, and by developing generic certification and quality assurance, continued supply of products can be made available without the need for repeated risk assessments that are expensive and consume significant resources. Once made, the risk assessment can be linked to appropriate manufacturing and testing parameters. Categories of biologicals for veterinary use into which generic risk assessments could apply may include (not in order of risk):

1. synthetic material;
2. amino acids, alcohols, esters, sugars and vitamins;
3. cosmetics;
4. plant extracts and processed biochemicals of plant origin;
5. products derived by microbial fermentation;
6. diagnostic, analytical and immunochemical kits for in-vitro use;
7. material of human origin;
8. therapeutics;
9. implantables of animal origin;
10. antibodies and immunoglobulins;
11. deoxyribonucleic acid (DNA), ribonucleic acid (RNA), restriction enzymes and other products of molecular biology;
12. cell-lines and hybridomas;
13. animal proteins, hormones, enzymes, albumins, tissue extracts and culture media containing animal material;
14. animal serum;
15. micro-organisms (conventional or genetically modified);
16. probiotics;
17. preserved specimens, microscope slides and smears.

All of these materials may contain pathogens depending on their source and processing procedures.
INFORMATION TO BE SUBMITTED WHEN APPLYING FOR AN IMPORT LICENCE

When undertaking risk analysis for biologicals, Veterinary Authorities should follow the *Terrestrial Manual*. The manufacturer or the Veterinary Authority of the exporting country should make available detailed information, in confidence if necessary, on the source of the materials used in the manufacture of the product (e.g. substrates). They should make available details of the method of manufacture (and where appropriate inactivation) of the substrates and component materials, the quality assurance procedures for each step in the process, final product testing regimes, and the pharmacopoeia with which the product must conform in the country of origin. They should also make available challenge organisms, their biotypes and reference sera, and other means of appropriate product testing.

RISK ANALYSIS PROCESS

Risk analysis should be as objective and transparent as possible and should be performed in accordance with Section 2 of the *Terrestrial Code*, and certification in line with Section 5 of the *Terrestrial Code*. Of necessity, assessment of the country and commodity factors and risk reduction measures will be based largely on manufacturers’ data. These data depend on quality assurance at all stages of manufacture, rather than on testing of the final product alone.

Domestic exposure may be influenced by the approved usage of the product. Veterinary Authorities may place limits on usage of some products (e.g. restricting usage to institutions of appropriate biosecurity).

BIOCONTAINMENT

Suitable biocontainment may be necessary for many forms of biologicals. In particular, the importation of exotic micro-organisms should be carried out in accordance with Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities*.

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CHAPTER 1.1.8.

MINIMUM REQUIREMENTS FOR VACCINE PRODUCTION FACILITIES

Chapter under study

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CHAPTER 1.1.9.

QUALITY CONTROL OF VACCINES

Chapter under study

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CHAPTER 1.1.10.
INTERNATIONAL STANDARDS FOR VACCINE BANKS

INTRODUCTION

Emergency vaccination is one of several measures that may be deployed to control outbreaks of disease as it provides a valuable adjunct to the application of the essential zoosanitary measures. These include rapid diagnosis, tracing, movement control and disinfection, and may also include slaughter of infected and in-contact animals.

The terms ‘emergency vaccine’ and ‘emergency vaccination’ can have different connotations, but are usually applied to differentiate between routine, prophylactic (preventive) vaccination and emergency vaccination, the latter being applied as an immediate response to an outbreak of disease. Emergency vaccination may be applied in a number of different circumstances and in a number of different ways, including the following:

a) Against an outbreak of disease in a country that is normally free of this disease and that does not normally vaccinate against this disease. It may be applied as Ring Vaccination or Barrier Vaccination, outside of and around a focus of the disease to inhibit outward spread.

b) Against an outbreak of disease in a neighbouring country or region when emergency Barrier Vaccination may be applied along the border in the country or region that is at risk.

c) As a complimentary measure in a stamping-out policy, when emergency vaccination is applied to the animal population around an outbreak location, usually within the protection zone in which outbreaks have occurred, by so-called Suppressive or Dampening Down Vaccination. This is a form of ring vaccination that is followed by killing of the vaccinated animals.

d) Against an outbreak of disease in a country that does normally vaccinate but where emergency vaccine is applied to boost existing immunity.

e) Against an outbreak of disease in a country that does normally practice preventative vaccination, but where the vaccine(s) employed do not provide protection against the strain involved in the outbreak.

Criteria that determine the successful application of emergency vaccination include rapid access to vaccine(s) that (i) contain virus strain(s) of sufficient antigenic relatedness to the outbreak strain(s) (ii) are of the required type of vaccine formulation (iii) have acceptable safety and potency (iv) have appropriate availability, including quantity and immediacy of supply and (v) meet considerations of cost. The evident need to hold strategic reserves, or banks, of such valuable commodities is best exemplified by foot and mouth disease (FMD) vaccines. They are specified in contingency plans for use in an FMD outbreak and have led to an escalation in the establishment of national and international FMD vaccine reserves for use all over the world (Forman & Garland, 2002), providing assurance that vaccine would be readily available and at the disposal of the country requiring it.

Emergency FMD vaccines are normally formulated to a higher potency than its conventional counterpart and there are banks who stipulate a requirement of at least 6 PD$_{50}$ (50% protective dose) per dose for cattle in contrast to the minimal statutory requirement of 3PD$_{50}$. Higher potency can be achieved by simply increasing the antigen payload per dose and its benefits can include rapidity, magnitude and duration of the protective response (Barnett et al., 2004; Rweyemamu et al., 1984). However, conventional vaccines may also be used in an emergency, particularly when vaccine of appropriate strain composition is immediately available or where revaccination might be desired in an already pre-immune population.
Chapter 1.1.10. — International standards for vaccine banks

The concept of vaccine banks, exemplified by FMD, and the increased reliance on such banks is indicative of it being a very practical adjunct to other control measures that could usefully be adopted for a number of other diseases such as bluetongue, classical swine fever and avian influenza.

DEFINITION OF A VACCINE BANK

Strategic reserves, or banks as they are more commonly referred, are of two types. They may hold the final end product, a ready-to-use formulated vaccine, and/or the antigen component, which can be stored for a very long time for subsequent formulation into vaccine as and when required. The latter has been more commonly adopted for FMD because of the economic benefits, and this avoids constantly replacing vaccines that exceed their shelf-life. Stockpiles of antigens, or ready-to-use vaccine, will be referred to as vaccine banks in this chapter.

TYPES OF VACCINE BANKS

A country may hold its own national bank and/or it may be part of a larger group of countries that have drawing rights and share a bank such as exemplified by the North American or European Union FMD vaccine banks. Such consortiums may share a common geographical region, or have similar disease status and approach to control. The bank may be held on the territory of one or several of its members or be retained by the manufacturer, and, if held as antigen, would be formulated for use either by the manufacturer, or in a dedicated facility maintained by the bank members. However, in the latter case, the recent increasing demands by licensing authorities to require the same standards of independent manufacturing facilities as those of the commercial sector with a marketable product, is making this option very difficult. In the case of an antigen bank, a contract between the authorities and the vaccine manufacturer (formulation and filling) has to clearly define the details of formulation of the vaccine, e.g. time between reception of order and delivery, availability of buffers and vials, etc.

The location of stored antigens is of vital importance since the need to formulate vaccine may require antigen to be returned to the original manufacturer, incurring a delay in supply. Even if the antigens are held by the commercial sector, delay following a request for the supply of emergency vaccine might still occur if the manufacturer is currently in the middle of production of a product. The time to produce the vaccine should be about 48–72 hours. Delays in the production and despatch of emergency vaccine to control an outbreak may lead to wider spread of the disease and further difficulty in its control. Formulated vaccine would of course allow for immediate access. However, beside the wasteful and uneconomic implications resulting from regular replacement of the vaccine, it may not always contain the most suitable strain to deal with an outbreak.

The economic benefits of sharing a bank are obvious, but they also provide potential to stockpile greater doses and a wider number of vaccine strains, and reduce the problem of deciding on the introduction of narrow spectrum vaccine strains. Collaboration between vaccine banks would also be an economic way of increasing the amount of emergency vaccine available. Care would be required to ensure that collaborating banks operate to the same standards that drawing rights were clearly defined and that regular contact was maintained between banks to confirm the safety, efficacy and availability of the vaccines. Issues related to regulatory compliance would also need to be addressed at an early stage to ensure that vaccine produced from the bank would be authorised for use in any of the participating countries.

SELECTION OF VACCINES FOR A BANK

Depending on the disease and the likely contingency requirements, a range of vaccine strains may be required. Disease control authorities in consultation with the vaccine bank administrators must decide upon the vaccine strains that should be held and on what basis they should be stored (i.e. as a separate antigen component for subsequent formulation, or as a ready to use formulation). The value of any vaccine bank is very much dependent upon the appropriateness of what it holds for field application, particularly in respect to diseases that are made up of several serotypes and have wider strain variation. The potential of an outbreak not adequately covered by a banked vaccine must be alleviated by continuous monitoring of the global disease situation and recognition that additional vaccine strains may need to be included in the banks’ portfolio or, in the case where no suitable vaccine strain is available, developed speedily for subsequent inclusion.

The world as an interdependent community that encompasses rapid and extensive movement of people, animals and animal products and the increasing awareness of the potential to deliberately introduce disease through bioterrorism heightens the risk of an incursion and makes prediction of specific threat difficult. To improve the process of vaccine selection, a continuous exchange of information and increased co-operation and collaboration
between different international, regional and national laboratories, vaccine manufacturers and the vaccine/antigen banks authorities should be encouraged. Risk analysis studies should be done to classify the virus strains to be stored with the priority level of high, medium and low. Close liaison with national and international reference laboratories is therefore recommended as some laboratories already provide periodic recommendations on strains that should be included in FMD vaccine banks. In the context of the risk of bio-terrorism, disease control authorities may consider it pertinent to restrict the information released relating to the storage of specific stockpiles of antigens and/or vaccines.

**QUANTITIES OF VACCINE REQUIRED IN A BANK**

The decision as to how many doses of vaccine are required is complex and problematic, embracing questions of serotypes, strains, use of mono or polyvalent vaccines, and type of formulation. Factors bearing on the decision include the type of disease, the different circumstances and ways of applying emergency vaccination (items a to e) described in the introduction), number, species and location of livestock that are to be protected, geographical considerations, knowledge of the current and predicted global epidemiological situation, and the analyses of risks of introduction and spread of disease, together with cost–benefit studies. In determining the supply of emergency vaccines, decisions on the quantity of the product inevitably involve a compromise between the cost of purchase and the likely number of doses required. The minimum vaccine requirement might therefore be based on the number of doses that could be distributed and applied in the first week of vaccination, the expectation would be that additional supplies could by then have been procured, either from other banks or from commercial sources. For example, 500,000 bovine doses of different FMD vaccine strains were routinely maintained by the International FMD Vaccine Bank (IVB), and drawing rights by member countries varied from 100,000 to 500,000 bovine doses. Nevertheless, this would soon be exhausted if used in an area of high livestock density.

**ACQUISITION OF VACCINES FOR A BANK**

According to both the type of vaccine bank and the disease concerned, the acquisition of the appropriate vaccine(s) or antigen(s) will depend on whether they are available from the commercial sector or government institutions or produced in-house.

Regulatory concerns on existing, or potential, immunological veterinary medicinal products (IVMPs) and the advisability to use approved, authorised medicines, will predispose a bank to acquire, or maintain, its vaccines and antigens selectively. It is recommended that appropriately licensed manufacturers that have the necessary Marketing Authorisation (MA) and internationally accepted standards of Good Manufacturing Practice (GMP), modern quality assurance (QA) and Qualified Person (QP) product release should be used as authorised sources.

This has certainly been exemplified in recent years by FMD vaccine banks in which there has been a strong preference for purchasing and holding antigens/vaccines within the commercial manufacturing sector and thus avoiding the expense and difficulties of maintaining a dedicated ‘licensed’ facility compliant with GMP for the purpose of formulation in an emergency.

Disease control authorities should consider the option of requesting a tender for antigens/vaccine from more than one supplier, particularly where regulatory considerations are of paramount importance, and they may wish to seek advice from appropriate licensing authorities on the necessary standards required. Request for tenders can then ensure not only a competitive price but a veterinary medicinal product manufactured to an acceptable level of quality. It should also establish suppliers that can produce the desired vaccines/antigens and dose amounts within a specified time period that meet necessary, or indeed mandatory, tests of compliance such as safety and efficacy.

Where the requirement is to hold antigens/vaccines at a site other than at the principle site of manufacture, disease control authorities may wish to consider only accepting them after they have been shown to have passed the necessary acceptance testing procedures such as safety and/or efficacy. Alternatively, if the antigen/vaccine has to be located in the bank prior to completion of any acceptance testing, then the antigen/vaccine should be stored apart and labelled as quarantined material until the testing shows full compliance to the vaccine banks requirements.

**REGULATORY STANDARDS — SAFETY, EFFICACY AND QUALITY**

Regulatory requirements for a veterinary medicinal product must be considered by any country wishing to have the necessary authorisation to use emergency vaccine in an outbreak situation. For example, all veterinary
medicinal products that are placed on the market in the European Union (EU) must hold a marketing authorisation and the EU lays down the requirements for such authorisations. The EU also has emergency provisions under Articles 7 and 8 that permit release of a vaccine without an authorisation in the country requiring it. However, a more recent EU Directive 2003/85/EC on current and future policy on control of FMD places more emphasis on the use of vaccines as part of a vaccinate-to-live policy. This makes the issue of an authorised product even more essential, particularly where vaccinated animals are intended for the food chain and require the support of agencies responsible for human health. Therefore, it is important that licensed products be used; unlicensed products are very much a last resort.

Quality, safety and efficacy are therefore all the more important and these will vary depending on the disease. Where particular immunologicals are covered by individual monographs in official Pharmacopoeias (e.g. FMD vaccine in the European Pharmacopoeia – Monograph 63) then the standards for Safety, Efficacy, Sterility and Quality are laid down. For the other case where the immunological comes under the Pharmacopoeia general section on Vaccines for Veterinary Use then those minimum standards should apply, and disease control authorities may wish to add, to the minimum standards, other individual requirements. These standards might include antigen strain identity, freedom from adventitious agents, innocuity, absence of toxicity, quantity of antigen payload per dose, safety, potency and sterility, and manufacture in officially approved quality assured (QA) good manufacturing practice (GMP) premises.

Any adjuvant or pharmacologically active ingredient used in a formulation must also conform to the necessary guideline requirements including residues in food-producing species.

Differentiating between animals that have been vaccinated and animals that have either recovered from infection or that have acquired sub-clinical infection post-vaccination may also be an important issue, as is the case for FMD. The detection of antibodies to non-structural polyproteins (NSPs) such as 3ABC of FMDV has been shown to be a sensitive and specific method to differentiate between infection and vaccination. This relies on manufacturing methods whereby the NSP component can be reduced to a level that will not cause detectable sero-conversion following vaccination making purity of vaccine an important consideration.

**STORAGE OF VACCINES/ANTIGENS IN A BANK**

It is important that the areas of storage chosen to hold emergency antigens/vaccines are suitable in the context of the required national or internationally accepted standards of GMP. This is usually covered when a bank is held in a "licensed" and routinely inspected vaccine plant. However, if the bank is located outside a nominated vaccine formulation facility, regulatory considerations again may be of paramount importance and Disease Control Authorities may wish to seek advice from appropriate licensing authorities on the necessary standards required.

If the vaccine bank is associated with a laboratory, or other facility, where pathogens are handled, this should be completely independent of the bank storage facilities, and maintenance and monitoring personnel should obey a quarantine period before entering the bank.

Appropriate storage of antigens/vaccines in an emergency reserve will be very much dependent on the disease to which they are targeted. The antigen may be a chemically inactivated or killed virus, for example such as that used in FMD vaccines, or it may be an attenuated vaccine such as that exemplified by Bluetongue vaccines. The antigens themselves may be concentrated and held at ultra-low temperature, over liquid nitrogen for example, or may be a freeze dried commodity where low temperature is not necessarily important. Whatever the method of storage, it is vitally important that they are optimally maintained and routinely monitored in order to have some assurance that they will be efficacious when needed. Managers of vaccine banks should therefore ensure that the necessary arrangements are in place to monitor their reserves on a routine basis and to include, where necessary and at appropriate time intervals, a testing regime to ensure integrity of the antigen component or acceptable potency of the final product. For example, 24-hour storage temperature may be recorded as well as periodic inspection of the bottles containing the antigen for cracks or leakage. In this context, managers may wish to also consider the possibility of independent testing, or of greater reliance on overseeing/auditing of the manufacturer's test procedures.

The need for routine testing of stocks for stability is evident, and therefore depositories of antigens/vaccines should include a large number of small samples that are representative of the larger stock for such purposes stored side by side with it.

Whilst not directly related to the establishment, storage and operation of vaccine or antigen banks, Countries should nevertheless recognise the importance of contingency planning to ensure that the stored vaccine, if required, is distributed and administered in an efficient and prompt manner. They should make certain that the necessary cold-chain facilities are available, that vaccination protocols are defined in advance, that vaccination teams are established and trained appropriately and that all the other necessary documentation, equipment,
reagents and clothing is stockpiled to sufficient levels to support any potential vaccination campaign. Therein the benefits of also performing periodic exercises and simulations should not be overlooked.

It would be advisable for member countries to monitor the literature published on important advances that are being made in subjects relating to vaccine bank technology. On-going research does lead to improvements of product, equipment, manufacture and distribution and therein more efficient and practical use of Banks. In this context there has been a recent study examining methods of prolonging the storage of fully formulated vaccine by a novel formulation procedure (Barnett & Statham, 2002).

REFERENCES


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PART 2

OIE LISTED DISEASES AND OTHER DISEASES OF IMPORTANCE TO INTERNATIONAL TRADE
SECTION 2.1.
MULTIPLE SPECIES

CHAPTER 2.1.1.
ANTHRAX

SUMMARY

Definition of the disease: Anthrax is primarily a disease of herbivorous animals, although all mammals, including humans, and some avian species can contract it. Mortality can be very high, especially in herbivores. The aetiological agent is the spore-forming, Gram-positive rod-shaped bacterium, Bacillus anthracis. The disease has world-wide distribution and is a zoonosis.

Description of the disease: The disease is mediated mainly by exotoxins. Peracute, acute, subacute and, rarely, chronic forms of the disease are reported. Ante-mortem clinical signs may be virtually absent in peracute and acute forms of the disease. Subacute disease may be accompanied by progressive fever, depression, inappetence, weakness, prostration and death. Acute, subacute, and chronic disease may show localised swelling and fever. In chronic disease, the only sign may be enlarged lymph glands.

Identification of the agent: Bacillus anthracis is readily isolated in relatively high numbers from blood or tissues of a recently dead animal that died of anthrax, and colony morphology of B. anthracis is quite characteristic after overnight incubation on blood agar. The colony is relatively large, measuring approximately 0.3–0.5 cm in diameter. It is grey-white to white, non-haemolytic with a rough, ground-glass appearance and has a very tacky, butyrous consistency. The vegetative cells of B. anthracis are large, measuring 3–5 µm in length and approximately 1 µm in width. Ellipsoidal central spores, which do not swell the sporangium, are formed at the end of the exponential cell growth phase. The cells stain strongly Gram positive, and long chains are often seen in vitro, while paired or short chains are seen in vivo. Visualisation of the encapsulated bacilli, usually in large numbers, in a blood smear stained with polychrome methylene blue (M’Fadyean reaction) is fully diagnostic.

Serological tests: Antibody detection in serum from infected animals is rarely used for diagnostic purposes and is essentially a research tool. The predominant procedure used is the enzyme-linked immunosorbent assay (ELISA).

Requirements for vaccines: The most widely used livestock anthrax vaccine developed by Max Sterne in 1937, is a live, non-encapsulated, spore former held in suspension. In Russia and Eastern Bloc countries, an equivalent type of vaccine is used (strain 55). A list of producers is given in the World Health Organization anthrax guidelines.

A. INTRODUCTION

Anthrax, an acute bacterial disease primarily of herbivores, is transmissible to humans. The aetiological agent, Bacillus anthracis, is a Gram-positive spore-forming rod-shaped bacterium. Anthrax is known by many names around the world including charbon, woolsorters disease, ragpickers disease, malignant carbuncle, malignant pustule and Siberian ulcer.

Animals become infected by ingesting spores, or possibly by being bitten by flies that have fed on an infected animal or carcass. Infected animals are usually found dead as death can occur within 24 hours. A careful post-
mortem examination of recently dead animals may show any number of lesions, none of which is pathognomonic or entirely consistent. To avoid environmental contamination, post-mortem examinations of carcasses of animals suspected to have died of anthrax are discouraged. Lesions most commonly seen are those of a generalised septicemia often accompanied by an enlarged spleen having a ‘blackberry jam’ consistency and poorly clotted blood. Haemorrhage from the nose, mouth, vagina and/or anus at death may be found.

Gram-positive rod-shaped \( B. \text{anthracis} \) is an obligate pathogen. Most of the other species of \( B. \text{bacillus} \) are common ubiquitous environmental saprophytes, although a number, notably \( B. \text{cereus}, B. \text{licheniformis} \) and \( B. \text{subtilis} \), are occasionally associated with food poisoning in humans and with other clinical manifestations in both humans and animals.

1. Zoonotic risk and biosafety requirements

More than 95% of human anthrax cases take the cutaneous form and result from handling infected carcasses or hides, hair, meat or bones from such carcasses. \( B. \text{anthracis} \) is not invasive and requires a lesion to infect. Protection for veterinarians and other animal handlers involves wearing gloves, and other protective clothing when handling specimens from suspected anthrax carcasses and never rubbing the face or eyes. The risk of gastrointestinal anthrax may arise if individuals eat meat from animals infected with anthrax.

The risk of inhaling infectious doses becomes significant in occupations involving the processing of animal by-products for manufacturing goods (industrial anthrax). These include the tanning, woollen, animal hair, carpet, bone processing, and other such industries, where the potential for aerosolisation of substantial numbers of spores increases the risk of exposure to infectious doses. It is important that industrial workers use appropriate personal protective clothing and equipment and follow standard operating procedures that minimise the risk of transmission. Efficient air extraction equipment should be positioned over picking, combing, carding and spinning machines. Air blowing machinery should never be used for cleaning equipment due to the risk of spore dispersal.

Clinical specimens and cultures of \( B. \text{anthracis} \) should be handled at biosafety level 3 as outlined in Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities. Vaccination of laboratory personnel is recommended.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Demonstration of encapsulated \( B. \text{anthracis} \) in smears of blood or tissues from fresh anthrax-infected carcasses and growth of the organism on blood agar plates is relatively uncomplicated and within the capability of most bacteriology laboratories. Difficulty may be encountered in the case of pigs and carnivores in which the terminal bacteraemia is frequently not marked, or in animals that received antibiotics before death.

Recovery of \( B. \text{anthracis} \) from old decomposed carcasses, processed specimens (bone meal, hides), or environmental samples (contaminated soil) is often difficult, requiring demanding and labour-intensive procedures. However live spores may be recovered from the turbinate bones of dead livestock and wildlife for an extended period after death (M. Hugh-Jones, personal communication).

a) Culture and identification of \( B. \text{anthracis} \)

i) Fresh specimens

\( B. \text{anthracis} \) grows readily on most types of nutrient agar, however, 5–7% horse or sheep blood agar is the diagnostic medium of choice. Blood is the primary clinical material to examine. Swabs of blood, other body fluids or swabs taken from incisions in tissues or organs can be spread over blood agar plates. After overnight incubation at 37°C, \( B. \text{anthracis} \) colonies are grey-white to white, 0.3–0.5 cm in diameter, non-haemolytic, with a ground-glass surface, and very tacky when teased with an inoculating loop. Tailing and prominent wisps of growth trailing back toward the parent colony, all in the same direction, are sometimes seen. This characteristic has been described as a ‘medusa head’ or ‘curled hair’ appearance. Confirmation of \( B. \text{anthracis} \) should be accomplished by the demonstration of a capsulated, spore-forming, Gram-positive rod in blood culture. Absence of motility is an additional test that can be done.

Anthrax-specific phages were first isolated in the 1950s, and the specifically named gamma phage was first reported in 1955 (Brown & Cherry, 1955) and quickly became the standard diagnostic phage for anthrax. Gamma phage belongs to a family of closely related anthrax phages (World Health Organization [WHO], 2008).

Two tests for confirming the identity of \( B. \text{anthracis} \) are gamma phage lysis and penicillin susceptibility. The typical procedure for these tests is to plate a lawn of suspect \( B. \text{anthracis} \) on a blood or nutrient
agar plate and place a 10–15 µl drop of the phage suspension on one side of the lawn and a 10-unit penicillin disk to the other side. Allow the drop of phage suspension to soak into the agar before incubating the plate at 37°C. A control culture, e.g. the Sterne vaccine or the NCTC strain 10340, should be tested at the same time as the suspect culture to demonstrate the expected reaction for gamma phage lysis and penicillin susceptibility. If the suspect culture is *B. anthracis*, the area under the phage will be devoid of bacterial growth, because of lysis, and a clear zone will be seen around the penicillin disk indicating antibiotic susceptibility. Note that some field isolates of *B. anthracis* may be phage resistant or penicillin resistant. As the performance of the gamma phage lysis assay may be affected by the density of bacterial inoculum, Abshire *et al.* (2005) recommend streaking the suspect culture on the agar plate over several quadrants instead of using a lawn format and inoculating a drop of gamma phage on the first and second quadrants on the plate. If antibiotic or phage resistant *B. anthracis* is suspected then polymerase chain reaction (PCR) diagnostic methods may be applied.

Phage suspensions may be obtained from central veterinary laboratories or central public health laboratories.

The phage can be propagated and concentrated by the following protocol. Store phage at 2–4°C and do not freeze phage as it will quickly become non-viable.

**Stage one**

- a) Spread a blood agar (BA) plate of the Sterne vaccine strain of *B. anthracis*. Incubate overnight at 37°C.
- b) Inoculate approximately 10 ml of nutrient broth (NB) with growth from the BA plate and incubate at 37°C for approximately 4 hours or until just cloudy, then refrigerate.
- c) Spread 100 µl of the culture from step b on three pre-dried BA plates and incubate at 37°C for 30–60 minutes.
- d) Spread 100 µl of the phage suspension to be amplified over the same plates. Incubate at 37°C overnight.
- e) Harvest the phage-lysed growth on the BA plate in 5 ml of NB followed by a second ‘wash’ of 5 ml NB. Incubate at 37°C overnight.
- f) Filter (0.45 µm) and count by dropping 20 µl drops (three drops per dilution) of tenfold dilutions of the filtrate in saline onto lawns of the *B. anthracis* culture prepared as in step c.

**Stage two**

This is essentially the same procedure as Stage one, only uses the filtrate from step f to harvest the phage from the plates.

- g) Prepare three Sterne strain lawns on BA, as in step c. Incubate at 37°C for 30–60 minutes.
- h) Spread 100 µl phage from step f. Incubate at 37°C overnight.
- i) To 9 ml of filtrate from step f, add 1 ml of 10× concentrated NB.
- j) Harvest the phage from step h with 5 ml of the solution from step i, followed by a second 5 ml wash with the rest of the solution from step f.
- k) Add 10 ml of 1× NB.
- l) Incubate at 37°C overnight, filter and count.

**Stage three**

- m) Inoculate 100 ml of brain–heart infusion broth with approximately 2.5 ml of the culture from step b. Incubate on a rotary shaker at 37°C until just turbid.
- n) Add the 20 ml of filtrate from step l and continue incubation overnight.
- o) The resultant filtrate is checked for sterility and titrated in tenfold dilutions on lawns of the vaccine strain as in step f to determine the concentration of the phage. This should be of the order of 10⁸–10⁹ plaque forming units per ml.

**ii) Capsule visualisation**

Virulent encapsulated *B. anthracis* is present in tissues and blood and other body fluids from animals that have died from anthrax. Thin smears may be prepared from blood from ear veins or other peripheral veins, exudate from orifices and, for horses and pigs, from oedematous fluid or superficial lymph nodes in the neck region. However if the animal has been dead more than 24 hours, the capsule may be difficult to detect. The bacteria should be looked for in smears of these specimens that have been dried, fixed either using heat or by dipping the smear in 95–100% alcohol for about 1 minute and...
air dried and then stained with polychrome methylene blue (MacFadyean’s reaction). The capsule stains pink, whereas the bacillus cells stain dark blue. The cells are found in pairs or short chains and are often square-ended (the chains are sometimes likened to a set of railway carriages – so-called ‘box-car’ or ‘jointed bamboo-rod’ appearance). Gram and Giemsa stains do not reveal the capsule. The capsule is not present on B. anthracis grown aerobically on nutrient agar or in nutrient broth, but can be seen when the virulent bacterium is cultured for a few hours in a few millilitres of blood (defibrinated horse or sheep blood seems to work best). Alternatively, the capsule is produced when the virulent B. anthracis is cultured on nutrient agar containing 0.7% sodium bicarbonate and incubated in the presence of CO₂ (20% is optimal, but a candle jar works well). The agar is prepared by weighing nutrient agar base powder required for a final volume of 100 ml but reconstituting the measured agar in only 90 ml of water. Autoclave and cool to 50°C in a water bath. Add 10 ml of a filter-sterilised (0.22–0.45 µm filter) 7% solution of sodium bicarbonate. Mix and pour into Petri dishes. The encapsulated B. anthracis will form mucoid colonies and the capsule can be visualised by making thin smears on microscope slides, fixing, and staining with polychrome methylene blue (MacFadyean’s stain).

Polychrome methylene blue can be prepared as follows: 0.3 g of methylene blue is dissolved in 30 ml of 95% ethanol; 100 ml of 0.01% potassium hydroxide (KOH) is mixed with the methylene blue solution. Ideally, this should be allowed to stand exposed to the air, with occasional shaking, for at least 1 year to oxidise and mature. Addition of K₂CO₃ (to a final concentration of 1%) hastens the ‘ripening’ of the stain, but before it is regarded as diagnostically reliable, its efficacy should be established by testing it in parallel with an earlier, functional batch of stain on bona fide samples. It has been found that stains that give positive reactions with cultures of B. anthracis cultured artificially in horse blood sometimes do not give positive results in the field.

In making smears for staining, only small drops of blood or tissue fluid are needed and a thin, small smear is best. After fixing (either using heat or by dipping the smear in 95–100% alcohol for about 1 minute) and drying, a small (approximately 20 µl) drop of stain is placed on the smear and spread over it with an inoculating loop. After 1 minute, the stain is washed with water, blotted, air-dried and observed initially using the ×10 objective lens under which the short chains appear like short hairs; once found, these can be observed under oil immersion (×1000) for the presence of the pink capsule surrounding the blue/black-staining bacilli. To avoid laboratory contamination, the slide and blotting paper should be autoclaved or left for some hours in a 10% sodium hypochlorite solution.

iii) Other specimens

Identification of B. anthracis from old, decomposed specimens, processed materials, and environmental samples, including soil, is possible but these samples often have saprophytic contaminants that outgrow and obscure B. anthracis on non-selective agars. The following procedure is suggested:

a) The sample is blended in two volumes of sterile distilled or deionised water and placed in a water bath at 62.5 ± 0.5°C for 30–60 minutes. Turnbull et al. (2007) have demonstrated that heat activation of spores can be conducted at a temperature range of 60–70°C with holding times not exceeding 15–30 minutes for best recovery.

b) Tenfold dilutions to 10⁻² or 10⁻³ are then prepared. From each dilution, 10–100 µl are plated on to blood agar and optionally 250–300 µl on to PLET agar (polymyxin, lysozyme, EDTA [ethylene diamine tetra-acetic acid], thallous acetate) (Knisely, 1966; WHO, 2008). All plates are incubated at 37°C.

c) Blood agar plates are examined for typical colonies as previously described after overnight incubation, and the PLET plates are examined after 40–48 hours. Confirmation of the identity of suspect colonies as B. anthracis is done as described above.

PLET medium (Knisely, 1966; WHO, 2008) is prepared by using heart-infusion agar base (DIFCO) made up to the manufacturer’s instructions with the addition of 0.25–0.3 g/litre EDTA and 0.04 g/litre thallous acetate. The mixture is autoclaved and uniformly cooled to 50°C before adding the polymyxin at 30,000 units/litre and lysozyme at 300,000 units/litre. After mixing thoroughly, the agar is dispensed into Petri dishes.

Reports of procedures for direct detection of B. anthracis in soils and other environmental specimens using the PCR are emerging. None of these has become routinely applicable at the present time.

Animal inoculation may be considered for recovery of B. anthracis if all other methods fail. Examples of when this might occur are specimens from animals that received antibiotic therapy before death or environmental samples containing sporostatic chemicals. Due to the increasing concern to eliminate the use of animals for biological testing, this approach should be used as a last resort and only if justified. Adult mice or guinea-pigs are the animals of choice. If the samples involved are soils, the animals should be pretreated, the day before testing, with both tetanus and gas gangrene antiserum. The samples are prepared as described for culturing, including heat-shocking at 62.5°C for 15 minutes. Mice are injected subcutaneously with 0.05–0.1 ml; guinea-pigs are inoculated intramuscularly with up
to 0.4 ml (0.2 ml in each thigh muscle). Any B. anthracis present will result in death in 48–72 hours and the organism can be cultured from the blood as described above.

b) Immunological detection and diagnosis

It needs to be borne in mind that B. anthracis is antigenically very closely related to B. cereus, which is considered a ubiquitous component of the environmental microflora. The only unshared antigens that lend themselves to differentiating these two species by immunological approaches are the anthrax toxin antigens, produced during the exponential phase of growth, and the capsule of B. anthracis. This places considerable constraints on the extent to which immunological methods can be used in routine detection methodology.

i) Ascoli test

Ascoli (1911) published a procedure for the detection of thermostable anthrax antigen in animal tissue being used for by-products. This uses antiserum raised in rabbits to produce a precipitin reaction. The test lacks high specificity, in that the thermostable antigens of B. anthracis are shared by other Bacillus spp., and is dependent on the probability that only B. anthracis would proliferate throughout the animal and deposit sufficient antigen to give a positive reaction. This test appears to be used only in Eastern Europe.

To perform the Ascoli test, put approximately 2 g of sample in 5 ml of saline containing 1/100 final concentration of acetic acid and boil for 5 minutes. The resultant solution is cooled and filtered through filter paper. A few drops of rabbit antiserum (see preparation below) are placed in a small test tube. The filtrate from the previous step is gently layered over the top of the antiserum. A positive test is the formation of a visible precipitin band in under 15 minutes. Positive and negative control specimen suspensions should be included.

Antiserum is prepared in rabbits by the subcutaneous inoculation of Sterne anthrax vaccine on days 1 and 14. On days 28 and 35, the rabbits receive 0.5 ml of a mixture of several strains of virulent B. anthracis not exceeding 10^5 colony-forming units (CFU)/ml suspended in saline. Alternatively, the live virulent bacteria can be inactivated by prolonged suspension in 0.2% formalised saline, but the antigen mass needs to be increased to 10^8–10^9 CFU/ml. The suspension should be checked for inactivation of the B. anthracis before animal inoculation by culture of 0.1 ml into 100 ml of nutrient broth containing 0.1% histidine and, after incubation at 37°C for 7 days, subculture on to blood or nutrient agar. The dose regimen for the formalised suspension after initial vaccination on days 1 and 14 is increasing doses of 0.1, 0.5, 1, and 2 ml given intravenously at intervals of 4–5 days. Following either procedure, a test bleed at 10 days after the last injection should determine whether additional 2 ml doses should be administered to boost the precipitin titre.

ii) Immunofluorescence

While some success has been achieved with immunofluorescence for capsule observation in the research situation (Ezzell & Abshire, 1996), it does not lend itself to routine diagnosis.

c) Confirmation of virulence with the polymerase chain reaction

Confirmation of virulence can be carried out using the PCR. The following instructions are taken from the WHO (2008). Template DNA for PCR can be prepared from a fresh colony of B. anthracis on nutrient agar by suspension of a loop of growth in 25 µl sterile deionised (or distilled) water and heating to 95°C for 20 minutes. Following cooling to approximately 4°C, and brief centrifugation, the supernatant can be used for the PCR reaction.

Examples of suitable primers (Beyer et al., 1996; Hutson et al., 1993) for confirming the presence of the pXO1 and pXO2 plasmids are given in the table below.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer ID</th>
<th>Sequence 5′–3′</th>
<th>Product size</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protective antigen (PA)</td>
<td>PA 5 3048–3029</td>
<td>TCC-TAA-CAC-TAA-CGA-AGT-CG</td>
<td>596 bp</td>
<td>1 mM</td>
</tr>
<tr>
<td></td>
<td>PA 8 2452–2471</td>
<td>GAG-GTA-GAA-GGA-TAT-ACG-GT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capsule</td>
<td>1234 1411–1430</td>
<td>CTG-AGC-CAT-TAA-TCG-ATA-TG</td>
<td>846 bp</td>
<td>0.2 mM</td>
</tr>
<tr>
<td></td>
<td>1301 2257–2238</td>
<td>TCC-CAC-TTA-CGT-AAT-CTG-AG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
PCR can be carried out in 50 µl volumes using the above primers, 200 µM each of dATP, dCTP, dTTP and dGTP, 1.5 mM MgCl₂ and 2.5 units of AmpliTaq DNA polymerase, all in NH₄ buffer, followed by the addition of 5 µl of template DNA. A 2% agarose gel has been found to work best with these small fragments.

Alternatively, ‘Ready-To-Go™’ beads are available. These are premixed, predispensed, dried beads, stable at room temperature, containing all the necessary reagents, except primer and template, for performing 25 µl PCR reactions. The template can be added in a 2.5 µl volume.

The following PCR cycle can be used: 1 × 95°C for 5 minutes; 30 × 95°C for 0.5 minute followed by 55°C for 0.5 minute followed by 72°C for 0.5 minute; 1 × 72°C for 5 minutes; cool to 4°C.

It should be noted that the primers given in the table above have proved successful for confirming the presence or absence of pXO1 and/or pXO2 in pure cultures of isolates from animal (including human) specimens or environmental samples. They may be unsuitable, however, for direct detection of B. anthracis in such specimens or samples. A choice of alternatives can be found in Jackson et al. (1998) and Ramisse et al. (1996). For the rare possibility that an isolate may lack both pXO1 and pXO2, a chromosomal marker should also be run; primers for these are also described in Jackson et al. (1998) and Ramisse et al. (1996).

C. REQUIREMENTS FOR VACCINES

1. Background

a) Rationale and intended use of the product

The most widely used vaccine for prevention of anthrax in animals was developed by Sterne (1937). He derived a rough variant of virulent B. anthracis from culture on serum agar in an elevated CO₂ atmosphere. This variant, named 34F2, was incapable of forming a capsule and was subsequently found to have lost the pXO2 plasmid, which codes for capsule formation. It has become the most widely used strain world-wide for animal anthrax vaccine production. In Central and Eastern Europe, an equivalent pXO2 – derivative, Strain 55, is the active ingredient of the current livestock vaccine. A list of manufacturers of anthrax vaccine for use in animals is given in Annex 5 of WHO (2008).

The following information concerning preparation of the anthrax vaccine for use in animals is based on Misra (1991) and the WHO (1967). Generalised procedures are given; national regulatory authorities should be consulted in relation to Standard Operating Procedures that may pertain locally.

2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed

i) Biological characteristics

Anthrax vaccine production is based on the seed-lot system. A seed lot is a quantity of spores of uniform composition processed at one time and maintained for the purpose of vaccine preparation. Each seed lot is no more than three passages from the parent culture and must produce a vaccine that is efficacious and safe for use in animals. It is recommended that a large seed lot be prepared from the parent strain and preserved by lyophilisation for future production lots. The parent culture can be purchased.

ii) Quality criteria

The seed lot is acceptable for anthrax vaccine if a vaccine prepared from the seed lot or a suspension harvested from a culture derived from a seed lot meets the requirements for control of final bulk with respect to freedom from bacterial contamination, safety and efficacy (immunogenicity).

b) Method of manufacture

i) Procedure

Preparation of the master seed

Seed lots are cultured on solid media formulated to promote sporulation of the organism. The solid medium formula for casein digest agar (sporulation agar) given in Misra (1991) is: 50 g tryptic digest of casein; 10 g...
yeast extract; 0.1 g CaCl₂·6H₂O; 0.01 g FeSO₄·7H₂O; 0.05 g MgSO₄·7H₂O; 0.03 g MnSO₄·4H₂O; 5.0 g K₂HPO₄; 1.0 g KH₂PO₄; 22 g agar; 1000 ml deionised or distilled water. The ingredients are dissolved in the water with the appropriate amount of heating; the solution is adjusted to pH 7.4, distributed into Roux bottles (120 ml per bottle) or other appropriate container, sterilised by autoclaving and cooled in the horizontal position. After the agar has solidified, excess liquid should be removed aseptically and the bottles left in an incubator (37°C) for at least 2 days to dry and to check the sterility.

Volumes of 2 ml of vaccine seed should be spread across the agar in Roux bottles, which should be incubated at 37°C until at least 80% sporulation is apparent by microscopic examination of aseptically extracted loopfuls (at least 72 hours). The growth is harvested with 10 ml per bottle of sterile deionised or distilled water and checked for purity. After washing three times in sterile deionised or distilled water with final suspension, also in sterile deionised or distilled water, sterilised lyophilisation stabiliser is added and the suspension is dispensed into lyophilisation vials and freeze-dried.

Attenuated vaccine strains can gradually lose their antigenicity over repeated subculturing conditions. Therefore, it is recommended that master seed lots be made in bulk and kept within three passages from the original seed culture. A large number of master seed stocks should be prepared.

**Preparation and testing of the working seed**

Reconstitute a vial of seed stock and inoculate several slants (approximately 10 ml) of sporulation (casein digest) agar. Incubate at 37°C for 72 hours and store in a refrigerator. Test the slants for purity by culture on to nutrient agar plates and in nutrient broth (0.1 ml in 100 ml of nutrient broth). The latter should be subcultured on to nutrient agar after incubation at 37°C for 7 days and should be a pure culture of *B. anthracis*. A sample of the broth culture should also be checked for lack of motility.

Volumes of seed needed for a production run should be calculated on the basis of harvesting the spores from each slant with 10 ml of sterile deionised or distilled water and using this to inoculate five Roux bottles.

**Preparation of vaccine concentrate**

Roux bottles with casein digest agar are prepared as for the master seed in Section C.1.b above. One Roux bottle can be expected to yield about 2000 doses of vaccine. Each Roux bottle is inoculated with 2 ml of working seed suspension and incubated at 37°C with porous plugs for several days until small loopfuls of culture from randomly selected bottles show at least 90% of the organisms to be in sporulated forms when examined in wet mounts by phase contrast (phase bright spores) or following staining for spores. The growth from each bottle is then harvested with 20 ml of physiological saline. Tests for contaminants should be carried out by subculture to nutrient agar plates and inoculation of 100 ml nutrient broth with 0.1 ml of harvested spores followed by subculture to nutrient agar after 7 days at 37°C and by tests for motility. Acceptable harvests (i.e. those showing no evidence of contaminants) are pooled.

**Glycerination**

Twice the volume of sterile, pure, neutral glycerol should be added to the bulk pool of vaccine concentrate. Saponin (0.1% final concentration) may also be added at this point if it is to be included as an adjuvant. Mix thoroughly (the inclusion of sterilised glass beads may be helpful). Carry out a purity test and hold for 3 weeks at ambient temperature to allow lysis of any vegetative bacteria, determine the viable spore count and store under refrigeration thereafter.

**Determining titre and dilution for use**

The number of culturable spores in the product is then calculated by spreading tenfold dilutions on nutrient agar plates. The suspension is diluted so that the final bulk contains the number of culturable spores desired. The diluent should contain the same proportions of saline, glycerol and (if being included) saponin as present in the vaccine concentrate. The vaccine should contain a minimum of 2–10 × 10⁸ culturable spores per dose for cattle, buffaloes and horses, and not less than 1–5 × 10⁶ culturable spores per dose for sheep, goats and pigs.

**Filling the containers**

Distribution of aliquots of vaccine into single and multidose containers is performed as outlined in WHO (1965). Basically, the final bulk is distributed to containers in an aseptic manner in an area not used for production, and any contamination or alteration of the product must be avoided. The vaccine may be lyophilised after distribution into appropriate dosage containers. Containers are sealed as soon as possible with a material that is not detrimental to the product and that is capable of maintaining a hermetic seal for the life of the vaccine.

**ii) Requirements for substrates and media**

Please refer to Misra (1991) for detailed information on substrates and media used for anthrax vaccine production.
iii) **In-process controls**

*Purity of the seed lot*

Purity tests consist of microscopic examination of stained smears with culture and motility tests as described in Section C.2.b.

*Safety of the seed lot*

Not less than $5 \times 10^9$ culturable spores should be injected subcutaneously into each of three healthy, 1–2-year-old, unvaccinated sheep, which must survive an observation period of at least 10 days.

*Immunogenicity of the seed lot*

At least 10 healthy guinea-pigs, 300–500 g in weight should be inoculated with $5 \times 10^6$ viable spores and observed for 21 days. At least 80% of the animals must survive. The immunised animals, together with three unimmunised controls, should then be challenged with 10 median lethal doses ($LD_{50}$) of the strain 17 JB of *B. anthracis*. During a 10-day observation period, none of the immunised animals should succumb to the challenge while all the controls should die from anthrax. The test should be repeated if one of the immunised animals dies.

iv) **Final product batch tests**

*Sterility and purity*

The vaccine is a live culture of *B. anthracis* spores; sterility does not apply, but the batches must be tested for freedom from contamination (see Chapter 1.1.7 *Tests for sterility and freedom from contamination of biological materials*).

*Safety*

Safety testing is performed on two healthy sheep or goats and consists of inoculating subcutaneously twice the recommended vaccination dose. The animals are observed for 10 days. The final bulk passes the test if no systemic reactions develop and if not more than a transient oedema is observed at the injection site. If the test is carried out in sheep only, a progressive oedema indicates that the vaccine may be unsuitable for goats.

*Batch potency*

Efficacy or immunogenicity is tested on the final bulk as follows: at least ten healthy 300–500 g guinea-pigs are inoculated with a sheep dose of the vaccine. The guinea-pigs are observed for 21 days, and at least 80% of the animals must survive the observation period. Surviving immunised guinea-pigs and three non-vaccinated controls are challenged with an appropriate dose of virulent *B. anthracis*. A recommended challenge is 200 $LD_{50}$ of the Pasteur II strain (17JB). If, by 10 days after challenge, all vaccinated guinea-pigs survive and control animals die, the final bulk is deemed to be satisfactory. If any vaccinated animals die during the post-challenge observation period from a cause other than anthrax, and death is not associated with the vaccine, the test may be repeated.

c) **Requirements for authorisation**

i) **Safety requirements**

*Target and non-target animal safety*

The vaccine has been shown to cause disease in some goats and llamas; this may be related to the saponin adjuvant. The vaccine is not recommended for use in pregnant animals, nor in animals destined for slaughter within 2–3 weeks of vaccination. Local regulations may specify other time periods in some countries or regions, but there is no scientific reason for regarding meat from clinically healthy animals as unfit for human handling or consumption after a holding period of 2 weeks following vaccination. Concurrent administration of antibiotics to vaccinated animals is contraindicated as the antibiotic will interfere with the vaccine. Antibiotics should not be given for several days before and after vaccination.

Accidental human inoculation is treated by expressing as much of the inoculum as possible from the injection site and washing the wound thoroughly with soap and water. Medical attention should be sought if infection develops.

*Reversion–to-virulence for attenuated/live vaccines*

The 34F2 strain of *B. anthracis* is known to be stable and cannot produce capsule *in vitro*.

*Environmental consideration*

Leftover vaccine, empty vials, and equipment used for vaccinating are contaminated with the live spores and should be autoclaved, disinfected, or incinerated.
Chapter 2.1.1. – Anthrax

ii) **Efficacy requirements**

*For animal production*

Not applicable.

*For control and eradication*

The recommended dose for cattle and horses is a minimum of \(2 \times 10^6\) culturable spores; for sheep, goats and pigs, it is \(1 \times 10^6\) culturable spores. The vaccine should contain these spores in an appropriate volume, e.g. \(2 \times 10^6/ml\). Immunity should be good for at least 1 year and it is recommended that an annual booster be given. Horses may be slow to develop immunity following initial vaccination; some manufacturers therefore recommend a two-dose initial vaccination, administered 1 month apart, followed by a single annual booster.

*Bacillus anthracis* spores are stable in unlyophilised or lyophilised vaccine and preservatives are not required. Storage under refrigeration is recommended (4°C).

As there is no generally acceptable test for stability of anthrax vaccines, it is recommended that, in each filling lot, the number of culturable spores be determined before and after holding at an appropriate temperature for an appropriate period. There should be no evidence of a fall in the number of culturable spores.

### 3. Vaccines based on biotechnology

a) **Vaccines available and their advantage**

There are no vaccines based on biotechnology available for anthrax.

b) **Special requirements for biotechnological vaccines, if any**

Not applicable.

REFERENCES


Chapter 2.1.1. – Anthrax


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NB: There are OIE Reference Laboratories for Anthrax
(see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).

Please contact the OIE Reference Laboratories for any further information on anthrax diagnostic tests, reagents and vaccines.
CHAPTER 2.1.2.
AUJESZKY’S DISEASE

SUMMARY

Aujeszky’s disease, also known as pseudorabies, is caused by an alphaherpesvirus that infects the central nervous system and other organs, such as the respiratory tract, in a variety of mammals except humans and the tailless apes. It is associated primarily with pigs, the natural host, which remain latently infected following clinical recovery (except piglets under 2 weeks old, which die from encephalitis). The disease is controlled by containment of infected herds and by the use of vaccines and/or removal of latently infected animals.

A diagnosis of Aujeszky’s disease is established by detecting the agent (virus isolation, polymerase chain reaction [PCR]), as well as by detecting a serological response in the live animal.

Identification of the agent: Isolation of Aujeszky’s disease virus can be made by inoculating a tissue homogenate, for example of brain and tonsil or material collected from the nose/throat, into a susceptible cell line such as porcine kidney (PK-15) or SK6, or primary or secondary kidney cells. The specificity of the cytopathic effect is verified by immunofluorescence, immunoperoxidase or neutralisation with specific antiserum. The viral DNA can also be identified using PCR; this can be accomplished using the real-time PCR techniques.

Serological tests: Aujeszky’s disease antibodies are demonstrated by virus neutralisation, latex agglutination or enzyme-linked immunosorbent assay (ELISA). A number of ELISA kits are commercially available world-wide. An OIE international standard serum defines the lower limit of sensitivity for routine testing by laboratories that undertake the serological diagnosis of Aujeszky’s disease.

It is possible to distinguish between antibodies resulting from natural infection and those from vaccination with gene-deleted vaccines.

Requirements for vaccines: Vaccines should prevent or at least limit the excretion of virus from the infected pigs. Recombinant DNA-derived gene-deleted or naturally deleted live Aujeszky’s disease virus vaccines, lack a specific glycoprotein (gG, gE, or gC), which enables the use of companion diagnostic tests to differentiate vaccinal antibodies from those resulting from natural infection.

A. INTRODUCTION

Aujeszky’s disease, also known as pseudorabies, is caused by *Suid herpesvirus 1* (SHV-1), a member of the subfamily *Alphaherpesvirinae* and the family *Herpesviridae*. The virus is generally handled in BSL-2 laboratories. The virus infects the central nervous system and other organs, such as the respiratory tract, of a variety of mammals (such as dogs, cats, cattle, sheep, rabbits, foxes, minks, etc.) except humans and the tailless apes. It is associated primarily with pigs, the natural host, which remain latently infected following clinical recovery (except piglets under 2 weeks old, which die from encephalitis). In consequence, the pig is the only species able to survive a productive infection and therefore, serves as the reservoir host. In pigs, the severity of clinical signs depends on the age of the pig, the route of infection, the virulence of the infecting strain and the immunological status of the animal. Young piglets are highly susceptible with mortality rates reaching 100% during the first 2 weeks of age. These animals show hyperthermia and severe neurological disorders: trembling, incoordination, ataxia, nystagmus to opisthotonos and severe epileptiform-like seizures. When pigs are older than 2 months (grower-finisher pigs), the respiratory forms become predominant with hyperthermia, anorexia, mild to severe respiratory signs: rhinitis with sneezing and nasal discharge may progress to pneumonia. The frequency of secondary bacterial infections is high, depending on the health status of the infected herd. In this group of pigs, the morbidity can reach 100%, but in cases of the absence of complicated secondary infections, mortality ranges from 1–2% (Pejsak & Truszczyński, 2006). Sows and boars primarily develop respiratory signs, but in pregnant sows, the virus can cross the placenta,
infect and kill the fetuses inducing abortion, return to oestrus, stillborn fetuses. In the other susceptible species, the disease is fatal, the predominant sign being intense pruritus causing the animal to gnaw or scratch part of the body, usually head or hind quarters, until great tissue destruction is caused. For that reason, the disease was named in the past: mad-itch.

Focal necrotic and encephalomyelitis lesions occur in the cerebrum, cerebellum, adrenals and other viscera such as lungs, liver or spleen. In fetuses or very young piglets, white spots on liver are pathognomonic of their infection by the virus. Intranuclear lesions are frequently found in several tissues.

Aujeszky’s disease is endemic in many parts of the world, but several countries have successfully completed eradication programmes, e.g. the United States of America, Canada, New Zealand and many Member States of the European Union.

The disease is controlled by containment of infected herds and by the use of vaccines and/or removal of latently infected animals (Pejsak & Truszczynski, 2006). Stamping out has been or is used in several countries usually when the infected farms are small or when the threat to neighbouring farms is very high in free countries.

Whereas isolation of the Aujeszky’s disease virus or detection of the viral genome by the polymerase chain reaction are used for diagnosis in the case of lethal forms of Aujeszky’s disease or clinical disease in pigs, serological tests are required for diagnosis of latent infections and after the disappearance of the clinical signs. Affected animals except pigs, do not live long enough to produce any marked serological response. Serological tests are the tests to be used to detect subclinically or latently infected pigs, especially in the case of qualification of the health status of the animals for international trade or other purposes.

### B. DIAGNOSTIC TECHNIQUES

#### 1. Identification of the agent

**a) Virus isolation**

The diagnosis of Aujeszky’s disease can be confirmed by isolating the virus from the oro-pharyngeal fluid, nasal fluid (swabs) or tonsil swabs from living pigs, or from samples from dead pigs or following the presentation of clinical signs such as encephalitis in herbivores or carnivores. For post-mortem isolation of SHV-1, samples of brain, tonsil, and lung are the preferred specimens. In cattle, infection is usually characterised by a pruritus, in which case a sample of the corresponding section of the spinal cord may be required in order to isolate the virus. In latently infected pigs, the trigeminal ganglia is the most consistent site for virus isolation, although latent virus is usually non-infective unless reactivated, making it difficult to recover in culture.

The samples are homogenised in normal saline or cell culture medium with antibiotics and the resulting suspension is clarified by low speed centrifugation at 900 g for 10 minutes. The supernatant fluid is used to inoculate any sensitive cell culture system. Numerous types of cell line or primary cell cultures are sensitive to SHV-1, but a porcine kidney cell line (PK-15) is generally employed. The overlay medium for the cultures should contain antibiotics (such as: 200 IU/ml penicillin; 100 µg/ml streptomycin; 100 µg/ml polymyxin; and 3 µg/ml fungizone).

SHV-1 induces a cytopathic effect (CPE) that usually appears within 24–72 hours, but cell cultures may be incubated for 5–6 days. The monolayer develops accumulations of birefringent cells, followed by complete detachment of the cell sheet. Syncytia also develop, the appearance and size of which are variable. In the absence of any obvious CPE, it is advisable to make one blind passage into further cultures. Additional evidence may be obtained by staining infected cover-slip cultures with haematoxylin and eosin to demonstrate the characteristic herpesviral acidophilic intranuclear inclusions with margination of the chromatin. The virus identity should be confirmed by immunofluorescence, immunoperoxidase, or neutralisation using specific antiserum.

The isolation of SHV-1 makes it possible to confirm Aujeszky’s disease, but failure to isolate does not guarantee freedom from infection.

**b) Identification of virus by the polymerase chain reaction**

The polymerase chain reaction (PCR) can be used to identify SHV-1 genomes in secretions or organ samples. Many individual laboratories have established effective protocols, but there is as yet no internationally agreed standardised approach.
Chapter 2.1.2. – Aujeszky’s disease

The PCR is based on the selective amplification of a specific part of the genome using two primers located at each end of the selected sequence. In a first step, the complete DNA may be isolated using standard procedures (e.g. proteinase K digestion and phenol–chloroform extraction) or commercially available DNA extraction kits. Using cycles of DNA denaturation to give single-stranded DNA templates, hybridisation of the primers, and synthesis of complementary sequences using a thermostable DNA polymerase, the target sequence can be amplified up to 10²-fold. The primers must be designed to amplify a sequence conserved among SHV-1 strains, for example parts of the gB or gD genes, which code for essential glycoproteins, have been used (Mengeling et al., 1992; Van Rijn et al., 2004; Yoon et al., 2006). A real-time PCR has been developed that can differentiate gE-deleted vaccine viruses from wildtype virus based on the specific detection of gB and gE genes (Ma et al., 2008). However, the gE specific real-time PCR has a lower sensitivity than the gB-specific real-time PCR.

The amplified product may be identified from its molecular weight as determined by migration in agarose gel, with further confirmation where possible by Southern hybridisation using a complementary probe. Recent techniques involve liquid hybridisation using enzyme-labelled probes, which give a colour reaction after incubation with the appropriate substrate. More recent techniques include the use of fluorescent probes linked to an exonuclease action and real-time monitoring of the evolution of product, enabling simultaneous amplification and confirmation of the template DNA thus increasing the rapidity and specificity of the PCR assays.

In all cases, the main advantage of PCR, when compared with conventional virus isolation techniques, is its rapidity; with the most modern equipment, the entire process of identification and confirmation can be completed within one day. However, because of the nature of the test, many precautions need to be taken to avoid contamination of samples with extraneous DNA from previous tests or from general environmental contamination in the laboratory (see Chapter 1.1.7 Tests for sterility and freedom from contamination of biological materials). This may limit the value of the test for many laboratories unless care is taken to avoid DNA carry-over contamination. The use of an internal control is necessary to avoid false negative results by ensuring adequate efficiency of DNA extraction and confirming the absence of PCR inhibitors in each sample. In practice, different systems can be used for detection of endogenous or exogenous gene (Hoffman et al., 2009).

2. Serological tests

Virus neutralisation (VN) has been recognised as the reference method for serology (Moennig et al., 1982), but for general diagnostic purposes it has been widely replaced by the enzyme-linked immunosorbent assay (ELISA) because of its suitability for large-scale testing (Moennig et al., 1982). The tests can be performed on a variety of matrices (e.g. serum, whole blood, milk, muscular exudates, and filter paper) but the preferred matrix is serum.

A latex agglutination test has also been developed and can be used for screening for antibodies. It can differentiate between the immune response of naturally infected pigs and those that have been vaccinated with gE deleted vaccines (Yong et al., 2005). Kits for the test are commercially available (Schoenbaum et al., 1990).

Serological tests are carried out only for pigs, as other animals (herbivores and carnivores) die too quickly to produce antibodies. In free areas where pigs are not vaccinated, an active epidemiological survey can be carried out using ELISA gB kits. As antibodies can be detected between 7 and 10 days post-infection, this serological tool can also be used in case of an outbreak suspicion, to confirm the infection of pigs. In area where pigs are vaccinated with gE deleted vaccines, the ELISA gE kits permit the differentiation between infected and vaccinated pigs (DIVA), but to assess the level of immunity induced by vaccination, gB ELISA kits or viral neutralisation should be used.

Any serological technique used should be sufficiently sensitive to give a positive result with the OIE International Standard Reference Serum. This serum can be obtained from the OIE Reference Laboratory for Aujeszky’s Disease in France (see Table given in Part 4 of this Terrestrial Manual). For international trade purposes, the test should be sensitive enough to detect the standard serum diluted 1/2. To authorise pig movement from an area where deleted gE vaccines are used to a free area, serological assays should be able to detect at least the dilution of 1/8 for ELISA gE of the OIE reference standard serum as prescribed by the European Commission (2008).

a) Virus neutralisation (a prescribed test for international trade)

VN in cell culture can be performed in several ways, which vary according to the length of incubation of the virus/serum mixtures (e.g. 1 hour at 37°C or 24 hours at 4°C), and the presence or absence of complement. Most laboratories use a reaction period of 1 hour at 37°C in the absence of complement, because this is easy and rapid. However, the sensitivity can be improved by increasing the incubation period to 24 hours at 4°C, which facilitates the detection of antibody levels 10–15 times lower than in the 1-hour method. For international trade purposes, the test method should be validated as being sensitive enough to detect the OIE Standard Reference Serum diluted 1/2.
VN cannot be used to differentiate antibodies of vaccinal origin from those caused by natural infection. It is one of the two tests available that complies with the requirement in the OIE Terrestrial Animal Health Code chapter when it refers to ‘a diagnostic test to the whole virus’.

**Cells:** Cells susceptible to infection with SHV-1 are used; they may be cell lines (e.g. PK-15, SK6, MDBK), or primary or secondary cell cultures.

**Cell culture medium:** The medium depends on the type of cells. For example, the medium for PK-15 cells is Eagle’s minimal essential medium (MEM) + 10% fetal bovine serum and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin, or alternatively, 50 µg/ml gentamycin).

**Maintenance of the cells:** The cells are cultured in cell culture vessels of, for example, 75 cm². They are trypsinised once or twice per week. For weekly trypsinisation, the cells are usually cultured in 50 ml of medium, with a multiplication rate of 5. For two trypsinisations a week, the cells are cultured in 30 ml of medium, with a multiplication rate of 3.

For trypsinisation, the growth medium is removed once the cell sheet is complete. The cell sheet is washed with about 5 ml of recently thawed trypsin/ethylene diamine tetra-acetic acid (EDTA) (0.25%) in an isotonic buffer. The washing fluid is discarded and the preparation is washed again, retaining only a few drops of trypsin. The container is placed in an incubator at 37°C for 5–10 minutes until the cells have become detached. Once the sheet is detached and the cells are well separated, for twice weekly passage they are suspended in 90 ml of growth medium, and this suspension is distributed into three 75 cm² cell culture bottles. For weekly trypsinisation the cells are suspended in 150 ml of growth medium and the suspension distributed into five 75 cm² cell culture bottles.

**Virus:** A suitable strain of SHV-1, such as the Kojnok strain, or NIA-3 strain, is stored at a temperature of –65°C or below, or in freeze-dried form at 4°C.

**Preparation of stock virus suspension:** The culture fluid is removed from a cell culture bottle containing a complete cell sheet. About 1 ml of stock virus suspension of known titre (about 10⁷ TCID₅₀/ml [50% tissue culture infective dose]) is added, and the bottle is incubated at 37°C±2°C for 1 hour. Then, 30 ml of culture medium is added and the bottle is again incubated at 37°C±2°C. The bottle is examined frequently until there is about 75% cell destruction (after about 36–48 hours). It is then frozen at a temperature of –20°C or lower in order to disrupt the cells.

The bottle is then thawed and shaken vigorously. Medium is collected and centrifuged at 1500 g for 15 minutes. The supernatant fluid is divided into portions (of about 0.5 ml) in small tubes that are labelled (date and virus reference) before being stored at a temperature of –65°C or lower until required.

**Titration of the stock virus suspension:** Titration of the stock suspension is performed by the method of Reed & Muench or that of Kärber, and the titre is expressed per 50 µl and per ml.

The VN test requires an internal quality control serum with a known titre of neutralising antibody to SHV-1 (it must be calibrated against an international standard serum or a secondary standard prepared from that serum), and a negative control serum (from a specific antibody free pig, e.g. from an official Aujeszky’s disease free herd). The test sera themselves should be of good quality, clearly labelled, of known provenance with clinical history, stored in refrigeration at all times, free from fungal or bacterial contamination, non-haemolysed and of sufficient quantity. Serum should be separated from the coagulum without delay, thereby preventing toxicity.

There are qualitative and quantitative procedures for VN, both of which are described below:

- **Qualitative technique**
  i) Complement in the serum samples is destroyed by heating in a water bath at 56–59°C for 30 minutes.
  ii) Each undiluted serum sample is placed in two to three wells, at 50 µl per well, of a 96-well cell-culture grade microtitre plate. Each serum can also be diluted 1/2 in the MEM, before being placed in two other wells.
  iii) 50 µl of virus suspension containing 100 TCID₅₀ (or 2 × 10³ TCID₅₀/ml), obtained by diluting stock virus suspension of known titre with MEM, is added to each well.
  iv) The plate is shaken and placed in an incubator for 1 hour at 37°C (±2°C) (5% CO₂ optional).
  v) 150 µl of cell suspension containing about 150,000 cells/ml is added to each well.
vi) The plate is covered (for incubation in CO₂), or a plastic sheet is sealed carefully around the edges of the plate (for incubation in air). The plate is shaken lightly to obtain an even distribution of cells at the bottom of the wells, and placed in the incubator at 37°C (±2°C) (CO₂ optional) for 3–5 days.

vii) Controls: Each set of plates must include the following controls:

Virus control: This is to verify the amount of virus actually used for the test. The virus dose used for virus neutralisation (target titre 100 TCID₅₀/50 µl) is diluted with MEM at 1/10, 1/100 and 1/1000. Of each dilution, 50 µl is placed in at least four wells, to which 50 µl of medium is added before the wells are incubated for 1 hour at 37°C (±2°C). The cell suspension is added in the same way as for the sera under test.

Cell control: 150 µl cell suspension and 100 µl MEM are placed in each of at least four wells.

Positive serum control: A serum of known SHV-1 neutralising antibody titre is used. Five dilutions are prepared in the same way as for the sera under test: a dilution corresponding to the serum titre, two-fold and four-fold dilutions, and 1/2 and 1/4 dilutions (equivalent to T, T/2, T/4, 2T and 4T, where T is the serum titre, i.e. undiluted serum for the qualitative test). To 50 µl of positive control sample dilutions, add 50 µl of virus suspension containing 100 TCID₅₀/50 µl. The cells are incubated and the cell suspension is added in the same way as for the sera under test.

Serum control: This is to verify the absence of a toxic effect of the sera on the cells. Wells containing 50 µl of each serum are incubated for 1 hour at 37°C in the presence of 50 µl of medium. Then, 150 µl of cell suspension is added in the same way as for the sera under test.

Negative serum control: This is done in the same way as for sera under test.

viii) Reading the results: An inverted-image microscope (×100) is used to examine the wells for toxic effects and CPEs after 48 and 72 hours. The controls must give the following results if the tests are to be considered valid:

Virus control: The titre of the viral suspension should be between 30 and 300 TCID₅₀/50 µl.

Cell control: The cell sheet must be intact.

Positive serum control: The titre obtained must be equal to the predicted titre, within one dilution.

Serum control: Examination for a CPE should take into account a possible toxic effect on cells.

Negative serum control: A CPE should be present.

ix) For the sera under test, the following results may be seen: presence of a CPE in three wells = negative result; absence of a CPE in three wells on day 3 = positive result; presence of a CPE in one well but not in the others = doubtful result, test must be repeated; small plaques indicating a CPE on day 3 = doubtful result, test must be repeated; toxicity in serum control and test wells = unreadable result, test must be repeated (NB replacement of medium with fresh medium after 16 hours’ incubation will reduce the toxicity without affecting the titre of specific antibody). Plates can be read until day 5 of incubation. If the serum was initially diluted 1/2, it is considered positive if CPE is absent in the two wells. Diluting the serum to 1/2 can prevent the toxicity effect of the tested sera.

x) Interpretation of the results: This test is capable of detecting the presence or absence of neutralising antibody to SHV-1. It is incapable of distinguishing vaccinated animals from infected animals.

The technique described (VN for 1 hour at 37°C) can give false-negative and false-positive results. The sensitivity can be increased (leading to fewer false negatives) by adopting a method based on neutralisation involving 24 hours of contact between virus and serum at 4°C, before the addition of cells.

A qualitative technique such as this one, which employs undiluted serum samples (1/2 final dilution), can give a false-positive result in certain cases due to nonspecific neutralisation of the virus. This problem can be addressed by carrying out a confirmatory test using the quantitative technique (see below).

• Quantitative technique

This is similar to the qualitative procedure, but each serum is used both undiluted and in a series of dilutions. Depending on the desired precision, the purpose of testing and the expected titre, two wells are used for each dilution of serum, and a greater or smaller range of dilutions. Ideally, the procedure may be described for a range of dilutions reaching an initial maximum of 1/256.

i) Complement in the serum samples is destroyed by heating in a water bath at 56–59°C for 30 minutes.

ii) 50 µl of MEM is added to wells A3 to A6 of a 96-well cell-culture grade microtitre plate.

iii) 50 µl of undiluted serum sample is added to wells A1 to A3, and continued for wells in rows B, C, etc., with other serum samples.
iv) Using a multichannel pipette, the contents of wells in row 3 are mixed, then 50 µl is transferred to row 4, and so on to row 6 or further to a predetermined row, using the same nozzles. The 50 µl portions remaining after the last row is discarded.

v) Controls are set up as described for the qualitative technique.

vi) 50 µl of MEM is added to row 1 instead of virus: this is a control row of serum samples. Viral suspension is deposited in the wells of the other rows. Subsequent manipulations are the same as described for the qualitative technique.

vii) **Reading the results:** The neutralising titre of a serum is expressed by the denominator of the highest initial dilution that brings about complete neutralisation of the CPE of the virus in 50% of the wells. Neutralisation at any dilution (even undiluted, equivalent to a final dilution of 1/2) is considered to be positive. If the serum shows neutralisation only when undiluted (with growth of virus and CPE at the 1/2 and subsequent dilutions), it would be advisable to apply alternative tests (ELISA or latex agglutination) to provide confirmation of the result, or to request another sampling of the animal, at least 8 days after the first.

**b) Enzyme-linked immunosorbent assay (a prescribed test for international trade)**

The sensitivity of the ELISA is generally superior to that of the VN test using 1-hour neutralisation without complement. Some weak positive sera are more readily detected by VN tests using 24-hour neutralisation, while others are more readily detectable by ELISA.

ELISA kits, which are available commercially, use indirect or competitive techniques for detecting antibodies. They differ in their mode of preparation of antigen, conjugate, or substrate, in the period of incubation and in the interpretation of the results. Their general advantage is that they enable the rapid processing of large numbers of samples. This can also be automated and the results analysed by computer. Some of these kits make it possible to differentiate between vaccinated and naturally infected animals when used with a ‘matching’ vaccine (Elriot *et al.*, 1989; Van Oirschot *et al.*, 1986). Alternatively, non-commercial ELISA protocols may be adopted (Toma & Eliot, 1986) provided they are shown to detect the OIE International Standard Reference Serum as positive at a dilution of 1/2 (the minimum sensitivity for international trade purposes). It is recommended to use a kit or in-house assay that has been validated to this standard by external quality control tests by an independent laboratory. A suitable test protocol for whole virus antibodies is presented below (Toma & Eliot, 1986).

**• Preparation of antigen**

i) A cell line sensitive to SHV-1 is used, such as PK-15 or fetal pig testis. It must be free from extraneous viruses, such as bovine viral diarrhoea virus. The cells should be split and seeded into fresh 75 cm² flasks the day before inoculation. A suitable medium such as MEM, without serum, is used to overlay the cultures.

ii) Virus inoculated, and control uninoculated flasks are processed in parallel throughout. A suitable well characterised strain of SHV-1 is used, e.g. Kojnock strain. When a confluent cell monolayer has developed (approximately 24 hours after seeding), it is inoculated with $10^8$ TCID₅₀ SHV-1 in 5 ml medium; and 5 ml medium (without virus) is placed in control flasks. The cultures are left for adsorption for 30 minutes at 37°C, and then overlaid with 20 ml medium.

iii) When CPE is just beginning, the supernatant medium is discarded and 4 ml KCl (4 mM solution) and glass beads are added. The flasks are shaken gently to detach cells.

iv) Cells are washed by centrifuging three times at 770 g in 4 mM KCl. The pellet is resuspended in 4 mM KCl with 0.2% Triton X-100 (1 ml per flask) by applying 60 strokes with a glass homogeniser.

v) The cell homogenate is layered on to 0.25 mM sucrose in 4 mM KCl and centrifuged for 10 minutes at 770 g.

vi) The pellet is resuspended in antigen-diluting buffer, pH 9.6 (0.1 M Tris, 2 mM EDTA, 0.15 mM NaCl) at 1/50 the volume of the original culture medium. It may then be stored in small aliquots at −70°C. Antigen is stable in this form for 2 years.

**• Coating microtitre plates**

i) Virus antigen and control (no virus) antigen are diluted in diluting buffer, pH 9.6 (see above) to a dilution predetermined in chequerboard titrations.

ii) 200 µl of antigen is dispensed into each well of 96-well ELISA-grade plates, coating alternate rows with SHV-1 positive and control antigen. Incubation is for 18 hours at 4°C.

iii) The plates are washed three times with washing solution (Tween 20, 0.5 ml/litre).

iv) Coated plates are stored at −20°C or −70°C. They are stable for several months.
Chapter 2.1.2. – Aujeszky’s disease

Test procedure

i) Test serum samples are diluted 1/30 in PBS/Tween buffer, pH 7.2 (137 mM NaCl, 9.5 mM phosphate buffer, 0.5 ml/litre Tween 20).

ii) Diluted samples are added to virus and control antigen coated wells, and incubated at 37°C for 30 minutes.

iii) The plates are washed three times with washing solution (0.5 ml/litre Tween 20).

iv) Protein A/peroxidase conjugate is added to all wells at a predetermined dilution in PBS/Tween buffer, pH 7.2 (see above), with added bovine serum albumen fraction V (10 g/litre), and the plates are incubated at 37°C for 30 minutes.

v) The plates are washed three times with washing solution (0.5 ml/litre Tween 20).

vi) A suitable chromogen/substrate mixture, such as tetra methyl benzidine (TMB)/hydrogen peroxide, is added to each plate.

vii) The reaction is stopped with 2 M sulphuric acid. The absorbance is read at 492 nm.

The test must be fully validated using known positive and negative sera, and calibrated against the OIE International Standard Reference Serum. All tests must include positive and negative internal controls, including a weak positive that, when diluted at the appropriate dilution for the test, has equivalent activity to a 1/2 dilution of the OIE International Standard Reference Serum. For further details see Tomà & Eloit, 1986 and Chapter 1.1.5 Principles and methods of validation of diagnostic assays for infectious diseases. Commercial ELISA kits also have to be validated in the setting in which they are going to be used.

As well as testing sera, the ELISA can be adapted to test pools of sera, filter paper disks that have been moistened with a small quantity of blood obtained by puncturing a superficial vein (Banks, 1985; Tomà et al., 1986), or muscle exudates (Le Potier et al., 1998). These techniques make it convenient to collect blood samples from large numbers of pigs (Vannier et al., 2007). The disks are air-dried before shipment to the laboratory.

Requirements for the detection of gE antibodies by ELISA in pigs destined for slaughter, that are to be introduced into zones free from Aujeszky’s disease, have been defined by several control authorities (European Commission, 2008). The OIE Terrestrial Animal Health Code specifies circumstances in which gE-specific tests may be used. The gE ELISAs can also be adapted to test blood on filter paper disks depending on its sensitivity.

C. REQUIREMENTS FOR VACCINES

1. Background

a) Rationale and intended use of the product

Aujeszky’s disease may be controlled by the use of vaccines containing either modified live or inactivated virus antigens. In addition, these conventional vaccines have been supplemented by recombinant DNA-derived gene-deleted or naturally deleted live SHV-1 vaccines. These vaccines, sometimes referred to as marker vaccines, are made with a virus that lacks a specific glycoprotein (most commonly gE-, although gG- or gC-deleted vaccines have also been described1). At least one commercially available vaccine has dual deletions. These gene-deleted marker vaccines have the advantage over conventional whole virus vaccines that it is possible to distinguish noninfected vaccinated animals from those with field infection. This is done by testing for the antibodies directed against the protein coded for by the deleted gene, which will be absent in noninfected marker-vaccinated pigs but present in field-infected pigs. Therefore, in countries with infected pigs, where the eradication of Aujeszky’s disease is planned, these marker vaccines are the vaccines of choice (Pensaert et al., 1992; 2004). Standards applicable to the manufacture of live and inactivated virus vaccines are described. For marker vaccines, the tests should include demonstrable absence of a serological response in vaccinated pigs to the protein coded for by the deleted gene, and in addition a demonstrable response to the same protein in vaccinated pigs that become infected by field virus.

Other vaccines are inactivated and constituted of adjuvanted, viral sub-unit of purified and concentrated immunogenic glycoproteins (except the gE) allowing differentiation of vaccinated from infected pigs.

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1 The nomenclature for the genes changed several years ago, but the old designation is still in the literature. The old and the new nomenclature is: gll = gB; glll = gC; gp50 = gD; gl = gE; gX = gG; gp63 = gl.
Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

2. **Outline of production and minimum requirements for conventional vaccines**

a) **Characteristics of the seed**

i) **Biological characteristics**

Vaccines are made using a seed-lot system in which a master seed virus (MSV) is prepared from a suitable strain of Aujeszky’s disease virus. A number of strains are used for vaccine manufacture. The antigen in an inactivated vaccine can be one of a number of wild-type strains, or the naturally deleted Bucharest virus. Modified live conventional vaccines use numerous strains, such as Bartha or are derived from Aujeszky’s original isolate or from other field isolates, such as the NIA-3 strain (Marchioli *et al*., 1987; McFerran & Dow, 1975; Van Oirschot *et al*., 1990; Visser & Lutticken, 1988).

It is recommended that for differentiating between infected and vaccinated animals, deleted strains should be used.

A virus identity test (using either a fluorescent antibody test, neutralisation test, [constant serum/decreasing virus method], or any other suitable identity test) must be conducted on the MSV.

ii) **Quality criteria (sterility, purity, freedom from extraneous agents)**

Most of the cell lines used to propagate SHV-1 are continuous lines, such as the PK-15 line. A master cell stock (MCS) is established at a specified passage level. The MCS and the highest passage level (MCS × n) intended for use in the preparation of a biological product is specified in an Outline of Production. Both MCS and MCS × n are monitored by a variety of procedures to characterise the cell line and to ensure freedom from adventitious agents. The extraneous agents to be detected are generally defined in monographs and/or guidelines (e.g. European Pharmacopoeia, US Code of Federal Regulations, EU guidelines, etc.). In general, the type of agents to be looked for is founded on a risk analysis depending on the history of the viral strain and cells on which the vaccinal strain was isolated and on which it is cultivated. The MCS must be monitored for species of origin. A minimum of 50 mitotic cells should be examined at both the MCS and MCS × n passage levels. The modal number in the MCS × n must not exceed 15% of the modal number of the MCS. Any marker chromosomes in the MCS must also be present in the highest cell passage.

If there is evidence that the cell line may induce malignancies in the species for which the product is intended, the cell line is tested for tumorgenicity and oncogenicity.

Both the MSV and the MCS must be shown to be free from mycoplasma, bacteria, fungi, cytopathogenic or haemadsorbing viruses, porcine parvovirus, cytopathic and noncytopathic ovine and bovine pestiviruses and other extraneous agents, such as circovirus, as determined by culturing and by fluorescent antibody procedures or others, such as PCR.

b) **Method of manufacture**

i) **Procedure**

Only MSV that has been established as pure, safe and immunogenic may be used as seed for a vaccine product. Cells from the MCS are propagated in a variety of growth media. All batches of vaccine must be from the first to the twentieth passage of MCS.

ii) **In-process controls**

It is necessary to carry out tests at each critical step of the manufacturing process. The control tests are also carried out on intermediate products with a view to verifying the consistency of the production process and the final product.

iii) **Final product batch tests**

It is essential to differentiate the tests that are carried out on a routine basis to release batches of final product from those that are performed to define the biological properties of a vaccine. The trials carried out for batch release are not the same as the ones carried out once only to determine the safety and efficacy of a vaccine. The batch release controls are always short-term trials, as inexpensive as possible, and not always carried out in pigs. Their purpose is mainly to attest the reproducibility of the quality of the finished product, which has to be in compliance with the quality initially defined in the application for marketing authorisation.

**Sterility and purity**

Tests must be carried out for sterility and freedom from contamination (see chapter 1.1.7 and Section C.2.a.ii of this chapter).
Each batch of SHV-1 vaccines must be tested for freedom from extraneous viruses. Using a minimum amount of a monospecific antiserum, the live vaccinal strain is neutralised and inoculated into cell cultures known to be sensitive to viruses pathogenic for pigs. No CPE and no haemadsorbing agents should be detected. The vaccines have to be free from pestiviruses.

Inactivation
For inactivated vaccines, inactivation must be checked using two passages in the same type of cell culture as used in the production of the vaccine. Tests can be carried out by vaccinating susceptible animals such as rabbits.

Identity
Where necessary, a specific test for virus identification should be carried out.

Safety
Safety of live vaccines is tested by administering ten doses of the reconstituted vaccine by the route stated on the leaflet to each of at least two piglets of the minimum age recommended for vaccination that are free from SHV-1 antibodies. Two piglets of the same origin and age are kept as controls. No abnormal local or systemic reaction should occur. The weight curve of the vaccinated piglets must not differ significantly from that of the controls.

For inactivated vaccines, safety is tested by injecting two doses into piglets under the same conditions as described previously.

Batch potency
The potency of the vaccine must be demonstrated using a suitable method, the results of which have to be correlated with the efficacy tests described previously.

In this kind of test, the most difficult point is to determine an acceptability threshold for using or rejecting the batch according to the results that are obtained.

Virus content tests should be carried out using each of at least three containers. The virus titre of the vaccine must be determined and must normally not be higher than 1/10 of the dose at which the vaccine has been shown to be safe, and not lower than the minimum release titre.

Preservatives
If no preservative is included in the final product, the manufacturer must demonstrate that the product remains acceptable for its recommended period of use after opening the vial.

The efficacy of preservatives in multidose containers must be demonstrated. The concentration of the preservative in the final filled vaccine and its persistence throughout shelf life must be checked.

Precautions (hazards)
All information about possible adverse reactions induced by the vaccine must be indicated. Any putative risk for human health if the user is accidentally given a small quantity of the product has to be indicated.

The manufacturer should indicate all the conditions of use of the vaccine: mixing, reconstitution, storage, asepsis, length of needle, route of administration and health status of the vaccinated animals.

Stability tests
Tests have to be carried out to verify the shelf life proposed by the manufacturer. These tests must always be real-time studies; they must be carried out on a sufficient number of batches (at least three) produced according to the described production process and on products stored in the final container, and normally include biological and physicochemical stability tests. The manufacturer has to provide the results of analyses that support the proposed shelf life under all proposed storage conditions. Usually, the proposed shelf life corresponds to the period for which the product is considered to be stable minus 3 months.

c) Requirements for authorisation
i) Safety requirements

Local and general reactions must be examined. When a live vaccine is used, it is necessary to differentiate the exact safety properties of the vaccinal strain from those of the finished product if this includes an adjuvant.

Objective and quantifiable criteria to detect and measure adverse reactions should be used; these would include temperature changes, weight gain, litter size, reproductive performance, etc., of vaccinated and control groups. The tests must be performed by administering the vaccine to the pigs in the recommended dose and by each recommended route of administration.
In general, safety is tested initially under experimental conditions, following the requirements of the OIE Terrestrial Animal Health Code, Chapter 7.8 Use of animals in research and education. When the results of these preliminary tests are known, it is necessary to increase the number of animals vaccinated in order to evaluate the safety of the vaccine under practical conditions.

- **Laboratory testing**

  All tests must be carried out on pigs that do not have antibodies against Aujeszky’s disease virus or against a subunit of the virus.

  **a. General effects**

  1. **Live vaccines**

     Intranasal tests and vaccination of 3–5-day-old piglets are very useful for ascertaining the degree of safety of a strain. At least five piglets should be used.

     It is also essential to assess the properties of a vaccine, especially live ones, in the target animals under normal conditions of use and at the youngest age intended for vaccination, e.g. fattening pigs, which are generally vaccinated when they are between 9 and 12 weeks old, and pregnant sows when this use of the vaccine is claimed by the manufacturer and is authorised. No clinical signs, including significant thermal reactions (data have to be recorded before vaccination and 6 hours, 24 hours and 48 hours later, then on a daily basis during the observation period), should be observed after vaccination. These assays have to be performed on at least ten vaccinated pigs, with five unvaccinated pigs as controls.

     Reversion to virulence following serial passage must be examined. Primary vaccination is done by the intranasal route. Series of at least four passages in piglets are made. No fewer than two fully susceptible animals must be used for each passage.

     The object of these assays is to test the genetic stability of live vaccine strains. The tests appear to be less necessary when a genetically modified live strain is concerned, especially if it is produced by gene deletion.

     It is recommended to test for possible excretion of the vaccine strain. For this purpose, no fewer than 14 piglets, 3–4 weeks old each receive one dose of vaccine by the recommended route and at the recommended site (except for vaccines administered by the intranasal route). Four unvaccinated piglets are kept as controls. Suitably sensitive tests for the virus are carried out individually on the nasal and oral secretions of vaccinated and in-contact pigs as follows: nasal and oral swabs are collected daily from 1 day before vaccination to 10 days after vaccination. Vaccine strains that are isolated from the nasal/oral secretion collected from pigs in which the vaccine was administered by the parenteral route are not recommended.

     The ability of the Aujeszky’s disease vaccine strain to spread from vaccinated pigs to unvaccinated ones (lateral spread) must be tested by using the recommended route of administration that presents the greatest risk of spreading (except for vaccines administered by the intranasal route). A repetition of the assays (four times) is necessary as this phenomenon is difficult to detect. Four piglets should be used each time for vaccination and placed in contact, 1 day later, with two unvaccinated piglets. It may also be necessary to examine the spread of the strain to nontarget species that may be susceptible to the vaccine strain.

     Live attenuated vaccine strains are tested with regard to their general effects by administering to 5–10-day-old piglets ten times the field dose. This administration of an overdose makes it possible to detect reactions not produced under normal conditions of use. Such reactions may be produced inadvertently when large numbers of animals are vaccinated. If vaccines are administered by the intranasal route, the manufacturer has to indicate clearly that the vaccine will spread from vaccinated pigs to unvaccinated ones.

  2. **Inactivated vaccines**

     It is essential to test inactivated vaccines in the target animals under normal conditions of use for fattening pigs and for sows when this use is claimed by the manufacturer and authorised (European Pharmacopoeia, 2008; Vannier et al., 2007). As described previously, it is fundamental to use objective and quantifiable criteria to detect and to measure adverse reactions, such as temperature changes, weight performance, litter size, reproductive performance, etc., on vaccinated and control groups. The tests must be performed by administering the vaccine in the recommended dose and by each recommended route of administration to the pigs for which it is intended.

     Pigs or sows are usually observed until there is no further evidence of vaccine reaction. The period of observation must not be fewer than 14 days from the day of administration. This period has to be extended when, for example, the vaccine is used in pregnant sows and it is necessary to assess the possible effects of the vaccine on reproductive performance. In this case, the period of observation lasts the full duration of the pregnancy.
Control authorities generally request vaccination with a double dose so that adverse reactions, which may be at the limit of detection when a single dose is administered, are more likely to be detected.

**b. Local reactions**

Local reactions are often associated with the use of inactivated vaccines, as these side-effects can be induced by adjuvants, particularly oil adjuvants (Vannier, 1986). However, some Aujeszky’s disease live vaccines are mixed with different adjuvants, which modify what has been observed in the past.

Local reactions are mainly inflammatory and can be more or less complicated (necrotic or suppurative), depending on the nature of the adjuvants used and the aseptic conditions of the vaccination. Oil adjuvants can induce a variety of effects including muscular degeneration, granuloma, fibrosis and abscessation. In addition to the nature of the oil used (the intensity of the reaction is reduced when metabolisable oils are used in the vaccine), the type of emulsion used (water/oil, oil/water, water/oil/water) induces these reactions to a greater or lesser extent. In consequence, it is necessary to observe the site of injection not only from the outside, but also by dissection after slaughter, especially for growing and finishing pigs.

- **Field testing**

  Field trials are necessary to assess the safety of an Aujeszky’s disease vaccine in a large number of pigs or sows. In Europe (European Pharmacopoeia, 2008), tests must be carried out in each category of animals for which the vaccine is intended (sows, fattening pigs). At least three groups of no fewer than 20 animals each are used with corresponding groups of no fewer than 10 controls. The rectal temperature of each animal is measured at the time of vaccination, 6, 24 and 48 hours later. At slaughter, the injection site must be examined for local reactions. If the vaccine is intended to be used in sows, reproductive performances have to be recorded. Field trials are supplemented by laboratory studies of efficacy correlated to vaccine potency.

- **Laboratory trials**

  All tests must be carried out on pigs that do not have antibodies against Aujeszky’s disease virus or against a subunit of the virus, except that some tests may be done using maternally immune animals.

  - **Assessment of passive immunity**

    To test the efficacy of vaccines, it is important to mimic the natural infection conditions (European Commission, 2008). SHV-1 infection gives rise to important losses of young piglets from nonimmune sows. Thus, when vaccinating sows, the main goal is to protect the young piglets through passive immunity conferred by the colostrum ingested immediately after birth, with the secondary objective of preventing abortion.

    To measure this passive immunity and the protection induced by vaccinating the sows, experimental models have been established. The sows are vaccinated according to the vaccinal protocol during pregnancy. When the piglets are, for example, 6–10 days old they are given an intranasal challenge exposure with a virulent SHV-1 strain. It is preferable to use a strain titrated in median lethal doses ($LD_{50}$). Pigs should be inoculated by the nasal route, $10^2 LD_{50}$ per pig in 1 ml. The efficacy of the vaccine is assessed by comparing clinical signs, but also and more importantly, mortality in piglets from unvaccinated dams with that observed in piglets from vaccinated sows.

    Piglets from vaccinated sows can be found to have 80% protection against mortality compared with those from the control sows. In order for the results to be significant, it is recommended that eight vaccinated sows and four control sows be used (subject to satisfactory numbers of piglets from each sow).

  - **Assessment of active immunity**

    1. **Clinical protection**

      Several criteria can be considered when measuring active immunity induced by vaccinating pigs. Generally, pigs are vaccinated at the beginning of the growing period, i.e. when they are between 9 and 12 weeks old. Laboratory trials are performed by challenging pigs at the end of the finishing period, when they weigh between 80 and 90 kg.

      In general, at least three criteria, such as rectal temperatures, weight losses and clinical signs, along with mortality, are used to measure the clinical protection of pigs after vaccination and challenge (De Leeuw & Van Oirschot, 1985). The antibody titre has little predictive value for the efficacy of the vaccines. Weight loss compared between the vaccinated and control groups is the most reproducible and reliable parameter when the challenge conditions are well standardised. The measure of the difference in weight gain or loss between the two groups of pigs and, in the interval of time between challenge (day 0 and
Chapter 2.1.2. — Aujeszky's disease

Aujeszky's disease day 7), has a very good predictive value for the efficacy of the vaccines (Stellmann et al., 1989). Significant results can be obtained when weight performances are compared between one group of at least eight vaccinated pigs and another group of eight unvaccinated control pigs.

For challenge, it is usually preferable to use a high titre of a virulent strain, as this makes it possible to obtain a more marked difference between vaccinated and control pigs. On the basis of previous work, a challenge dose with at least $10^6$ TCID$_{50}$/ml virulent strain having undergone not more than three passages on primary cells can be sufficient, but a higher titre ($10^{7.5}$ TCID$_{50}$/ml) is recommended. The oro-nasal route should be used to challenge the pigs by introducing the virulent strain in an appropriately high volume ($\geq$ 4 ml).

This method of evaluating the efficacy of SHV-1 vaccines is now well tested and has made it possible to establish an objective index for determining the efficacy of a vaccine. This index, which compares the relative weight losses between vaccinated and control pigs, can also be used for potency testing batches before release and for batch efficacy testing. However, the value of the cut-off index will be different as the conditions of the assay will not be identical. The influence of passively acquired, maternally derived antibodies on the efficacy of a vaccine must be evaluated adequately.

2. Virulent virus excretion

Additionally, it is desirable that vaccines should prevent or at least limit viral excretion from infected pigs (Vannier et al., 1991). When a control programme against Aujeszky's disease is based on large-scale vaccination, it is essential to choose the vaccines or the vaccinal scheme that best limits the replication of virulent virus in infected pigs. Several assays have been performed to compare vaccines on that basis.

Generally, the pigs are vaccinated and challenged at different periods. It is better, but more time-consuming, to infect pigs at the end of the finishing period. To measure the virus excretion, nasal swabs (taken at 10 cm depth in the nostrils) are taken daily from each pig from the day before challenge to at least 12 days after challenge. The swabs can be weighed before the sampling and immediately after to calculate the exact weight of collected mucus. Medium is then added to each tube containing a swab. The virus is titrated from the frozen and thawed medium.

Different indexes can be used to express the quantity of virulent virus excreted by pigs, taking into consideration the duration and the level of viral excretion, and the number of pigs excreting virulent virus.

3. Duration of immunity

It is recommended that any claims regarding the onset and duration of immunity should be supported by data from trials. Assessment of duration of immunity can be based on challenge trials or, as far as it is possible, on immunological and serological tests.

- Field trials

In general terms, it is extremely difficult to assess vaccine efficacy in animal populations. In order to do this, it would be necessary to vaccinate the animals in the absence of the pathogen that the vaccine protects against, then to await the moment of infection and to compare the effects of infection in vaccinated animals (or the offspring of vaccinated dams) with the effects in the unvaccinated animals of the same age, in the same building and in the same batch as the vaccinated animals (or those protected passively). As all these conditions are difficult to achieve in the field, field trials are certainly more appropriate to safety testing than to efficacy testing, except for the development of marker vaccines that offer the opportunity to evaluate the effectiveness of vaccines under field conditions (Bouma, 2005).

- Stability

Tests have to be carried out to verify the shelf life proposed by the manufacturer. These tests must always be real-time studies; they must be carried out on a sufficient number of batches (at least three) produced according to the described production process and on products stored in the final container, and normally include biological and physicochemical stability tests. The manufacturer has to provide the results of analyses that support the proposed shelf life under all proposed storage conditions. Usually, the proposed shelf life corresponds to the period for which the product is considered to be stable minus 3 months.

3. Vaccines based on biotechnology

a) Vaccines available and their advantages

Biotechnology combined with a better knowledge of the functions and characteristics of the SHV-1 glycoproteins helped to develop new vaccines. For example, Quint et al. (1987) deleted glycoprotein E-coding
sequence from the NIA3 strain. This resulted in an efficient marker vaccine against Aujeszky’s disease, allowing differentiation of vaccinated from infected animals (DIVA vaccines). Most of the vaccines used at the moment are obtained from rDNA-derived gene-deleted virus. The deletion of the genes coding for the glycoprotein E is the most commonly used allowing an attenuated live virus vaccine to be obtained but still protecting against the clinical signs and reducing significantly the level of the viral excretion by the pigs vaccinated and infected. Because of the ability of some glycoproteins of SHV-1 to induce strong immune responses, efficiencies of DNA vaccines, consisting of plasmids encoding these glycoproteins, were tested. Indeed, DNA vaccination has a number of advantages: ease of construction and standardised production of plasmids, no handling of infectious particles, induction of humoral and cellular immune responses, bypass of the maternal derived immunity. The pioneering study on DNA vaccination against Aujeszky’s disease infection was published in 1997 (Gerdts et al., 1997). The use of a novel generation of plasmid amplifying the level of gene transcription of the proteins of interest (Dory et al., 2005) have been shown to be efficient strategies. These vaccines are not yet commercialised.

b) Special requirements for biotechnological vaccines, if any

Criteria to assess quality, safety and efficacy of the vaccines derived from the biotechnology are the same as the ones defined for conventional vaccines (see section 2). Nevertheless special attention has to be paid to the stability of the rDNA construction.

REFERENCES


* * *

**NB:** There are OIE Reference Laboratories for Aujeszky’s disease (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ ). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Aujeszky’s disease.
CHAPTER 2.1.3.

BLUETONGUE AND EPIZOOTIC HAEMORRHAGIC DISEASE

SUMMARY

Bluetongue virus (BTV) infection involves domestic and wild ruminants such as sheep, goats, cattle, buffaloes, deer, most species of African antelope and various other Artiodactyla as vertebrate hosts. This non-contagious, insect-borne viral infection is inapparent in the vast majority of infected animals but causes fatal disease in a proportion of infected sheep, deer and wild ruminants. Infection in cattle mostly has not resulted in clinical signs, with the recent exception of BTV8 infection of cattle in Europe. Cattle are particularly significant in the epidemiology of the disease due to the prolonged viraemia in the absence of clinical disease. Clinical signs range from mild to severe and vary not only between species but between breeds and within the flock or herd. Clinical signs of BT disease are mainly attributable to vascular permeability and include fever, hyperaemia and congestion, facial oedema and haemorrhages, and erosion of the mucous membranes. However in mild cases of the disease, a transitory hyperaemia and slight ocular and nasal discharge may be observed. In very severe cases the tongue may show hyperaemia, become oedematous and protrude from the mouth, or become cyanotic. Hyperaemia may extend to other parts of the body, particularly the coronary band of the hoof, the groin, axilla and perineum. In severe cases there is additionally skeletal and cardiac muscle degeneration. Wool breaks may occur. Sheep may become lame as a result of laminitis and skeletal myopathy. A similar severe disease of wild ruminants is caused by epizootic haemorrhagic disease virus (EHDV), which, like BTV, is a member of the Orbivirus genus, but is classified in a separate species or serogroup.

Identification of the agent: BTV is a member of the Orbivirus genus of the family Reoviridae, one of 20 recognised species or serogroups in the genus. The BTV species, or serogroup, contains 24 recognised serotypes and EHDV contains at least seven. The orbiviral species are differentiated by immunological tests that detect viral proteins that are conserved within each, and hence are distinguishable by serogrouping tests. However there may be considerable cross-reaction between related species, and this is the case with the BT and EHD serogroups. The serotype of individual viruses in each species is identified on the basis of neutralisation tests and different strains within a serotype are identified by sequence analysis. Complete BTV particles are double-shelled icosahedral double-stranded RNA virus. The outer layer contains two proteins, one of which, VP2, is the major determinant of serotype specificity. The inner shell and core contains two major and three minor proteins and ten double-stranded RNA genetic segments. VP7 located in the inner shell is the major core protein possessing the species or serogroup-specific antigens. Virus identification traditionally requires isolation and amplification of the virus in embryonated hens’ eggs, tissue culture or inoculations of susceptible ruminants and the subsequent application of serogroup- and serotype-specific tests. Reverse-transcription polymerase chain reaction (RT-PCR) technology has permitted rapid amplification of BTV and EHDV RNA in clinical samples, and RT-PCR-based procedures are now available. Real-time PCR is allowing development of even more rapid and sensitive tests, and procedures are currently being validated and published. These procedures can augment the classical virological techniques to provide information on virus serogroup, serotype and topotype.

Serological tests: Serological responses appear some 7–14 days after BTV infection and are generally long-lasting. Historically, tests such as agar gel immunodiffusion and indirect enzyme-linked immunosorbent assay (ELISA) were used to detect BTV and EHDV species-specific antibodies, but have the major drawback of being unable to consistently distinguish between antibodies to viruses in the two species. A monoclonal antibody-based competitive ELISA has
solved this problem and competitive ELISAs to specifically detect anti-BTV antibodies are recommended. Procedures to determine the serotype-specificity of antibodies in sera are more complex and time-consuming because they assess whether the sera inhibit the infectivity of panels of known virus serotypes in neutralisation tests.

**Requirements for vaccines and diagnostic biologicals:** Vaccination is used in several countries to limit direct losses, minimise the circulation of BTV and allow safe movement of animals. However, there is no vaccine to protect against EHDV infection. For many years South Africa has used live, attenuated BT vaccines that are serotype-specific. Live attenuated vaccines are produced by adapting BTV field isolates to growth in vitro through serial passages in tissue culture or in embryonated hens’ eggs. Stimulation of a strong antibody response by these vaccines is directly correlated with their ability to replicate in the vaccinated host. Live attenuated vaccines are cheap to produce in large quantities, they generate protective immunity after a single inoculation and have proven effective in preventing clinical BT disease. Adverse consequences are depressed milk production in lactating sheep, and abortion/embryonic death and teratogenesis in offspring from pregnant females that are vaccinated during the first half of gestation. Another risk associated with the use of live attenuated vaccines is their potential for spread by vectors, with eventual reversion to virulence and/or reassortment of vaccine virus genes with those of wild-type virus strains. The frequency and significance of these events remain poorly defined but transmission of vaccine strains by vector Culicoides in the field has already been documented in Europe. The fact that attenuated viruses are teratogenic makes determination of transmissibility very important. Vaccine efficacy, teratogenic potential and transmissibility should be tested. Hence inactivated or recombinant vaccines would be preferred if effective. The inactivated vaccines are not teratogenic and have been used under government supervision since 2004 in the management of recent European outbreaks.

### A. INTRODUCTION

Midges of certain species in the genus *Culicoides* (the insect host) (Standfast *et al*., 1985) transmit bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) among susceptible ruminants, having become infected by feeding on viraemic animals (the vertebrate host). After a replication period in the insect’s salivary glands, that depends upon ambient temperature, the virus can be transmitted to a new vertebrate host during feeding. Infected midges remain infective for life. The central role of the insect in BT and EHD epidemiology ensures that distribution and prevalence of the infection is governed by ecological factors, such as high rainfall, temperature, humidity and soil characteristics, which favour insect survival (Calistri *et al*., 2003). In many parts of the world therefore, infection has a seasonal occurrence (Verwoerd & Erasmus, 2004). It is accepted that BTV and EHDV do not establish persistent infections in ruminants, and that survival of BTV and EHDV in the environment is associated with insect factors (Lunt *et al*., 2006; MacLachlan, 2004). Globally the distributions of BTV and EHDV have been considered on the basis of epidemiological systems (episystems) based on the vector species present and their natural history (Tabachnick, 2004). However, recent information suggests that BTV and EHDV have moved between episystems and with different species of vector midges (Johnson *et al*., 2007; Mellor *et al*., 2009a).

The vertebrate hosts for BTV include both domestic and wild ruminants, such as sheep, goats, cattle, buffaloes, deer, most species of African antelope and other Artiodactyla such as camels. Although antibodies to BTV, and in some cases virus antigen and/or live virus has been demonstrated in some carnivores, felines, black and white rhinoceroses and elephants, the role of non-ruminant species in the disease in the wild is not known. The outcome of infection ranges from inapparent in the vast majority of infected animals, especially wild Africa ruminants and cattle, to fatal in a proportion of infected sheep, goats, deer and some wild ruminants (Verwoerd & Erasmus, 2004). However a higher incidence of clinical disease has been observed in cattle infected with BTV8 in Europe. Some breeds of sheep are more susceptible to disease than others, with the result that in some countries BTV infections of livestock can occur unobserved, and be detected only by active surveillance (Daniels *et al*., 2004). Epizootic haemorrhagic disease virus (EHDV) can produce a disease in wild ruminants with clinical manifestations identical to those observed in response to BTV infection. As EHD has not been studied in detail, there will be less information provided on this virus and disease. Clinical signs of disease in sheep vary markedly in severity, influenced by the type or strain of the infecting virus, husbandry factors as well as by breed (Verwoerd & Erasmus, 2004). In severe cases there is an acute febrile response characterised by hyperaemia and congestion, leading to oedema of the face, eyelids and ears, and haemorrhages and erosions of the mucous membranes. The tongue may show intense hyperaemia and become oedematous, protrude from the mouth and, in severe cases become cyanotic. Hyperaemia may extend to other
parts of the body particularly the coronary band of the hoof, the groin, axilla and perineum. There is often severe muscle degeneration. Breaks in the wool may occur associated with pathology in the follicles. A reluctance to move is common and torticollis may occur in severe cases. In fatal cases the lungs may show interalveolar hyperaemia, severe alveolar oedema and the bronchial tree may be filled with froth. The thoracic cavity and pericardial sac may contain several litres of plasma-like fluid. Most cases show a distinctive haemorrhage near the base of the pulmonary artery (Verwoerd & Erasmus, 2004).

Taxonomically, BTV is classified as a species or serogroup in the *Orbivirus* genus in the family *Reoviridae*, one of 22 recognised species in the genus that also includes EHDV, equine encephalosis and African horse sickness (AHS) viruses (Monaco *et al*., 2006). Within species, individual members are differentiated on the basis of neutralisation tests, and currently 24 serotypes of BTV and 7 serotypes of EHD are recognised (Attoui *et al*., 2009). There is significant immunological cross-reactivity between members of the BT and EHD viruses (Monaco *et al*., 2006). Recently, a novel BT like virus (Toggenburg orbivirus) was isolated from goats in Switzerland and proposed as a new BTV serotype (Hofmann *et al*., 2008). The broader significance of the finding has yet to be established.

BTV and EHDV particles are composed of three protein layers. The outer layer contains two proteins, VP2 and VP5. VP2 is the major neutralising antigen and determinant of serotype specificity. Removal of the outer VP2/VP5 layer leaves a bi-layered icosahedral core particle that is composed of two major proteins, VP7 and VP3, three minor proteins and the ten species of double-stranded RNA. VP7 is a major determinant of serogroup specificity and the site of epitopes used in competitive enzyme-linked immunosorbent assay (C-ELISA) to detect anti-BTV and EHDV antibodies (Mertens *et al*., 2005). VP7 can also mediate attachment of BTV to insect cells (Xu *et al*., 1997).

Genetic sequencing of BTVs and EHDVs allows differentiation and analysis of strains separately from serotype (Gould, 1987; McColl & Gould, 1991; Pritchard *et al*., 1995; Wilson *et al*., 2000). Even for strains within the one serotype it is possible to identify the likely geographical origin (topotype) (Gould, 1987; Potgieter *et al*., 2005). Such studies have led to the detection of international movements of BTV strains. Natural movements of vectors by climatic forces are believed to result in some intercontinental movements of BTV and EHDV. Particularly important in recent times as examples of such vector movements have been the incursions of BTV into southern Europe, (Mellor *et al*., 2009b); northern Australia (Daniels *et al*., 2009) and southern States of the USA (Johnson *et al*., 2007). However the origins of the recent (2006–2008) incursions of BTV directly into northern Europe remain uncertain. Identification of apparent associations between some genotypes of virus and some vector species has led to the concept of viral-vector ecosystems being proposed (Daniels *et al*., 2004; 2009; MacLachlan, 2004; Tabachnick, 2004) Recent movements of several BTV serotypes between vector species and into new geographic regions indicates a more complete understanding of BTV and EHDV epidemiology is required.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the BTV agent (a prescribed test for international trade)

a) Virus isolation

The same diagnostic procedures are used for domestic and wild ruminants. A number of virus isolation systems for BTV are in common use, but the most sensitive method is by inoculation of embryonated hens’ eggs (ECE). Inoculation of sheep may also be a useful approach if the titre of virus in the sample blood is very low, as may be the case several weeks after virus infection. Attempts to isolate virus in cultured cells in vitro may be more convenient, but the success rate is frequently much lower than that achieved with in vivo systems (Gard *et al*., 1988). EHDV may be isolated in the same isolation system as for BTV but primary inoculation of cell cultures such as C6/36 cells, deleting the first passage in ECE, has proven to be equally sensitive (Gard *et al*., 1988).

- Isolation in embryonated hens’ eggs
  i) Blood is collected from febrile animals into an anticoagulant such as EDTA (ethyamine diamine tetra-acetic acid), heparin or sodium citrate, and the blood cells are washed three times with sterile phosphate buffered saline (PBS). Washed cells are re-suspended in PBS or isotonic sodium chloride and either stored at 4°C or used immediately for attempted virus isolation.
  ii) For long-term storage where refrigeration is not possible blood samples are collected in oxalate–phenol–glycerin. If samples can be frozen, they should be collected in buffered lactose peptone or 10% dimethyl sulphoxide (Thomas, 1984) and stored at −70°C or colder. The virus is not stable for long periods at −20°C.
iii) In fatal cases, spleen and lymph nodes are the preferred organs for virus isolation attempts. Organs and tissues should be kept and transported at 4°C to a laboratory where they are homogenised in PBS or isotonic saline, and used as described below, for blood cells.

iv) Washed blood cells are re-suspended in distilled water or sonicated in PBS and 0.1 ml amounts inoculated intravascularly into 5–12 ECE that are 9–12 days old. This procedure requires practice. Details are provided by Clavijo et al. (2000).

v) The eggs are incubated in a humid chamber at 32–33.5°C and candled daily. Any embryo deaths within the first 24 hours post-inoculation are regarded as nonspecific.

vi) Embryos that die between days 2 and 7 are retained at 4°C and embryos remaining alive at 7 days are killed. Infected embryos may have a haemorrhagic appearance. Dead embryos and those that live to 7 days are homogenised as two separate pools. Whole embryos, after removal of their heads, or pooled organs such as the liver, heart, spleen, lungs and kidney are homogenised and the debris is removed by centrifugation.

vii) Virus in the supernatant may be identified either directly by antigen-capture ELISA (Hawkes et al., 2000) or reverse transcription polymerase chain reaction (RT-PCR), or indirectly by antigen-detection methods such as immunofluorescence or immunoperoxidase after further amplification in cell culture, as described in the next section.

viii) If no embryos are killed following inoculation of sample material, an inoculum made from the first egg passage material may be re-passaged in ECE or in cell culture.

• Isolation in cell culture

Virus isolation may be attempted in BTV in susceptible cell cultures such as mouse L, baby hamster kidney (BHK-21), African green monkey kidney (Vero) or Aedes albopictus clone C6/36 (AA). The efficiency of isolation is often significantly lower following inoculation of cultured cells with diagnostic samples compared with that achieved in ECE. Highest recovery rates are achieved by primary isolation of virus in ECE, followed by passage in C6/36 (AA) cells for further replication of virus. Additional passages in mammalian cell lines such as BHK-21 or Vero are usually performed. A cytopathic effect (CPE) is not necessarily observed in AA cells but appears in mammalian cells. Cell monolayers are monitored for the appearance of a CPE for 5 days at 37°C in 5% CO₂ with humidity. If no CPE appears, a second passage is made in cell culture. The identity of BTV in the culture medium of cells manifesting a CPE may be confirmed by a number of immunological methods described below, including antigen-capture ELISA, immuno-fluorescence, immunoperoxidase, virus neutralisation (VN) tests, or by RT-PCR.

• Isolation in sheep

This procedure is used for isolation of BTV and less sensitive for the isolation of EHDV and not routinely used.

i) Sheep are inoculated with washed cells from 10 ml to 500 ml of blood, or 10–50 ml tissue suspension. Inocula are administered subcutaneously in 10–20 ml aliquots. Large volumes may aid in the virus isolation attempts and should be administered intravenously.

ii) The sheep are held for 28 days and checked daily for pyrexia and weekly for antibody response using serological tests such as the C-ELISA as described below. Sheep blood collected at 7–14 days post-inoculation will usually contain the isolated virus, which can be stored viable at 4°C or –70°C.

b) Immunological methods

• Serogrouping of viruses

Orbivirus isolates are typically serogrouped on the basis of their reactivity with specific standard antisera that detect proteins, such as VP7, that are conserved within each serogroup. The cross-reactivity between members of the BT and EHD serogroups raises the possibility that an isolate of EHDV could be mistaken for BTV on the basis of a weak immunofluorescence reaction with a polyclonal anti-BTV antiserum. For this reason, a BT serogroup-specific MAb can be used. A number of laboratories have generated such serogroup-specific reagents (Anderson, 1984; Lunt et al., 1988). Commonly used methods for the identification of viruses to serogroup level are as follows.

i) Immunofluorescence

Monolayers of BHK or Vero cells on chamber slides (glass cover-slips) are infected with either tissue culture-adapted virus or virus in ECE lysates. After 24–48 hours at 37°C, or after the appearance of a mild CPE, infected cells are fixed with agents such as paraformaldehyde, acetone or methanol, dried and viral antigen detected using anti-BTV antiserum or BTV-specific MAbs and standard immunofluorescent procedures.
ii) Antigen capture enzyme-linked immunosorbent assay

Viral antigen in ECE and culture medium harvests (Hawkes et al., 2000) infected insects (Mecham et al., 1990) and sheep blood may be detected directly. In this technique, virus derived proteins are captured by antibody adsorbed to an ELISA plate and bound materials detected using a second antibody. The capture antibody may be polyclonal or a serogroup-specific MAb. Serogroup-specific MAbs and polyclonal antibody raised to Baculovirus-expressed core particles have been used successfully to detect captured virus (Hawkes et al., 2000).

iii) Immunospot test

Small volumes (2 µl) of infected cell culture supernatant or lysed or sonicated infected cells are adsorbed to nitrocellulose and air-dried. Nonspecific binding sites are blocked by incubation in a solution containing skim milk protein. After incubation with a BTV serogroup-reactive MAb, bound antibody is detected using horseradish peroxidase-conjugated anti-mouse IgG (Gard & Kirkland, 1993).

• Serotyping by virus neutralisation

Neutralisation tests are type specific for the currently recognised 24 BTV and 7 EHDV serotypes and can be used to serotype a virus isolate or can be modified to determine the specificity of antibody in sera. In the case of an untyped isolate, the characteristic regional localisation of BTV and EHDV serotypes can generally obviate the need to attempt neutralisation by all 24 antisera, particularly when endemic serotypes have been identified.

There is a variety of tissue culture-based methods available to detect the presence of neutralising anti-BTV antibodies. Cell lines commonly used are BHK, Vero and L929. Four methods to serotype BTV are outlined briefly below. BTV and EHDV serotype-specific antisera generated in guinea-pigs or rabbits have been reported to have less serotype cross-reactivity than those made in cattle or sheep. It is important that antiserum controls be included.

i) Plaque reduction

The virus to be serotyped is diluted to contain approximately 100 plaque-forming units (PFU), and incubated with either no antiserum or with dilutions of individual standard antisera to a panel of BTV serotypes. Virus/antiserum mixtures are added to monolayers of cells. After adsorption and removal of inoculum, monolayers are overlaid with agarose. The neutralising antibody titres are determined as the reciprocal of the serum dilution that causes a fixed reduction (e.g. 90%) in the number of PFU. The unidentified virus is considered serologically identical to a standard serotype if the latter is run in parallel with the untyped virus in the test, and is similarly neutralised.

ii) Plaque inhibition

Tests are performed in 90 mm diameter Petri dishes containing confluent cell monolayers that are infected with approximately 5 × 10⁴ PFU standard or untyped virus. After adsorption and removal of inoculum, monolayers are overlaid with agarose. Standard anti-BTV antisera are added to individual filter paper discs and placed on the agarose surface. Dishes are incubated for at least 4 days. A zone of virus neutralisation, with concomitant survival of the cell monolayer, will surround the disc containing the homologous antiserum.

iii) Microtitre neutralisation

Approximately 100 TCID₅₀ (50% tissue culture infective dose) of the standard or untyped virus is added in 50 µl volumes to test wells of a flat-bottomed microtitre plate and mixed with an equal volume of standard antiserum diluted in tissue culture medium. Approximately 10⁴ cells are added per well in a volume of 100 µl, and after incubation for 4–6 days, the test is read using an inverted microscope. Wells are scored for the degree of CPE observed. Those wells that contain cells only or cells and antiserum, should show no CPE. In contrast, wells containing cells and virus should show convincing CPE. The unidentified virus is considered to be serologically identical to a standard BTV serotype if both are neutralised in the test to a similar extent.

iv) Fluorescence inhibition test

This rapid and simple neutralisation assay (Blacksell & Lunt, 1996) requires varying concentrations of an unknown virus and standard concentrations of reference antisera. Virus isolates grown in cell culture are serially diluted starting and mixed with individual reference antisera in the wells of a Lab-Tek slide for 1 hour prior to addition of cells. After incubation for 16 hours, cells are fixed and probed by an immunofluorescent procedure using a BT serogroup-specific MAb. The serotype of the virus is indicated by the specificity of the antiserum causing the largest reduction in the number of fluorescent cells.
c) Reverse-transcription polymerase chain reaction (a prescribed test for international trade)

Primer-directed amplification of viral nucleic acid has revolutionised BTV and EHDV detection in tested samples (Dangler et al., 1990; McColl & Gould, 1991; Wade-Evans et al., 1990). RT-PCR techniques have allowed the rapid identification of BT viral nucleic acid in blood and other tissues of infected animals. RT-PCR-based diagnostics should be interpreted with caution. The RT-PCR procedure will detect virus-specific nucleic acid, but this does not necessarily indicate the presence of infectious virus (MacLachlan et al., 1994). RT-PCR can also be used to ‘serogroup’ Orbiviruses and may ultimately be possible to ‘serotype’ BTV within a few days of receipt of a clinical sample, such as infected sheep blood (Mertens et al., 2007). Traditional approaches, which rely on virus isolation followed by virus identification serologically, may require up to 4 weeks to generate information on serogroup and serotype.

Oligonucleotide primers used so far have been derived from RNA 7 (VP7 gene) (Wade-Evans et al., 1990), RNA 6 (NS1 gene) (Dangler et al., 1990), RNA 3 (VP3 gene) (Pritchard et al., 1995), RNA 10 (NS3 gene) (Billinis et al., 2001) and RNA 2 (VP2 gene) (McColl & Gould, 1991). The size of the amplified transcripts is usually small — in the order of several hundred nucleotides — but can also be a full-length gene. In the procedure described in detail below, a 101-nucleotide stretch of RNA 6 is amplified. Primers derived from the more highly conserved genes, such as VP3, VP7 and NS1, may be used for serogrouping (i.e. will react with all members of the BT serogroup), while primers for which the sequence was determined from VP2 gene sequences provide information on virus serotype. A multiplex RT-PCR assay that depends on the size of the amplified products has been used to identify the five North American BTV serotypes, both alone and in mixtures, in a single reaction (Johnson et al., 2000).

The nucleic acid sequence of cognate BTV genes may differ with the geographical area of virus isolation (Gould, 1987). This has provided a unique opportunity to complement studies of BTV epidemiology by providing information on the potential geographical origin of virus isolates, a process termed genotyping or topotyping. Thus, determination of the nucleic acid sequence of portions of RNA may provide information on where the virus came from. It appears likely that sequencing of BTV isolates from other parts of the world may permit finer discrimination of geographical origin. However, the relationship between sequence and geographical origin may not be straightforward. This sequencing information is important and all data regarding BTV segment sequences should be made widely available by submitting the data to officially recognised web sites.

http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ and
http://eubtnet.izs.it/btnet/index.htm

The web sites provide phylogenetic tree analyses of BTV isolates based on the sequence of RNA segments. These compiled data will provide a resource for epidemiological studies, the identification of new isolates and the design of new primers for further RT-PCR and possibly serotype-specific assays for BTV.

It has been observed that BTV nucleic acid can be detected by RT-PCR from the blood of infected calves and sheep at least 30 days, and sometimes over 90 days, after the virus can be isolated. The presence of virus-specific nucleic acid does not necessarily indicate the presence of infectious virus.

The capacity of RT-PCR assays to detect very small numbers of nucleic acid molecules means that such tests are exquisitely sensitive to contamination by extraneous nucleic acids. The latter may include any primers in use in the laboratory or previously amplified polynucleotides. It is critical therefore to have a ‘clean’ area containing all equipment necessary for reagent and test preparation and a separate area with its own equipment for amplification. Impervious gloves should be worn and changed frequently at all stages of the procedure, particularly after working with sample RNA or amplified DNA. This will help protect reagents and samples from contamination by ubiquitous RNases and other agents and from cross-contamination by DNA. The possibility of false positives, due to sample contamination, highlights the importance of sequencing RT-PCR products to determine, for example, if the amplified sequence is identical to or different from that of the positive control. False negatives, due for example to poor sample quality or inappropriate primers, may be identified following the failure to amplify both BTV and a host gene, such as globin, from extracts of infected cells. This is covered in more detail in Chapter 1.1.4 Principles and methods of validation of diagnostic assays for infectious diseases.

There are many RT-PCR assays currently in use that use different extraction methods, reverse transcriptases, amplification enzymes, primers and conditions. Technology is changing rapidly. Also the genetic diversity of the BTV genes makes the choice and validation of RT-PCR assays conditional on its application in a regional setting. This is also the case for real-time PCR, which will be developed for use in routine molecular diagnosis of BTV in the future. Therefore the procedures listed below are examples only. Increasingly it will be more important to maintain diagnostic testing under accreditation to an international
standard such as ISO/ISEC 17025 and participate in proficiency testing. Systems for offering proficiency testing for RT-PCR tests are being developed in a number of countries.

The RT-PCR assay described here involves three separate procedures. In the first, BTV RNA is extracted from blood using a chaotropic agent such as guanidine thiocyanate (GuSCN) to denature protein and release viral RNA. A number of commercial kits are available for this purpose and the protocol below describes the use of one such kit, IsoQuick\(^1\). The reagents provided with the kit are numbered and their use is indicated in the protocol below. Other kits are available and one, TRIZOL\(^2\), is particularly useful for the extraction of viral nucleic acid from spleen or blood clots. Operators should follow the procedures specified in each kit and use reagent solutions either provided or recommended for the kit of their choice. The second procedure is the denaturation of viral double-stranded RNA and reverse transcription to generate cDNA, which is amplified by RT-PCR. In the procedure described below, the Superscript\textsuperscript{TM} Preamplification System\(^3\) is used to transcribe viral RNA, and reagents\(^4\) are used for the RT-PCR. Equivalent kits and reagents are available from other sources. The final step of the process is the analysis of the RT-PCR product by electrophoresis. Procedures used to determine the sequence of the amplified product are not described here.

- **Extraction of viral RNA**
  
  i) Whole blood is collected from test and uninfected control animals in EDTA tubes and centrifuged at 800–1000 \(g\) for 10 minutes. The plasma is aspirated and the red blood cells (RBCs) are gently resuspended in sterile PBS. RBCs are pelleted by centrifugation at 1000 \(g\) for 10 minutes and the supernatant is removed.
  
  ii) Next, 400 µl of test RBCs is added to each of four 1.7 ml microcentrifuge tubes, and 400 µl of control RBCs is added to each of two microcentrifuge tubes. An equal volume of RNase-free water is added to each tube and the tubes are vortexed briefly to mix and lyse the cells. Two tubes containing test RBCs are frozen at –70°C for repository purposes and the extraction is continued in duplicate.
  
  iii) Lysed test and control RBCs are centrifuged at 12,000–16,000 \(g\) for 10 minutes and the supernatant is discarded. Next, 800 µl RNase-free water is added and the tubes are vortexed and centrifuged again at the same speed for 10 minutes. The supernatant is removed and the RBC pellet is drained.
  
  iv) A small volume of BTV (e.g. 5 µl containing from 10\(^3\) to 10\(^7\) PFU) is added to one of two control RBC pellets. This is the positive control. The other control RBC pellet remains as the negative control.
  
  v) Next, 75 µl of sample buffer (IsoQuick reagent A) is added to each pellet, and the pellets are then vortexed vigorously, followed by the addition of 125 µl of the GuSCN-containing lysis solution (IsoQuick reagent 1). The mixture is vortexed vigorously for 30 seconds.
  
  vi) Before use the extraction matrix provided with the kit (IsoQuick reagent 2 plus dye 2A) is shaken vigorously and 500 µl is added to the sample lysates. Then, 400 µl extraction buffer (IsoQuick reagent 3) is added and the tubes are vortexed for 10 seconds.
  
  vii) The tubes are incubated at 65°C for 10 minutes, vortexed briefly after 5 minutes and centrifuged at 12,000 \(g\) for 5 minutes.
  
  viii) The aqueous phase (500 µl) is transferred to a new microcentrifuge tube and an equal volume of extraction matrix (IsoQuick reagent 2) is added. The tubes are vortexed for 10 seconds and centrifuged at 12,000 \(g\) for 5 minutes.
  
  ix) The aqueous phase (330 µl) is transferred to a new microcentrifuge tube and a 10% volume (33 µl) of sodium acetate (IsoQuick reagent 4) and 365 µl isopropanol are added. After gentle mixing, the tubes are placed at –20°C for from 20 minutes to 1 hour.
  
  x) The RNA is pelleted by centrifugation at 12,000 \(g\) for 10 minutes. The supernatant is decanted and 1.0 ml 70% ethanol is added and mixed gently. After centrifugation at 12,000 \(g\) for 5 minutes, the supernatant is decanted and 1.0 ml 100% ethanol is added. The tubes are stored at –70°C until ready for use in the RT-PCR.

- **Reverse-transcription polymerase chain reaction**
  
  i) RNA in ethanol is centrifuged at 12,000 \(g\) for 5 minutes. The ethanol is decanted and the tubes are inverted and allowed to drain. The pellet, which may not be obvious, must not be allowed to dry out because this makes resuspension difficult. A dry pellet is also likely to fall out of the inverted tube.

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\(^1\) IsoQuick: Orca Research, Bothell, Washington, United States of America (USA).

\(^2\) TRIZOL: Life Technologies, Grand Island, New York, USA.

\(^3\) Superscript\textsuperscript{TM} Preamplification System: Life Technologies, Grand Island, New York, USA.

\(^4\) Available from Perkin-Elmer.
ii) Next, 12 µl RNase-free water is added to each tube, mixed and heated at 65°C for 5–10 minutes. The samples are placed in ice.

iii) In a ‘clean’ biohazard hood, stock solutions containing 200 pmol/µl of primers A, B, C and D are prepared in RNase-free water and stored at –70°C.

First stage RT-PCR primers (to amplify RNA 6 from nucleotide 11 to 284)
Primer A: 5'-GTT-CTC-TAG-TTG-GCA-ACC-ACC-3'
Primer B: 5'-AAG-CCA-GAC-TGT-TTC-CCG-AT-3'

Nested RT-PCR primers (to amplify RNA 6 from nucleotide 170 to 270)
Primer C: 5'-GCA-GCA-TTT-TGA-GAG-AGC-GA-3'
Primer D: 5'-CCC-GAT-CAT-ACA-TTG-CTT-CCT-3'

iv) Primer stock solutions are diluted to a concentration of 15–20 pmol/µl. Primers for the first stage RT-PCR reaction are prepared by mixing equal volumes of A and B. Primers for the nested RT-PCR reaction are prepared by mixing equal volumes of C and D. Small aliquots of pooled primer mixes are frozen at –20°C.

v) RT-PCR reaction tubes are labelled and, for first stage synthesis, 4.0 µl of primer (A + B) mix is added to each tube. The tubes are held on ice.

vi) In a ‘clean’ fume hood methylmercuric hydroxide is diluted to 50 mM (1/20 dilution) and 2-mercaptoethanol is diluted to 350 mM (1/40 dilution) in RNase-free water. Methylmercuric hydroxide and 2-mercaptoethanol are considered to be extremely and highly toxic, respectively. Use both chemicals with extreme care and dispose of them and pipette tips as required by safety regulations. Alternative methods using heat denaturation have been described (Maan et al., 2007; Mertens et al., 2007).

vii) Next, 4 µl of test and positive and negative control RNA samples (step ii) are added to 4 µl of the primer mix in RT-PCR tubes (Wilson et al., 2000).

viii) To each RT-PCR tube 2.0 µl of the 1/20 dilution of methylmercuric hydroxide is added with gentle mixing and allowed to sit at room temperature for 10 minutes prior to adding 2.0 µl of the 1/40 dilution of 2-mercaptoethanol. For safety reasons, some laboratories use formamide instead of methylmercuric hydroxide for double-stranded RNA denaturation. However, for optimum sensitivity, methylmercuric hydroxide is preferred.

ix) In a ‘clean’ hood a cDNA mix is prepared containing the following reagents in sufficient volume for the number of samples being tested. The amount given is per sample and the reagents are contained in the kit5.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 × Superscript™ buffer (200 mM Tris/HCl, pH 8.4, and 500 mM KCl)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)</td>
<td>1.25 µl</td>
</tr>
<tr>
<td>Dithiothreitol (DTT) (0.1 M)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Reverse transcriptase (200 units/µl)</td>
<td>0.75 µl</td>
</tr>
</tbody>
</table>

x) Then, 8.0 µl of the mix is added to each RT-PCR tube to a final volume of 20.0 µl.

xi) The RT-PCR tubes are placed in a thermal cycler, such as GeneAmp™ PCR System 9600, which is programmed for reverse transcription as follows:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold 44°C</td>
<td>50 minutes</td>
</tr>
<tr>
<td>Hold 4°C</td>
<td>For ever</td>
</tr>
</tbody>
</table>

xii) The tubes are removed from the thermal cycler and 1.0 µl RNase H and a wax bead are added to each tube. The cycler is programmed as follows:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold 37°C</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Hold 98°C</td>
<td>4 minutes</td>
</tr>
<tr>
<td>Hold 4°C</td>
<td>For ever</td>
</tr>
</tbody>
</table>

xiii) In a ‘clean’ hood a first stage amplification mix is prepared containing the following reagents and in a volume sufficient for the number of samples being tested. All these reagents except water are available6. The amount given is per sample.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase-free water</td>
<td>62.0 µl</td>
</tr>
<tr>
<td>10 × PCR Perkin-Elmer buffer (100 mM Tris/HCl, pH 8.3, and 500 mM KCl)</td>
<td>7.0 µl</td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>7.0 µl</td>
</tr>
<tr>
<td>dNTP mix (2.5 mM each dATP, dCTP, dGTP, dTTP)</td>
<td>4.0 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 units/µl)</td>
<td>0.85 µl</td>
</tr>
</tbody>
</table>

5 Superscript™ Preamplification System: Life Technologies, Grand Island, New York, USA.
6 Available from Perkin-Elmer.
xiv) The first stage mix is removed from the ‘clean’ area to the thermal cycling area and 80 µl is overlaid in each sample tube. The wax layer must not be pierced. Each tube should now contain 101 µl.

xv) The tubes are placed in the thermal cycler, which is programmed as follows (correct for GeneAmp PCR System 9600 – programmes for other thermal cyclers would need to be determined) for first stage amplification:

<table>
<thead>
<tr>
<th>Cycle Type</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>One cycle</td>
<td>95°C</td>
<td>3 min</td>
</tr>
<tr>
<td>Hold 58°C</td>
<td>20 s</td>
<td></td>
</tr>
<tr>
<td>Hold 72°C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>40 cycles</td>
<td>95°C</td>
<td>20 s</td>
</tr>
<tr>
<td>Hold 58°C</td>
<td>20 s</td>
<td></td>
</tr>
<tr>
<td>Hold 72°C</td>
<td>20 s</td>
<td></td>
</tr>
</tbody>
</table>

xvi) RT-PCR reaction tubes are prepared for the nested reaction in a ‘clean’ hood 15 minutes before cycling is complete, and held on ice:

\[
\begin{align*}
\text{Rnase-free water} & \quad 17 \mu l \text{ per tube} \\
\text{Nested primer mix (C+D)} & \quad 4.0 \mu l \text{ per tube} \\
\text{Wax bead} & \\
\end{align*}
\]

xvii) When first stage amplification is complete, the tubes are removed from the thermal cycler and placed in a biological safety cabinet (not the ‘clean’ hood). Then, 1.5 µl of the first stage product is transferred to the corresponding nested RT-PCR tube containing primer, water and a wax bead.

xviii) The tubes are placed in the thermal cycler, which is programmed as follows for wax layer formation:

<table>
<thead>
<tr>
<th>Cycle Type</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>One cycle</td>
<td>98°C</td>
<td>4 min</td>
</tr>
<tr>
<td>Hold 4°C</td>
<td>For ever</td>
<td></td>
</tr>
</tbody>
</table>

xix) In a ‘clean’ hood the nested mix of the following reagents is prepared in sufficient volume for the number of samples being tested. The reagents used are the same as in the first stage (step xii). The amount given is per sample.

\[
\begin{align*}
\text{RNase-free water} & \quad 17.0 \mu l \\
\text{10 × PCR buffer} & \quad 5.0 \mu l \\
\text{MgCl}_2 & \quad 3.5 \mu l \\
\text{dNTP mix} & \quad 4.5 \mu l \\
\text{Taq DNA polymerase} & \quad 0.5 \mu l \\
\end{align*}
\]

xx) The nested mix is removed from the ‘clean’ hood to the thermal cycler and 30 µl is overlaid into each sample tube. Each tube should now contain 52 µl.

xxi) The tubes are placed in the thermal cycler, which is programmed as follows for nested amplification. After completion, the tubes are held at 4°C or at –20°C until electrophoresis:

<table>
<thead>
<tr>
<th>Cycle Type</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>One cycle</td>
<td>95°C</td>
<td>3 min</td>
</tr>
<tr>
<td>Hold 58°C</td>
<td>20 s</td>
<td></td>
</tr>
<tr>
<td>Hold 72°C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>40 cycles</td>
<td>95°C</td>
<td>20 s</td>
</tr>
<tr>
<td>Hold 58°C</td>
<td>20 s</td>
<td></td>
</tr>
<tr>
<td>Hold 72°C</td>
<td>20 s</td>
<td></td>
</tr>
</tbody>
</table>

Electrophoretic analysis of RT-PCR product

i) First, 1 × TBE buffer (0.045 mM Tris/borate, pH 8.6, and 1.5 mM EDTA) is prepared from a ×10 stock solution. For the Bio-Rad Wide Mini-Sub cell system, 700 ml buffer is prepared (100 ml for the gel and 600 ml for the tank buffer).

ii) A 3% solution of NuSieve 3/1 agarose\(^7\) or an equivalent is prepared in TBE buffer. The solution is boiled until the agarose is completely dissolved, and then allowed to cool to 40°C. Whereas ethidium

\(^7\) NuSieve 3/1 agarose: FMC Bioproducts, Rockland, Maine, USA.
blomide was used previously to stain the gel, as it is a mutagen and is toxic, its use has been replaced by SYBR® Safe DNA gel stain8 according to the manufacturer’s instructions.

iii) The ends of the electrophoresis tray are taped and the agarose solution is poured. The comb is inserted and the agarose is allowed to solidify on a level surface for 30–60 minutes. The comb and the tape are gently removed from the electrophoresis tray.

iv) Pour the tank buffer into the electrophoresis apparatus and insert the tray with the agarose so that the buffer covers the agarose.

v) Test and positive and negative control samples are prepared for electrophoresis in 0.65 ml microcentrifuge tubes as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel-loading solution9</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>Amplified DNA from each of the RT-PCR tubes and an extra tube is set up for a DNA ladder</td>
<td>15.0 µl</td>
</tr>
<tr>
<td>Gel-loading solution9</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>100 base-pair ladder10</td>
<td>1.0 µl</td>
</tr>
</tbody>
</table>

vi) Samples are loaded into the appropriate wells in the gel and run at 65–95 volts for 30–90 minutes or until the dye has travelled about half the length of the gel. The gel is transferred to a transilluminator and photographed for a permanent record. Use protective eye-wear to visualise the gel bands.

vii) BT-positive samples will have a band of 101 base pairs. For the test to be valid, the positive control must show a band of the correct size, and the negative and ‘no RNA’ controls show no band. Samples are considered to be positive if there is a band of the same size as the positive control. Duplicate samples should show the same reaction. If there is disparity, the test should be repeated.

viii) A destaining bag11 is placed in the tank buffer overnight to remove the ethidium bromide. The buffer can then be poured down the drain and the destaining bag, after reuse 10–15 times, should be placed in a properly identified ethidium bromide waste container and ultimately incinerated.

d) Real-time reverse-transcription polymerase chain reaction tests

Real-time RT-PCR is a sensitive method that can be used for the laboratory detection of viral RNA. During the one-step real-time RT-PCR reaction, viral RNA is reverse transcribed into cDNA and subsequently amplified. A fluorogenic signal from dual-labelled probe is released during amplification and recorded in real time. The primers and probe combination for each real-time RT-PCR reaction are designed to amplify and detect a targeted region of the viral genome segment, in this case of BTV.

There are two published real-time assays that have been shown to detect all 24 serotypes (Shaw et al., 2007; Toussaint et al., 2007). Of the two, the assay of Shaw et al. (2007) has been tested against more serotypes and topotypes to date. So far the real-time RT-PCR assays have not been validated to the level required by the OIE validation template, although ring trials have been conducted (Batten et al., 2008). A further development is of triplex and duplex assay kits that detect a combination of group, BTV-8, BTV-1, BTV-6 and internal control. Serotype-specific assays against all the other serotypes are in the process of development.

It may be expected that after the validation processes have been completed, the real-time RT-PCR assays will become the tests of choice for diagnosis and in support of international trade.

2. Serological tests

Anti-BTV and anti-EHDV antibodies generated in infected animals can be detected in a variety of ways that vary in sensitivity and specificity. Both serogroup-specific and serotype-specific antibodies are elicited and if the animal was not previously exposed to BTV, the neutralising antibodies generated are specific for the serotype of the infecting virus. Multiple infections with different BTV serotypes lead to the production of antibodies capable of neutralising serotypes to which the animal has not been exposed.

a) Complement fixation

A complement fixation test to detect BTV antibodies was widely used until 1982, when it was largely replaced by the AGID test although the CF test is still used in some countries.

8 SYBR® Safe DNA gel stain: Available from Invitrogen (Cat No. S33102).
9 Gel-loading solution: Cat. G-2526, Sigma, St Louis, Missouri, USA.
10 100 base-pair ladder: Available from Life Technologies, Grand Island, New York, USA (Cat. 15268-019).
11 Destaining bag: Available from Ameresco, Solon, Ohio, USA.
b) Agar gel immunodiffusion (an alternative test for international trade)

The AGID test to detect anti-BTV and anti-EHDV antibodies is simple to perform and the antigen used in the assay relatively easy to generate. Since 1982, the test has been one of the standard testing procedures for international movement of ruminants. However, one of the disadvantages of the AGID used for BT or EHD is its lack of specificity in that it cannot differentiate between the BT and EHD serogroup. However, one of the disadvantages of the AGID used for BT or EHD is its lack of specificity in that it cannot differentiate between the BT and EHD serogroup. Thus AGID positive sera may have to be retested using a BT or EHD serogroup-specific assay. The lack of specificity and the subjectivity exercised in reading the results have encouraged the development of ELISA-based procedures for the specific detection of anti-BTV antibodies. The preferred format, a C-ELISA is described in the Section B.2.c.

- Test procedure
  
  i) A 2.8 mm thick layer of 0.9% agarose in 0.85% NaCl is prepared and circular wells, 4.0 mm in diameter and 2.4 mm apart, are cut out with six wells arranged around a central well.
  
  ii) Viral antigen is prepared by generating a crude soluble preparation from BHK or Vero cells infected with a single BTV serotype 24–48 hours previously. Antigen can be concentrated by precipitation or ultrafiltration.
  
  iii) A reference positive serum and three test sera are placed in alternate wells in a six-well pattern surrounding antigen in a central well and the plates are incubated at 20–25°C in a humid environment for 24 hours.
  
  iv) A series of precipitin lines form between the antigen and known positive sera and lines generated by strong positive test sera will join up with those of the positive controls. With weak positive samples the control lines bend toward the antigen and away from the test sample well, but may not form a continuous line between the control test wells. With negative samples, the precipitin lines will continue into the sample wells without bending toward the antigen.
  
  v) All weak positive samples and other samples that produce questionable results should be repeated using wells that are 5.3 mm in diameter placed 2.4 mm apart or retested using the C-ELISA as described below.

c) Competitive enzyme-linked immunosorbent assay (a prescribed test for international trade)

The BT competitive or blocking ELISA was developed to measure BTV-specific antibody without detecting cross-reacting antibody to other Orbiviruses (Afshar et al., 1989; Billinis et al., 2001; Lunt et al., 1988; Naresh & Prasad, 1995; Reddington et al., 1991). The specificity is the result of using one of a number of BT serogroup-reactive MAbs, such as MAb 3-17-A3 (Anderson, 1984) or MAb 20E9 (Lunt et al., 1988) or MAb 6C5F4D7 (Martyn et al., 1990). The antibodies were derived in a number of laboratories, and although different, all appear to bind to the amino-terminal region of the major core protein VP7. In the C-ELISA, antibodies in test sera compete with the MAbs for binding to antigen. The following procedure for the C-ELISA has been standardised after comparative studies in a number of international laboratories.

- Test procedure
  
  There are several test procedures described; this is an example of one BT ELISA procedure.
  
  i) First, 96-well microtitre plates are coated at 4°C overnight or at 37°C for 1 hour with 50–100 µl of either tissue culture-derived sonicated cell culture antigen (Anderson, 1984) or the major core antigen VP7 expressed in either Baculovirus (Oldfield et al., 1990) or yeast (Martyn et al., 1990) and diluted in 0.05 M carbonate buffer, pH 9.6.
  
  ii) The plates are washed five times with PBST (0.01 M PBS containing 0.05% or 0.1% Tween 20, pH 7.2).
  
  iii) Next, 50 µl of test sera is added in duplicate at a single dilution, either 1/5 (Afshar et al., 1989) or 1/10 (Lunt et al., 1988) in PBST containing 3% bovine serum albumin (BSA).
  
  iv) Immediately, 50 µl of a predetermined dilution of MAb diluted in PBST containing 3% BSA is added to each well. MAb control wells contain diluent buffer in place of test serum.
  
  v) Plates are incubated for 1 hour at 37°C or 3 hours at 25°C, with continuous shaking.
  
  vi) After washing as described above, wells are filled with 100 µl of an appropriate dilution of horseradish peroxidase-labelled rabbit anti-mouse IgG (H+L) in PBST containing 2% normal bovine serum.
  
  vii) Following incubation for 1 hour at 37°C, the conjugate solution is discarded and plates are washed five times using PBS or PBST. Wells are filled with 100 µl substrate solution containing 1.0 mM ABTS (2,2’-azino-bis-[3-ethylbenzothiazoline-6-sulfonic acid]), 4 mM H2O2 in 50 mM sodium citrate, pH 4.0, and
the plates are shaken at 25°C for 30 minutes. (Other substrates may be used and the reaction continued with shaking for an appropriate length of time to permit colour development.)

viii) The reaction is stopped by addition of a stopping reagent, such as sodium azide.

ix) After blanking the ELISA reader on wells containing substrate and stopping reagent, the absorbance values are measured at 414 nm. Results are expressed as per cent inhibition and are derived from the mean absorbance values for each sample by the following formula:

\[
\% \text{ inhibition} = 100 - \left( \frac{\text{Mean absorbance test sample}}{\text{Mean absorbance MAb control}} \times 100 \right).
\]

NB: Some laboratories prefer to use a negative control serum that has previously been shown to have a percentage inhibition of zero as an alternative to the MAb control.

x) Percentage inhibition values >50% are considered to be positive. Inhibition between 40% and 50% is considered to be suspicious. The results of the test sera duplicates can vary as long as the results do not lie either side of the positive cut-off.

xi) Strong and weak positive sera and a negative serum should be included on each plate. The weak positive should give 60–80% inhibition and the negative should give less than 40% inhibition.

A number of commercially produced cELISAs based on recombinant VP-7 and anti-VP-7 MAb are now available. These commercial assays are routinely used in many laboratories across the world and have been proved to be fit for purpose in ring-trials (Batten et al., 2008). Formal acceptance for trade purposes should depend on adoption of individual kits to the OIE Register.

d) Indirect ELISA

An indirect ELISA for bulk milk samples has been shown to be reliable and useful for surveillance purposes (Kramps et al., 2008). It should be validated for relevant serotypes before use.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Both live attenuated and killed BTV vaccines are currently available for use. Vaccines to protect against EHDV infection are not yet readily available. Recombinant BT vaccines based on various approaches are under development but none has been licensed and these vaccines will not be addressed here. In South Africa live attenuated vaccines have been used for over 40 years and are known to induce an effective and lasting immunity (Erasmus, 1975). Live attenuated vaccines are produced by adapting BTV field isolates to growth in vitro through serial passages in tissue culture or in ECEs. Stimulation of a strong antibody response by these vaccines is directly correlated to their ability to replicate in the vaccinated host. Live attenuated vaccines are cheap to produce in large quantities; they generate protective immunity after a single inoculation and have proven effective in preventing clinical BT disease in the areas where they are used (Dungu et al., 2004). However, live attenuated BTV vaccines suffer from a variety of documented or potential drawbacks, including under-attenuation, the impact of which may vary with different breeds of sheep. Potential adverse consequences are depressed milk production in lactating ruminants, and abortion/embryonic death and teratogenesis in offspring when used on pregnant females in the first period of pregnancy. Another risk associated with the use of live attenuated vaccines is that of their potential for spread by vectors, with eventual reversion to virulence and/or reassortment of modified live virus (MLV) genes with those of wild-type virus strains. The frequency and significance of these events remain poorly defined but transmission of vaccine strains by vector midges has already been documented in the USA, South Africa and Europe (Ferrari et al., 2005; MacLachlan et al., 1985; Monaco et al., 2006; Venter et al., 2004). Therefore inactivated vaccines, if effective, are preferred. Virus inactivation eliminates risks of vector transmission, reversion to virulence, fetal abnormalities and the possibility of viral reassortment.

1. Seed management

a) Characteristics of seed

For live, attenuated vaccines the master or primary virus seed is prepared from a single plaque of serially passaged, attenuated BTV. Vaccine viruses have been attenuated by either passage in ECE, tissue culture cells or a combination of both. Each primary seed virus lot should also be tested for transmissibility and reversion to virulence prior to vaccine manufacture. Samples of vaccine prepared from secondary seed virus at the maximum permitted passage level should be tested in sheep for avirulence, safety and immunogenicity.

For killed vaccines the issues of attenuation do not apply, and the approach adopted has been to use field strains of low passage level with the intent of achieving high antigenicity.
Primary seed virus must be free of contaminating bacteria, viruses, prions, fungi and mycoplasmas, particularly pestivirus contamination. For the latter, particular attention should be paid to the fetal bovine serum used in cell cultures, as it may be contaminated. Seed viruses must be shown to have the desired serotype specificity.

BTV seed lot viruses should be sequenced and the data made available to relevant databases (Osburn, 2004).

Secondary seed lots, which are used as inocula for vaccine production, are usually not more than three passages beyond the primary seed lot.

b) Method of culture
Although the first attenuated BT vaccines were propagated in ECE subsequently cell cultures have been used for tissue culture adaptation and serial passage. These include primary bovine embryo, lamb and fetal lamb kidney cells, and the continuous BHK cells. Cell cultures must be thoroughly checked for the presence of contaminating viruses.

BTV for inactivated vaccines is produced in large-scale suspension cell systems that have been shown to be susceptible to the virus.

c) Validation as a vaccine
Attenuated BT vaccines must be safe and efficacious, and a brief description of appropriate tests for these parameters is given below. In addition, attenuated viruses should not revert to virulence during replication in vaccinated animals or be able to be transmitted from such animals by insect vectors. The latter criterion is very important because insect-mediated transmission of attenuated virus from vaccinated to non-immune animals, with the subsequent replicative steps in each host species, increases the possibility of reversion to virulence. Although tests for reversion to virulence and transmissibility are rarely, if ever performed, a brief description of what may be necessary is outlined.

There is a variation in BT susceptibility between breeds of sheep; it is important that sheep that have been proven to be susceptible to infection with BTV be used for vaccine validation.

i) Safety
All vaccines must be safety tested. Safety tests for attenuated vaccines do not address the issue of their teratogenicity. Attenuated virus vaccines are teratogenic and should not be administered to pregnant sheep during the first half of pregnancy as this may cause fetal abnormalities and embryo death (Flanagan & Johnson, 1995; Lunt et al., 2006).

Demonstration of avirulence is necessary for live, attenuated vaccines. A number of sheep, seronegative by an appropriate, sensitive serological test (that will reliably detect antibodies even in vaccinated animals), are inoculated with the primary seed stock. Temperatures are noted twice daily. The animals are monitored at regular intervals over a period of 28 days for clinical signs and any local or systemic reactions to ensure avirulence and innocuity. Blood samples removed at regular intervals can be used to measure level of viraemia and antibody responses. The test shall be valid if all of the vaccinated sheep show evidence of virus replication and do not display signs of disease other than mild transient illness. In South Africa, a clinical reaction index is calculated for each animal between days 4 and 14 and must be below a specific standard value.

ii) Efficacy

Vaccinated and unvaccinated sheep should be challenged with virulent homologous serotype. It is recommended that the challenge model use virus passaged only in ruminant animals and with no ECE or cell culture passages. Passage in such an isolation system results in viral cultures that might induce clinical BT disease that is milder than the natural disease (Flanagan & Johnson, 1995). Animals are monitored for clinical signs of BT, rectal temperatures are taken twice daily and blood samples removed at regular intervals to measure viraemia and antibody responses. Unvaccinated control sheep should show clinical signs of BT and viraemia. However, it is difficult to be certain of the appearance of clinical disease following inoculation of sheep with certain BTV serotypes and isolates, and consequently, evidence of infection of unvaccinated control sheep may rest on the appearance of a temperature rise of at least 1.7°C over the pre-challenge mean and a viraemia. As a further evidence of infection pre- and post-vaccination sera are checked for the presence of neutralising antibody.

iii) Transmissibility

Transmissibility is an issue with live attenuated vaccines but not with killed vaccines. Procedures to determine if attenuated virus can be transmitted by insects that feed on vaccinated, viraemic sheep are difficult to perform and analyse statistically, and consequently, this criterion of vaccine validation is rarely sought. Laboratory data indicate that laboratory-adapted viruses can be transmitted by insect
vectors (Ferrari et al., 2005; Monaco et al., 2006; Standfast et al., 1985). A suitable procedure to determine attenuated virus transmissibility requires that sheep be vaccinated and, during viraemia, that they be exposed to competent, uninfected Culicoides, which are then permitted to feed on uninfected animals that are monitored for the presence of BTV and anti-BTV antibody. As the titre of attenuated virus in the blood of vaccinated sheep is usually low, very large numbers of Culicoides may be needed and only a small proportion of these would become infected and live long enough to feed on and potentially transmit the virus to other uninfected sheep. It is difficult to design a laboratory experiment that takes account of the large numbers of vaccinated sheep and insects that would be present in field situations. Although virus titres in blood less than $10^3\text{TCID}_5/\text{ml}$ have traditionally been considered a “safe” threshold, authentic instances of insects acquiring BTV from animals with viraemic titres much less than $10^3\text{TCID}_5/\text{ml}$ have been reported (Bonneau et al., 2002). Given the complex interaction of BTV, Culicoides vectors and animal hosts in the life cycle of infection, virus titres induced by live attenuated vaccine should be kept to an absolute minimum especially if field transmission of vaccine strains is a concern.

Current data indicate that during viraemia and in contrast to wild-type virus, laboratory-adapted strains of BTV may be found in the semen of bulls and rams (Kirkland et al., 2004; Roberts et al., 1992). The implications of these observations for virus transmissibility are unclear. A recent study of semen from rams vaccinated with BTV2 live attenuated vaccine showed that even if BTV was not detected in the semen, the vaccine caused a decrease in the quality of the semen (Bréard et al., 2007).

iv) Reversion to virulence

Validation studies confirm that attenuated viruses do not revert to virulence in vaccinated sheep. However, if attenuated viruses can be transmitted from vaccinated animals, reversion to virulence during a number of sheep-insect replication cycles becomes a distinct prospect. The only appropriate way to monitor for reversion to virulence under these circumstances is to compare the virulence of the vaccine virus with that which had been subject to several sheep–insect replication cycles as described above. As indicated, this is difficult to achieve. Consequently, the effect of a number of sheep–insect passages on the virulence of attenuated viruses has not been determined. In South Africa, it is accepted that if blood from vaccinated animals during the viraemic stages is serially passaged three times in sheep without reversion to virulence, the chances of reversion in the field will be infinitely small. In Europe, five passages are required.

2. Method of manufacture

Attenuation of field isolates of BTV was first achieved by serial passage in ECE. Because of the concern about transmission of the egg propagated attenuated virus, it has been recommended that animals receiving vaccines produced in ECE should not be moved internationally (Osburn, 2004). More recently, it is clear that passage in cultured cells will also result in attenuation of virulence. No studies have been done to precisely relate passage number and extent of attenuation for individual virus isolates or serotypes. To prepare attenuated virus, field isolates are adapted to cell culture and passaged in vitro up to 40 times or more. Ideally, a number of plaque-purified viruses are picked at this stage and each is examined to determine the level of viraemia they generate and their ability to elicit a protective immune response in vaccinated sheep. The most suitable virus is one that replicates to low titre but generates a protective immune response, and this may represent the source of vaccine primary seed stock virus.

BTV for killed vaccines is produced in large-scale suspension cell systems under aseptic and controlled conditions. Cell lines adapted for large scale industrial cultures are used and these are proven to be free from contaminating microorganisms. When the viral suspension virus reaches its maximum titre, cell disruption is performed and the culture is clarified and filtered. Subsequently inactivation is performed according to processes adopted by the manufacturer, such as by addition of binary ethyleneimine (BEI) or other inactivants. The process must comply with legislation relevant for the intended market, be validated to ensure complete inactivation and be supported by the appropriate documentation. The inactivation process should not significantly alter the immunogenic properties of the viral antigens. Purification is carried out by chromatography. The inactivated virus is then concentrated by ultrafiltration and stored. The inactivated, chromatography-purified and concentrated BTV antigens are made into vaccine by dilution in a buffer solution and addition of adjuvants.

3. In-process control

All ingredients of animal origin, including serum and cells must be checked for the presence of viable bacteria, viruses, fungi or mycoplasmas.

Virus concentration of attenuated vaccines is assessed by infectivity and ELISAs.

For inactivated vaccines, during inactivation of the virus, timed samples are taken at regular intervals for the purpose of monitoring the rate and linearity of the inactivation process. Virus titres in the samples are determined by inoculation of BHK-21 or other appropriate cell cultures. At the end of the inactivation process, the vaccine is checked to ensure that there is no live virus.
4. Batch control

a) Sterility

Every batch of vaccine should be tested for the presence of contaminant viruses of viable bacterial, fungal or mycoplasmal contamination. For example, in South Africa a pool of ten randomly selected ampoules are inoculated into soya broth and thioglycollate broth, and incubated at room temperature and 37°C, respectively, for 14 days. If contaminated, the batch is disqualified.

b) Safety

Every batch of attenuated vaccine is safety tested in newborn and adult mice, guinea-pigs and sheep. If any adverse reactions or significant signs are noted, the test is repeated. Any increase in the body temperature of the target animal that is above the level expected for the particular strain of attenuated virus under test should be regarded as symptomatic. If the results are unsatisfactory after a second attempt, the batch is disqualified.

Safety testing of inactivated vaccines is conducted in sheep to ensure side effects are not observed.

c) Potency

Each batch is tested by inoculation of susceptible sheep. Pre-vaccination and 21- and 28-day post-vaccination sera are tested by VN assay to determine neutralising antibody levels. To be passed, the antibody titre must be equal to or higher than a set standard based on international vaccine standards.

d) Duration of immunity

Studies with live attenuated BTV vaccine have shown that antibodies in sheep may appear before day 10 post-vaccination, reach a maximum approximately 4 weeks later and persist for well over a year. There is a temporal relationship between the increase in neutralising antibody titre and clearance of virus from the peripheral circulation. Live attenuated BTV vaccines have been in use for over 40 years and are known to induce an effective and lasting immunity in sheep (Verwoerd & Erasmus, 2004). Many serotypes of BTV may be present in endemic areas of South Africa, and polyvalent vaccines are used. The inclusion of 15 serotypes in three polyvalent vaccines that are administered sequentially sometimes means that an effective immune response is not generated to all serotypes, presumably because of the antigenic mass of individual serotype-specific antigens is small. In an attempt to broaden the response, vaccination is repeated annually (Erasmus, 1975).

Initial studies with inactivated vaccines show that antibody against BTV can be detected by day 7 post-vaccination and increase in titre to days 14–21. A second dose of vaccine boosts the titre. Data to demonstrate the expected duration of immunity are being acquired.

e) Stability

Procedures have been developed for attenuated vaccines. Stability should be tested over a period of 2 years. Vaccines in liquid and lyophilised forms are deemed to have shelf lives of 1 and 2 years, respectively. Each batch of vaccine is subjected to an accelerated shelf-life test by storing it at 37°C for 7 days. It is then titrated and evaluated according to a set standard, as determined in the initial testing of the vaccine.

Inactivated vaccines have been used to the present time in emergency situations where shelf life has not been an issue. Requirements and procedures for routine commercial use have not been developed.

f) Precautions (hazards)

Attenuated vaccines should be used in the cooler months when adult Culicoides vector populations are at a minimum. They should not be used in ewes during the first half of pregnancy and in rams 2 months before the breeding season.

5. Tests on the final product

a) Safety

See C.4.b.

b) Potency

See C.4.c.
REFERENCES


* * *

**NB:** There are OIE Reference Laboratories for Bluetongue
(see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)).

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for bluetongue.
CHAPTER 2.1.4.

ECHINOCOCCOSIS/HYDATIDOSIS

SUMMARY

Diagnosis of echinococcosis in dogs or other susceptible carnivores relies on the demonstration of adult cestodes of the Echinococcus genus or their eggs in their faeces or small intestine. Recently, coproantigen and copro-DNA assays have proven useful for safe, fast and accurate diagnosis. In intermediate hosts, diagnosis depends on detection of the larval cyst form that can infect almost any organ, particularly the liver and lungs.

Identification of the agent: At present, five species of the genus Echinococcus are regarded as taxonomically valid. These are E. granulosus, E. multilocularis, E. oligarthrus, E. vogeli and E. shiquicus. Echinococcus oligarthrus and E. vogeli occur less frequently than the first two species. Until recently E. shiquicus had been discovered only in a specific region of the People’s Republic of China. These five species are morphologically distinct in both adult and larval stages. A number of intraspecific variants have been described for E. granulosus, which exhibit morphological and biological characteristics, and these can reliably be differentiated by DNA analysis. Some of the E. granulosa genotypes have been recommended for elevation to species status.

Larval forms of Echinococcus can usually be visually detected in organs. Special care has to be taken for a specific diagnosis of E. granulosus in instances where Taenia hydatigena in sheep is also a problem. Histological examination may confirm the diagnosis after formalin-fixed material is processed by conventional staining methods. The presence of a periodic-acid-Schiff positive, acellular laminated layer with or without an internal cellular, nucleated germinal membrane can be regarded as a specific characteristic of metacestodes of Echinococcus. The identification of larval E. multilocularis in rodents and other hosts is possible by macroscopic or microscopic examination and by DNA detection using the polymerase chain reaction (PCR).

The small intestine is required at necropsy for the detection of adult Echinococcus spp. in wild or in domestic carnivores. The technique of carrying out surveys with the use of arecoline has been generally adopted for determining the prevalence of E. granulosus in dogs. Handling infected material presents a risk to the operator of contracting a potentially fatal disease. Significant progress is being made in the development of immunological tests for the diagnosis of intestinal Echinococcus infections by use of coproantigen detection. The technique has been used successfully in surveys of E. granulosus in dogs and is currently used in surveys for E. multilocularis in populations of dogs, foxes, and cats. Coproantigen detection is possible in faecal samples collected from dead or living animals or from the environment.

PCR/DNA methods for the detection of E. multilocularis and more recently E. granulosus in definitive hosts have now been established in specialised laboratories as diagnostic techniques.

Serological tests: Antibodies directed against oncosphere, cyst fluid and protoscolex antigens can be detected in the serum of infected dogs and sheep, but this approach is presently of limited practical use as it does not distinguish between current and previous infections. Cross-reactivity between Echinococcus and Taenia species also may occur.

Requirements for vaccines and diagnostic biologicals: Progress has been made in the development of an effective vaccine against infection with the larval stage of E. granulosus in sheep and cattle.
A. INTRODUCTION

Species under genus *Echinococcus* are small tapeworms of carnivores with larval (metacestode) stages known as hydatids proliferating asexually in various mammals including humans. There were four morphologically distinct species in this genus until recently when *Echinococcus shiquicus* was added to the previously known species: *E. granulosus*, *E. multilocularis*, *E. oligarthrus*, and *E. vogeli*. Discovered in the Shiqu County, the Qinghai-tibet plateau region of western Sichuan, the People’s Republic of China (Xiao *et al.*, 2005; 2006), *E. shiquicus* is morphologically distinct both in adult and larval stages from other species.

A number of interspecific and intraspecific variants have been described for *E. granulosus*. Some genotypes of *E. granulosus* exhibit characteristic features that would justify the recognition as separate species according to some authors. Recently other species and genotypes of *Echinococcus* have been proposed (Thompson & McManus, 2002). Further studies are needed to define the full range of genetic diversity (Lymbery, 1995; McManus & Bryant, 1995; Rausch, 1995; Thompson, 1995). *Echinococcus granulosus* has a global distribution; *E. multilocularis* occurs in wide areas of the Northern Hemisphere, *E. shiquicus* is found in the People’s Republic of China and *E. oligarthrus* and *E. vogeli* are confined to Central and South America. All five species are infective to humans causing various forms of echinococcosis. Human cystic echinococcosis, caused by *E. granulosus* and alveolar echinococcosis, caused by *E. multilocularis*, are important public health threats in many parts of the world (WHO/OIE, 2001).

*Table 1.* Useful characteristics for identification of *Echinococcus* species. Source: Xiao *et al.* (2006)

<table>
<thead>
<tr>
<th></th>
<th><em>E. granulosus</em></th>
<th><em>E. multilocularis</em></th>
<th><em>E. oligarthrus</em></th>
<th><em>E. vogeli</em></th>
<th><em>E. shiquicus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Distribution</td>
<td>Cosmopolitan</td>
<td>Holoarctic region</td>
<td>Neotropical region</td>
<td>Neotropical region</td>
<td>Tibet plateau</td>
</tr>
<tr>
<td>Definitive Host</td>
<td>Dogs</td>
<td>Foxes</td>
<td>Wild felids</td>
<td>Bush dog</td>
<td>Tibetan fox</td>
</tr>
<tr>
<td>Intermediate Host</td>
<td>Ungulates</td>
<td>Microtine rodents</td>
<td>Neotropical rodents</td>
<td>Neotropical rodents</td>
<td>Plateau pika</td>
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<tr>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body length (mm)</td>
<td>2.0–11.0</td>
<td>1.2–4.5</td>
<td>2.2–2.9</td>
<td>3.9–5.5</td>
<td>1.3–1.7</td>
</tr>
<tr>
<td>No. segments</td>
<td>2–7</td>
<td>2–6</td>
<td>3</td>
<td>3</td>
<td>2–3</td>
</tr>
<tr>
<td>Length of large hooks (µm)</td>
<td>25.0–49.0</td>
<td>24.9–34.0</td>
<td>43.0–60.0</td>
<td>49.0–57.0</td>
<td>20.0–23.0</td>
</tr>
<tr>
<td>Length of small hooks (µm)</td>
<td>17.0–31.0</td>
<td>20.4–31.0</td>
<td>28.0–45.0</td>
<td>30.0–47.0</td>
<td>16.0–17.0</td>
</tr>
<tr>
<td>Position of genital pore</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Mature segment</td>
<td>Near to middle</td>
<td>Anterior to middle</td>
<td>Anterior to middle</td>
<td>Posterior to middle</td>
<td>Near to upper edge</td>
</tr>
<tr>
<td>b. Gravid segment</td>
<td>Posterior to middle</td>
<td>Anterior to middle</td>
<td>Near to middle</td>
<td>Posterior to middle</td>
<td>Anterior to middle</td>
</tr>
<tr>
<td>Gravid uterus</td>
<td>Branching laterally</td>
<td>Sac-like</td>
<td>Sac-like</td>
<td>Tubular</td>
<td>Sac-like</td>
</tr>
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<td>Metacestode</td>
<td>Unilocular cysts in viscera</td>
<td>Multilocular cysts in viscera</td>
<td>Polycystic cysts in muscles</td>
<td>Polycystic cysts in viscera</td>
<td>Unilocular cysts in viscera</td>
</tr>
</tbody>
</table>

- *Echinococcus granulosus*

The parasite is transmitted between the domestic dog and a number of domestic ungulate species. The dog/sheep cycle is most important. Sylvatic definitive and intermediate hosts also occur, e.g. wolf/cervid. The adult varies between 2 and 11 mm in length and usually possesses from two to seven segments, averaging from three to four segments. The penultimate segment is mature, and the genital pore normally opens posterior to the
middle in both mature and gravid segments. The last (gravid) segment is usually more than half the length of the entire worm. There are rostellar hooks of various sizes on the protoscolex in two rows. The size of the hooks varies between 25 to 49 µm in the first row, and between 17 and 31 µm in the second row. The gravid uterus has well-developed sacculations.

The larval stage is a fluid-filled bladder or hydatid cyst that is unilocular, although communicating chambers also occur. Growth is expansive, and endogenous daughter cysts may be produced. Individual bladders may reach up to 30 cm in diameter and occur most frequently in liver and lungs, but may develop in other internal organs. The infection with this stage is referred to as cystic echinococcosis.

The strain specificities of *E. granulosus* in domestic cycles include, dog/sheep in the Mediterranean region, South America (Argentina, Brazil, Chile, Peru and Uruguay), Africa (Ethiopia, Kenya and Sudan), the Middle East and Levant regions, Russia, Central Asia (Kazakhstan, Kyrgyzstan and Uzbekistan), Mongolia, the People’s Republic of China, Oceania and the United Kingdom; dog/horse in Belgium, Ireland and the United Kingdom; dog/cattle in Belgium, Germany, South Africa and Switzerland; dog/swine in Poland; and dog or wolf/reindeer in sub-Arctic regions of Norway, Finland and Alaska. The status of dog/camel strains requires further elucidation. This strain has recently been identified in human cases in Argentina, Nepal, the People's Republic of China and Iran (Bart et al., 2006; Fasihi et al., 2002; Rosenzvit et al., 1999; Zhang et al., 2000). To date, all genotypes of *E. granulosus* except the dog/horse (G4) and the Finnish cervid (G10) strains have been found to infect humans.

• **Echinococcus multilocularis**

The parasite is transmitted primarily between wild definitive hosts (e.g. *Vulpes vulpes*, *V. ferrilata*, *V. corsac*, *Alopex lagopus*, *Canis latrans*) and small arvicolid rodents (voles and lemmings). The adult varies between 1.2 and 4.5 mm in length and usually possesses from two to six segments, with an average of four to five. The penultimate segment is characteristically mature, and the genital pore is anterior to the midline in both mature and gravid segments. The gravid uterus is sac-like. On the rostellum, the larger hooks of the first row vary in size between 24.9 and 34.0 µm and the smaller hooks of the inner row between 20.4 and 31.0 µm.

The metacestode is a multivesicular structure consisting of conglomerates of small vesicles, usually not exceeding a few millimetres in diameter. Unlike *E. granulosus*, the larval mass often contains a semisolid rather than a fluid matrix. It proliferates by exogenous budding, which results in infiltration of tissues. Infection with this stage is commonly referred to as alveolar echinococcosis. There is no clear evidence for distinct strains or genotypes of *E. multilocularis*, though regional variations at the continental scale have been described (WHO/OIE, 2001).

This zoonotic parasite is found in the Northern Hemisphere, and its life cycle is mainly maintained in wildlife (Kamiya et al., 2007). The sylvatic cycle involves foxes and many species of wild rodents. Coyotes, raccoon dogs, wolves, wild cats, domestic dogs and cats (Hildreth et al., 2000; Kapel et al., 2006), however, may serve as definitive hosts while pigs, horses, primates and humans can be infected as intermediate hosts (Kamiya et al., 2007).

• **Echinococcus oligarthrus**

The parasite typically uses neotropical wild felids as definitive hosts (e.g. *Felis concolor*, *F. jaguarundi*) and large rodents (e.g. *Dasyprocta* sp., *Cuniculus paca*) as intermediate hosts. The adult varies between 2.2 and 2.9 mm in length, and normally possesses three segments, the penultimate of which is mature. The genital pore is anterior to the middle in mature segments and approximately at the middle in gravid segments. The gravid uterus is sac-like.

The metacestode is polycystic and fluid-filled with a tendency to become septate and multichambered. The rostellar hooks of the protoscolex vary in length between 25.9 and 37.9 µm. The hooks are described in more detail in the next section and compared with those of *E. vogeli*. The single cyst may reach a diameter of approximately 5 cm. Predilection sites are internal organs and muscles. To date, there have only been a few reports of human disease. The parasite appears not to mature in dogs.

• **Echinococcus vogeli**

The parasite typically uses the South American bush dog (*Speothus venaticus*) as a wild definitive host, but the domestic dog is susceptible, as are large rodents (e.g. *Cuniculus paca*) as intermediate hosts. The adult varies between 3.9 and 5.5 mm in length, and usually has three segments, the penultimate of which is mature. The genital pore is situated posterior to the middle in both the mature and gravid segments. The gravid uterus has no
lateral sacculations and is characterised by being relatively long and tubular in form, compared with the other segments, which are sac-like.

The metacestode is similar to that of *E. oligarthrus*. It has been reported that the two species can be distinguished by comparing differences in the dimensions and proportions of the rostellar hooks on the protoscolex. The hooks of *E. oligarthrus* vary in length between 25.9 and 37.9 µm (average 33.4 µm) and between 22.6 and 29.5 µm (average 25.45 µm) for large and small hooks, respectively. Those of *E. vogeli* vary between 19.1 and 43.9 µm (average 25.45 µm) and between 30.4 and 36.5 µm (average 33.6 µm) for the large and small hooks, respectively. Also the hook-guard for *E. oligarthrus* divides the hook 50:50, compared with 30:70 for *E. vogeli*.

*Echinococcus vogeli* is a zoonotic agent with approximately 100 human cases reported in South America. The infection caused by the larval stage of this species is commonly referred to as polycystic echinococcosis.

- **Echinococcus shiquicus**

The parasite was found in the Tibetan fox (*Vulpes ferrilata*) its definitive host and the plateau pika (*Ochotona curzoniae*), the intermediate host. In most species of *Echinococcus*, the gravid segment is connected to a mature segment; however, a strobila consisting of only two segments (a gravid segment directly attaching to a premature segment) is unique to this species (WHO/OIE, 2001). The adult stage is morphologically similar to *E. multilocularis* but differs by its smaller hooks, fewer segments, upper position of genital pore in the premature segment and fewer eggs in the gravid segment. It is easily distinguishable from *E. granulosus* by its shorter length, branchless gravid uterus and anterior position of genital pore in the gravid segment. The adult measures 1.3 to 1.7 mm.

The metacestode is found in the liver and is essentially a unicellular minicyst containing fully developed brood capsules; however, oligovesicular forms have also been observed. It is differentiated from *E. granulosus* having no daughter cysts appearing within the fertile cyst (WHO/OIE, 2001).

A detailed description of echinococcosis in humans and animals can be found in the WHO/OIE Manual on echinococcosis (WHO/OIE, 2001).

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

In the intermediate host, diagnosis depends on the detection of the larval cyst form, which can occur in almost any organ, but particularly in the liver and lungs. The diagnosis of echinococcosis in dogs or other carnivores requires the demonstration of the adult cestodes of *Echinococcus* spp. in their faeces or the small intestine or the detection of specific coproantigens or coproDNA.

Investigators carrying out these procedures are exposed to the risk of infection and severe disease, which must be minimised by appropriate procedures. Infective material can be decontaminated by freezing at –80°C (core temperature) for 48 hours, or –70°C for 4 days or by heating to 70°C for 12 hours (Nonaka *et al.*, 2008; Sakai *et al.*, 1998). Face masks, disposable gloves and an apron must be worn. Chemical disinfection is not reliable, although sodium hypochlorite may destroy a proportion of eggs (Craig, 1997). Contaminated material must be destroyed by heat; hot water, at temperature of 85°C or above, is very effective. The decontamination of laboratories can be achieved at reduced humidity (40%) combined with increased room temperature (30°C) for at least 48 hours.

**a) Diagnosis of Echinococcus eggs in environmental samples**

- **Faecal samples** (Ito, 1980; Thienpoint *et al.*, 1979)

This is a concentration method in which a saturated solution is used to separate *Echinococcus* eggs from faeces. A faecal sample of 0.5–2 g is mixed with water or 0.3% Tween 20 in 1% formalin (Nonaka *et al.*, 1998) in a 10–15-ml test tube and centrifuged (1000 g for 10 minutes) once or twice until the supernatant is clear. Sediment is mixed with either zinc sulphate 33% (1.18 sp. Gr.) or sucrose solution (1.27 sp. Gr.) and centrifuged at 1000 g for 5–10 minutes. The test tube is filled to the top and a cover-glass is placed on the tube. The cover-glass is examined microscopically 2–16 hours later.

- **Soil samples** (Matsuo & Kamiya, 2005)

A 20-g soil sample is placed in a 50-ml conical tube to which is added 40 ml of 0.05% Tween 80. The mixture is stirred vigorously and sieved through a 100-µm mesh. The suspension is centrifuged at 1000 g for
5 minutes and the supernatant is discarded. The remaining procedure follows the concentration method used for faecal sample examination.

b) Diagnosis of larval echinococcosis

• Necropsy

Whereas surveillance for *E. granulosus* in domestic animals may take place in licensed slaughter houses, that for *Echinococcus* sp. in wildlife must be done by field surveys. Specimens should be preserved by removal of tissue and fixation in 4% formal saline or kept cool at +4°C and deep-frozen at –20°C for subsequent examination. When undertaking surveillance work with *E. granulosus* in intermediate hosts, it is vitally important that data are stratified and reported according to the age of animals slaughtered. Prevalence rates are strongly age dependent (Torgerson & Heath, 2003) and reports from abattoirs that may slaughter only young animals will substantially under-represent the true situation. This is because older animals may be heavily infected even when animals have very few larvae.

Larvae can be observed in many organs, but in large animals, such as sheep and cattle, palpation or incision should be done. Pigs, cattle, sheep and goats may be infected with larval *Taenia hydatigena*, and it is sometimes difficult to differentiate between these two parasites when they occur in the liver. In wild animals, such as ruminants and rodents, several other larval cestodes should be considered for differential diagnosis.

Formalin-fixed material can be stained by conventional histological techniques. The presence of a periodic-acid-Schiff (PAS) positive acellular laminated layer, underlying a connective tissue layer, and with or without an internal cellular, nucleated germinal membrane can be regarded as a specific characteristic of the metacestodes of *Echinococcus* spp. The presence of protoscoleces within brood-capsules or in hydatid sand is also diagnostic for the genus. Genotyping of *E. granulosus* or *E. multilocularis* is usually done on DNA derived from protoscoleces or larval tissue material that is frozen, refrigerated or preserved in 90% ethanol.

c) Diagnosis of adult parasites in carnivores

• Necropsy

Necropsy is invariably employed in studies of echinococcosis in wildlife and is useful if domestic dogs are humanely culled. It should be emphasised that it is necessary to isolate and identify the adult *Echinococcus*, because under normal conditions of faecal examination, the eggs of *Echinococcus* cannot be differentiated from those of *Taenia* spp. The eggs of *E. granulosus* and *E. multilocularis* can now be identified and differentiated from other taeniid eggs by polymerase chain reaction (PCR).

The small intestine is removed as soon as possible after death, and tied at both ends. If the material is not frozen or formalin fixed (4–10%), it should be examined quickly, as the parasite can be digested within 24 hours. Formalin does not kill eggs. The fresh intestine is divided into several sections and immersed in 0.9% saline at 37°C for examination. Worms adhering to the intestinal wall may be observed and counted by means of a hand lens (for *E. granulosus* and *E. vogeli*). For accurate counts, the unfixed intestine is best divided into four or six sections, opened up and immersed in 0.9% saline at 37°C for 30 minutes to release the parasites. The contents are washed into another container for detailed examination, and the intestinal wall is scraped with a spatula. All material is boiled and washed by sieving to eliminate most of the particulate material and to make it noninfectious. The washed intestinal contents and scrapings are placed on a black tray, and the worms are counted with the aid of a hand lens or stereoscopic microscope. *Echinococcus granulosus* is usually found in the first third of the small intestine of dogs and *E. multilocularis* in the mid/posterior sections.

Necropsy is considered to be the most reliable form of diagnosis for *E. multilocularis* in definitive hosts. It is an inexpensive method for determining the prevalence in a population and the best way to determine worm burden (Duscher et al., 2005). Carcasses or intestines of definitive hosts for examination should be deep frozen at between –70°C and –80°C for 3–7 days before necropsy to kill any eggs. Eggs of *E. multilocularis* are resistant to freezing to –50°C.

Methods for quantitative determination of *E. multilocularis* in definitive host’s intestine.

• Sedimentation and counting technique (SCT; Eckert, 2003; Hofer et al., 2000)

This technique can be regarded as the ‘gold standard’ for assessing the sensitivity and specificity of other techniques.

i) The small intestine is incised longitudinally and cut into 20 cm long segments or into 5 pieces of approximately the same length. These pieces are transferred to a glass bottle containing 1 litre physiological saline (0.9% NaCl) solution.
ii) The glass bottle is shaken vigorously for a few seconds and the pieces of intestine are removed. The superficial mucosal layer is stripped by exerting pressure between thumb and forefinger to dislodge attached helminths.

iii) The glass bottle is left for 15 minutes for sedimentation to occur; the supernatant is then decanted. The glass bottle is refilled with physiological saline solution. This procedure is repeated 2–6 times until the supernatant is cleared of coloured particles.

iv) The sediment fraction is examined in small portions of about 5–10 ml in rectangular plastic or Petri dishes with a counting grid (9 x 9 cm) in transmission light under a stereomicroscope at a magnification of ×120.

v) If up to 100 worms are found, the entire sediment fraction is checked; if higher numbers are present, the total worm burden is calculated from the count of one subsample.

• **Intestinal scraping technique (IST; Deplazes & Eckert, 1996; Eckert et al., 2001)**

  i) Deep mucosal scrapings are taken at nearly equal distances from the small intestine using microscope slides (75 x 25 x 1 mm). Five mucosal scrapings from proximal, middle and posterior thirds of the small intestine (total 15) are recommended. Adherent materials are transferred to a square plastic Petri dish.

  ii) Scrapings are squashed between slides and examined under a stereoscopic light microscope (×120). Echinococcus multilocularis is usually found in the second half of the small intestine.

• **‘Shaking in a vessel’ technique (SVT; Duscher et al., 2005)**

  i) A plastic vessel (1 litre), which has a plastic screw-on lid with a central hole 6–7 cm in diameter is used. The hole is covered with a high-grade steel mesh (mesh size 500 µm) fixed into the remaining plastic ring with a hot soldering iron. Silicone is applied to seal the edges of the steel mesh.

  ii) The longitudinally opened small intestine is transferred to the vessel with all its contents; the vessel is closed with the lid and filled with water.

  iii) The vessel is inverted and shaken; the water is decanted. Vessel is refilled with water, and the process is repeated until the decanted water is clear.

  iv) The half-filled vessel is opened and the intestines are removed. The intestines are stripped between the thumb and forefinger to dislodge parasites stuck to the mucosa into the vessel.

  v) The vessel is closed again, refilled and shaken one last time draining as much water from it as possible.

  vi) The remaining sediment is filled into a 1 litre plastic jug and stored at 4°C. For prolonged storage, a 0.9% NaCl solution is added to the sediment to prevent the parasites from shrinking.

  vii) For analysis, the materials are placed into small glass Petri dishes and scanned along engraved lines using the stereomicroscope as above.

• **Preserving specimens**

Intact worms are fragile and for morphological studies are best handled in normal saline with a Pasteur pipette. They are washed free of other material and left for approximately 30 minutes for all movement to cease. After removal of the fluid, cold 5–10% formalin (5°C) or FAA fixative (95% ethanol [80 ml], 37–40% formaldehyde [10 ml], and glacial acetic acid [5 ml]) is added and the worms are left for a further 12 hours. For staining, the worms are washed in water for 15 minutes and transferred to Mayer’s paracarmine (carminic acid [1.0 g], aluminium chloride [0.5 g], calcium chloride [4.0 g], and 70% ethanol [100 ml]) for 12–24 hours. Excess stain is removed by immersion in 0.5–1.0% hydrochloric acid solution for a few seconds. Dehydration is accomplished by serial passage in ascending concentrations of alcohol (41, 50, 70, 85, 95, and 100%) for at least 15 minutes in each, with two changes in 100%. The alcohol is removed by xylol (10 minutes) and cleared with methyl salicylate or creosote. Prior to mounting in any suitable medium such as balsam, picolyte, etc., the specimens should be returned to the xylol for a few minutes. Persons involved in such examinations should receive serological screening for anti-Echinococcus serum-antibodies at least once a year (WHO/OIE, 2001).

Recently, some methods have been developed with the aim of simplifying and improving epidemiological investigations in final host populations and of allowing diagnosis in living animals. These methods include the detection of coproantigens and PCR DNA detection (see below).

d) **Arecoline surveys and surveillance**

Arecoline has been used to perform surveys of tapeworm infections in dog populations. Its use as a control agent has now been superseded by praziquantel. Arecoline is a parasympathomimetic agent. Its action results in sweating, and stimulation of salivary, lachrymal, gastric, pancreatic, and intestinal glands. It
increases intestinal tonus and the mobility of smooth muscle, and this effect is responsible for purgation. The liver is the principal site of detoxification. Arecoline also has a direct action on the worm itself, by causing paralysis, but not death, and thereby making it relax its hold on the intestinal wall. Thus, it must be administered by the oral route. The accompanying purgation carries the worms out with the faeces. It is particularly suitable for baseline surveys of *E. granulosus*, however, 15–25% of dogs may not purge. Arecoline may also be used to purge dogs infected with *E. multilocularis*. In animals, arecoline purgation has been useful; again, the recovered tapeworms are identified morphologically. Products containing arecoline are no longer available as an anthelmintic, but can be obtained from chemical supply companies. As it has side-effects, old, infirm and pregnant animals should be excluded from treatment. A dose of 4 mg/kg should result in purgation in under 30 minutes. Walking and abdominal massage of recalcitrant cases or enema for constipated dogs may avoid the use of a second dose (2 mg/kg), which should be given only sparingly.

Dogs that are purged successfully may produce at least two motions; the first will be formed faeces and can be ignored (or collected for laboratory tests as described later), but the mucus that follows may be productive. This can be divided into several samples and each examined separately, but this method is not recommended as the worms will be difficult to detect. Preferably, the mucus sample (about 4 ml) is diluted with 100 ml of tap water, covered with a thin layer of 1 ml of kerosene (paraffin) and boiled for 5 minutes. The kerosene prevents foaming and reduces the smell.

Investigators carrying out these procedures are exposed to risk of infection and severe disease. Personnel should wear whole body coveralls, boots, disposable gloves and a face mask. Coveralls should be boil washed after use, and boots disinfected in 10% sodium hypochlorite solution. The purge should be boiled as soon as possible after collection. Dogs may continue to pass eggs, proglottides and worms after the first purge, therefore, they should remain tethered for 2 hours after purgation and given access to drinking water. After arecoline testing, the area of ground used to tether dogs should be sprayed with kerosene and flamed.

e) Coproantigen tests

An alternative to arecoline testing, based on a faecal antigen-detection antibody sandwich enzyme-linked immunosorbtent assay (ELISA), has been developed and has shown particular promise as coproantigens can be detected shortly after infection (10–14 days) and the level declines rapidly following expulsion of the worms. The sensitivity and specificity of the test have been estimated at 70% and 98%, respectively (Allan et al., 1992; Buishi et al., 2005a; Craig, 1997; Deplazes & Eckert, 1996; Deplazes et al., 1992).

Both qualitative and quantitative results can be obtained from arecoline testing, which is most useful for base-line epidemiological studies on the comparative rates of infection with Taeniidae in dogs. Further studies may show that the coproantigen test may be more cost-effective than arecoline testing during routine surveillance of *E. granulosus* in the dog population (Buishi et al., 2005b).

ELISAs for specific coproantigen have now been developed that have sufficient specificity and sensitivity to replace arecoline testing for detecting *Echinococcus* in dogs and other definitive hosts (Craig, 1997). When testing for genus-specific *Echinococcus* coproantigens (against necropsy as a gold standard), specificity is around 98% and overall sensitivity approximately 70%; however, when mean worm burdens are >50–100, sensitivity approaches 100% (Allan et al., 1992; Craig, 1997; Craig et al., 1995; Deplazes et al., 1992). Dogs, dingoes, foxes and wolves have been screened successfully for coproantigen ELISAs and, importantly, *E. multilocularis* worm infestations are also detectable in red foxes and domestic dogs (Deplazes et al., 1992; Sakashita et al., 1995). When the capture ELISA uses either anti-ES or anti-somatic proglottid antibodies to *E. granulosus*, the sensitivity for *E. multilocularis* infection may be reduced, though genus specificity remains intact. Polyclonal- or monoclonal-antibody-based ELISAs for coproantigens exhibit high sensitivity and specificity to *E. granulosus* (~80%), even though they were developed for *E. multilocularis* (Deplazes et al., 1999; Rosenzvit et al., 1999). However, for low worm burdens (<50), the sensitivity of the *E. multilocularis* coproantigen ELISA is below that of the mucosal smear method at necropsy (Deplazes et al., 1999).

The exact nature of *Echinococcus* antigens released in faeces for coproantigen detection has not been fully characterised. However, their stability in 5% formal saline after boiling and susceptibility to periodate treatment suggest involvement of large (>150 KDa) of carbohydrate antigen(s) with α-D-mannose, α-D-glucose, β-galactose and N-acetyl-β-glucosamine residues (Craig, 1997; Elayoubi & Craig, 2004).

Coproantigens can be detected prior to release of eggs by *Echinococcus* worms, and therefore are not related to egg antigen(s) (Deplazes et al., 1992; Rosenzvit et al., 1999). This has the advantage of detection of prepatent infections. Furthermore, coproantigen levels return to the preinfection baseline within 5 days of anthelmintic treatment of infected dogs (Deplazes et al., 1992). More importantly, it reduces the biohazardous risk of exposure of personnel to potentially infective eggs during purgation or necropsy (Kamiya et al., 2004).

For detection of *E. multilocularis* infection of foxes, necropsy is time-consuming. Coproantigen testing by ELISA offers a specific practical alternative. Fox faecal samples should be taken at post-mortem from the
rectum rather than from the small intestine. *Echinococcus* coproantigens are also stable in fox or dog faeces left at 20°C for 1 week and in frozen dog faeces. Coproantigen testing has also been successfully used to evaluate the efficacy of deworming wild foxes infected with *E. multilocularis* using praziquantel-laced bait, which proved to be a successful combination of eliminating the source of infection (Kamiya et al., 2006).

- **Coproantigen test procedure** (*Echinococcus granulosus*) (Allan et al., 1992; Craig et al., 1995)
  i) The faecal sample (collected per rectum or from the ground) is mixed with an equal volume of phosphate buffered saline (PBS), pH 7.2, containing 0.3% Tween 20 (PBST), in a capped 5 ml disposable tube. This is shaken vigorously and centrifuged at 2000 g for 20 minutes at room temperature. Faecal supernatants can be tested immediately or stored at −20°C or lower. Supernatants that appear very dark or viscous are still acceptable for use.
  ii) A 96-well ELISA microtitre plate (Immulon #4, Thermo Electron Corporation) is coated with optimal concentration (typically 5 µg per ml) of a protein A purified IgG fraction of rabbit anti-*E. granulosus* proglottid extract (Allan et al., 1992) in 0.05 M bicarbonate/carbonate buffer, pH 9.6 (100 µl per well). The plate is covered and incubated overnight at 4°C.
  iii) The wells are rinsed three times in PBST with 1 minute between washes; 100 µl of the same buffer is added to each well, and the plate is incubated for 1 hour at room temperature.
  iv) The PBST is discarded and 50 µl of neat fetal calf serum is added to all wells. This is followed by the addition of 50 µl per well of faecal sample supernatants is added (in duplicate wells). The plate is incubated at room temperature for 1 hour with clingfilm seal covering the plate.
  v) The wells are rinsed as in step iii, but the contents are discarded into a 10% bleach (hypochlorite) solution.
  vi) An optimal dilution concentration of around 1 µg/ml of an IgG rabbit anti-*E. granulosus* proglottid extract peroxidase conjugate (Allan et al., 1992) in PBST is prepared and 100 µl per well is added to all wells. The plate is incubated for 1 hour at room temperature (22–24°C).
  vii) The wells are rinsed as in step iii.
  viii) Next, 100 µl per well of tetramethyl benzidine substrate (TMB, KPL Labs) is added and the plate is left in the dark for 20 minutes at room temperature (22–24°C).
  ix) Absorbance of wells is read at 630 nm. The enzyme-substrate reaction is stopped by adding 100 µl of 1 M phosphoric acid (H₃PO₄) to each well. The colour turns from blue to yellow if positive.
  x) Laboratories should establish their own end-point criteria using standard positive and negative samples. Standards can also be obtained from the OIE Reference Laboratories (see Table given in Part 4 of this *Terrestrial Manual*). Usually, the positive to negative threshold is taken as 3 standard deviations above the mean absorbance value of control negatives, or against a reference standard control positive using absorbance units equivalence.

- **Coproantigen test procedure** (*Echinococcus multilocularis*) (Morishima et al., 1999; Nonaka et al., 1998)

Sandwich ELISA using a monoclonal antibody EmA9 raised against adult *E. multilocularis* somatic antigen (Kohno et al., 1995).

  i) 0.5 g of each faecal sample is placed in a centrifuge tube and a 1% formalin solution containing 0.3% Tween 20 is added to a total volume of 15 ml.
  ii) After adequate mixing, the faecal solution is centrifuged at 1200 g for 10 minutes at room temperature. A supernatant fraction is used for the coproantigen detection assay.
  iii) Flat-bottomed microtitre plates (Immulon 600, Greiner, Germany) are coated with 50 µl/well of 1 µg/ml rabbit IgG directed against adult *E. multilocularis* excretory/secretory (ES) products in 0.05 M NaHCO₃/Na₂CO₃ buffer (pH 9.6) and are left overnight at 4°C.
  iv) The plates are washed three times with 250 µl/well PBS (pH 7.4) containing 0.05% Tween 20 (PBST), and blocked using 100 µl/well 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature (22–24 C).
  v) The plates are washed three times (with the wash disinfected with 10% bleach) and 50 µl of faecal supernatant is added to each well and the plates are incubated for 2 hours.
  vi) The plates are again washed four times and 0.5 µg/ml of the biotinylated monoclonal antibody in 0.5% BSA/0.5% casein in PBST is added to each well and the plates are incubated for 1 hour.
vii) The plates are washed four times and streptavidine-biotinylated horseradish peroxidase complex (Amersham Life Science), diluted 1/1000 in 0.5% BSA/0.5% cCasein in PBST is added to each well and the plates are incubated for 1 hour.

viii) The plates are washed five times and 100 µl/well of substrate solution (20 mg of phenylenediamine (Wako) in 50 ml of 0.1 M citric phosphate buffer with 10 µl of H₂O₂) is added.

ix) The plates are shaken immediately and placed in a 37°C incubator for 30 minutes. The reaction is stopped by adding 50 µl/well of 4 N H₂SO₄. The optical densities (OD) of the plates are read at 490 nm.

x) The cut-off value is calculated as the mean OD value plus 3 standard deviations of samples from uninfected animals.

This procedure was also used in a sandwich ELISA for *E. granulosus* coproantigen detection (Sakai et al., 1998). In 2008, a latex agglutination test and immunochromatography in-house kit using EmA9 became available for coproantigen detection (Kamiya, 2007; Nonaka et al., 2008).

f) **DNA recognition methods**

**Definitive hosts:** Copro-DNA has proven to be of value for the diagnosis of Echinococcosis in animal definitive hosts. DNA isolation from the faeces, however, is laborious.

PCR is a technically demanding and expensive technique. It is currently used mainly for confirmatory testing of coproantigen-positive samples or for identification of taenid eggs recovered from faeces. Table 2 presents the different PCR primers used for identification of copro-DNA from faeces in definitive hosts of genus *Echinococcus*.

**Table 2. PCR primers used for copro-DNA detection (modified from Mathis & Deplazes, 2006)**

<table>
<thead>
<tr>
<th>Primer designation: primer sequences (5’–3’)</th>
<th>Ref.</th>
<th>Target, comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. multilocularis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTG-AGG-CGA-TGT-GTG-ATG-GAG-AGG</td>
<td>Bretagne et al., 1993</td>
<td>U1 sRNA gene: may yield non-specific products when used with metacestode material containing host DNA (unpublished observation) Mitochondrial 12S RNA gene; used in two-tube nested PCR</td>
</tr>
<tr>
<td>CAA-GTG-GTC-AGG-GGC-AGT-AG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Outer primers:</strong></td>
<td></td>
<td>Mitochondrial 12S RNA gene; modified from Dinkel et al., 1998 for use in one-tube nested PCR</td>
</tr>
<tr>
<td>(P60 forward)</td>
<td>Dinkel et al., 1998</td>
<td></td>
</tr>
<tr>
<td>TTA-AGA-TAT-ATG-TGG-TAC-AGG-ATT-AGA-TAC-CC</td>
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<tr>
<td>(P375 reverse)</td>
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<td>AAC-CGA-GGG-TGA-CGG-GCG-GTG-TGT-ACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Inner primers:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Pnest forward)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACA-ATA-CCA-TAT-TAC-AAC-AAT-ATT-CCT-ATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Pnest reverse)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATA-TTT-TGT-AAG-GTT-GTT-CTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Outer primers:</strong></td>
<td></td>
<td>Mitochondrial 12S RNA gene; modified from Dinkel et al., 1998 for use in single PCR</td>
</tr>
<tr>
<td>(Em-1)</td>
<td>Van der Giessen et al., 1999</td>
<td></td>
</tr>
<tr>
<td>TAA-GAT-ATA-TGT-GGT-ACA-GGA-TTA-GAT-ACC-C</td>
<td></td>
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<tr>
<td>(Em-2)</td>
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<td></td>
</tr>
<tr>
<td>GGT-GAC-GGG-CGG-TGT-TGT-A</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Inner primers:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Em-3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATA-TTA-CAA-CAA-TAT-TCC-TAT-C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Em-4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATA-TTT-TGT-AAG-GTT-GTT-CTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer designation: primer sequences (5’–3’)</td>
<td>Ref.</td>
<td>Target, comments</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>------</td>
<td>------------------</td>
</tr>
<tr>
<td>(EM-H15) CCA-TAT-TAC-AAC-AAT-ATT-CCT-ATC</td>
<td>Stieger et al., 2002</td>
<td>NADH dehydrogenase subunit 1 (ND1) of mtDNA; cleavage with enzyme Cfo1 distinguish E. multilocularis from E. granulosus</td>
</tr>
<tr>
<td>(EM-H17) GTG-AGT-GAT-TCT-TGT-TAG-GGG-AAG</td>
<td>Moks et al., 2005</td>
<td>Repeated sequences from E. granulosus 'sheep strain'; yields banding pattern upon electrophoresis Mitochondrial 12SRNA gene; specific for E. granulosus 'sheep strain' Amplify a fragment of the coxl genespecific for E. granulosus</td>
</tr>
<tr>
<td>E. multilocularis and E. granulosus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ND1 (NDfor2-) AGT-TTC-GTA-AGG-GTC-CTA-ATA (NDrev2-) CCC-ACT-AAC-TAA-CTC-CTC-TTC</td>
<td>Moks et al., 2005</td>
<td></td>
</tr>
<tr>
<td>E. granulosus (Eg1121a) GAA-TGC-AAG-CAG-ATG (Eg1122a) GAG-ATG-AGT-GAG-AAG-GAG-TG</td>
<td>Abbasi et al., 2003</td>
<td></td>
</tr>
<tr>
<td>(Eg1f) CATTAATGATTTTGTAAAGTTG (Eg1r) CAC-ATC-ATC-TTA-CAA-TAA-CAC-C</td>
<td>Stefanic et al., 2004</td>
<td></td>
</tr>
<tr>
<td>(EgO/DNA-IM1) forward TCA-TAT-TTG-TTT-GAG-KAT-YAG-TKC reverse GTA-AAT-AAM-ACT-ATA-AAA-GAA-AYM-AC</td>
<td>Naidich et al., 2006</td>
<td></td>
</tr>
<tr>
<td>(Eg1f) CAT-TAA-TGT-ATT-TTG-TAA-AGT-TG (Eg1r) CAC-ATC-ATC-TTA-CAA-TAA-CAC-C</td>
<td>Trachsel et al., 2007</td>
<td>Echinococcus granulosus (sheep strain)</td>
</tr>
<tr>
<td>(Cest1) TGC-TGA-TTT-GTT-AAA-GTT-AGT-GAT-C (Cest2) CAT-AAA-TCA-ATG-GAA-ACA-ACA-ACA-AG</td>
<td>Trachsel et al., 2007</td>
<td>E. multilocularis</td>
</tr>
<tr>
<td>(Cest4) GTT-TTT-GTG-TGT-TAC-ATT-AAT-AAG-GGT-G</td>
<td>Trachsel et al., 2007</td>
<td>E. granulosus</td>
</tr>
</tbody>
</table>
**Table 2 cont.** PCR primers used for copro-DNA detection (modified from Mathis & Deplazes, 2006)

<table>
<thead>
<tr>
<th>Primer designation: primer sequences (5’–3’)</th>
<th>Ref.</th>
<th>Target, comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Cest5) GCG-GTG-TGT-ACM-TGA-GCT-AAA-C</td>
<td>Trachsel et al., 2007</td>
<td>Taenia spp.</td>
</tr>
<tr>
<td>(Cest3) YGA-YTC-TTT-TTA-GGG-GAA-GGT-GTG</td>
<td>Trachsel et al., 2007</td>
<td>Taenia spp. (Sequencing primer for the 267 bp amplicon of the multiplex PCR)</td>
</tr>
<tr>
<td>(Cest5) GCG-GTG-TGT-ACM-TGA-GCT-AAA-C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Cest5seq) GAT-TCT-TTT-TAG-GGG-AAG-G</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Differential diagnosis of *E. granulosus* and *E. multilocularis* infections in definitive hosts may be achieved by specific detection of PCR-amplified DNA from *E. multilocularis* eggs present in faeces (Bretagne et al., 1993; Lymbery, 1995). In practice, it is recommended to screen definitive hosts (e.g. foxes) using the coproantigen test and confirm with the PCR DNA test. In Europe, transmission of *E. multilocularis* generally occurs in regions where *E. granulosus* is not endemic or appears very infrequently. In other regions, including parts of the Near East (Turkey and Iran), Central Asia, Russia and the People’s Republic of China, these two species may occur together (Craig et al., 1996). Further evaluation of *E. multilocularis* infection is required to investigate intermittent shedding and duration of shedding of parasite DNA. Recently PCR has been developed for the detection of copro-DNA for *E. granulosus* and for genotypic differentiation. (McManus, 2006; Varcasia et al., 2007)

As PCR is generally used as a confirmatory test, it is suggested to concentrate the taeniid eggs by sequential sieving and an in-between concentration method step. DNA isolation from these eggs can be achieved using a simplified protocol of the alkaline lysis method combined with a commercial kit with no need for organosolvent extractions (Lightowlers & Gottstein, 1995).

**Intermediate hosts:** DNA hybridisation methods are not currently used for the detection of *E. granulosus* in livestock intermediate hosts. Molecular methods are, however, important in identification of isolates or strains of *E. granulosus* for epidemiological purposes (McManus & Bryant, 1995).

For the identification of small, degenerated or calcified lesions of *E. multilocularis* in intermediate or aberrant hosts, PCR is of great value (Mathis et al., 1996).

### 2. Serological tests

**a) Intermediate hosts**

Immunological tests, useful in humans, are less sensitive and specific in livestock and at present cannot replace necropsy (Craig, 1997; Lightowlers & Gottstein, 1995).

**b) Definitive hosts**

An extensive programme has been initiated to develop immunodiagnostic tests to control canine echinococcosis. Following ingestion of a cyst, dogs will be exposed at the intestinal level to various antigens during the establishment of the parasite and its development and oogenesis. Specific antibodies against oncosphere and protoscolex antigens can be readily detected in the serum of infected dogs. This methodology has not reached a practical stage as it does not differentiate between current and previous infections and false positives may occur with infections of *Taenia* species.

### C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

#### 1. Intermediate hosts

Application of an effective vaccine to reduce hydatid infection in livestock would be likely to have a substantial impact on the rate of transmission of the disease to humans (Lightowlers, 2006). As *E. granulosus* belongs to the *Taeniid* family, many aspects of its immunological relationship with its intermediate host are similar to that occurring in *Taenia* species. Moreover, it was considered that the vaccine development approach used in *Taenia*
species such as the native host-protective antigens of *T. ovis* would also be successful for *E. granulosus*. Using recombinant DNA technology, an oncosphere antigen vaccine EG95 was shown to be capable of inducing a high level of protection against experimental challenge infection with *E. granulosus* eggs in sheep (Lightowlers et al., 1996).

The EG95 vaccine has been licensed by the University of Melbourne and AgResearch New Zealand to a commercial group in the People’s Republic of China (Lightowlers, 2006).

### 2. Definitive hosts

While considerable research has been undertaken with crude antigens to protect dogs from echinococcosis, only limited evidence has been demonstrated so far. Recent studies using recombinant protoscolex antigens, however, look encouraging (Chabalgoity et al., 2001). Basic research on canine mucosal immunology and *Echinococcus* infection is required for progress.

### REFERENCES


Chapter 2.1.4. – Echinococcosis/Hydatidosis


*  
* * *

NB: There are OIE Reference Laboratories for Echinococcosis/Hydatidosis (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Echinococcosis/Hydatidosis
CHAPTER 2.1.5.

FOOT AND MOUTH DISEASE

SUMMARY

Foot and mouth disease (FMD) is the most contagious disease of mammals and has a great potential for causing severe economic loss in susceptible cloven-hoofed animals. There are seven serotypes of FMD virus (FMDV), namely, O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1. Infection with one serotype does not confer immunity against another. FMD cannot be differentiated clinically from other vesicular diseases, such as swine vesicular disease, vesicular stomatitis and vesicular exanthema. Laboratory diagnosis of any suspected FMD case is therefore a matter of urgency.

Typical cases of FMD are characterised by a vesicular condition of the feet, buccal mucosa and, in females, the mammary glands. Clinical signs can vary from mild to severe, and fatalities may occur, especially in young animals. In some species the infection may be subclinical, e.g. African buffalo (Syncerus caffer). The preferred tissue for diagnosis is epithelium from unruptured or freshly ruptured vesicles or vesicular fluid. Where collecting this is not possible, blood and/or oesophageal–pharyngeal fluid samples taken by probang cup in ruminants or throat swabs from pigs provide an alternative source of virus. Myocardial tissue or blood can be submitted from fatal cases, but vesicles are again preferable if present.

It is vital that samples from suspected cases be transported under secure conditions and according to international regulations. They should only be dispatched to authorised laboratories.

Diagnosis of FMD is by virus isolation or by the demonstration of FMD viral antigen or nucleic acid in samples of tissue or fluid. Detection of virus-specific antibody can also be used for diagnosis, and antibodies to viral nonstructural proteins (NSPs) can be used as indicators of infection, irrespective of vaccination status.

Identification of the agent: The demonstration of FMD viral antigen or nucleic acid is sufficient for a positive diagnosis. Due to the highly contagious nature and economic importance of FMD, the laboratory diagnosis and serotype identification of the virus should be done in a laboratory that meets the OIE requirements for Containment Group 4 pathogens.

Enzyme-linked immunosorbent assays (ELISA) can be used to detect FMD viral antigens and for serotyping. Lateral flow devices (LFD) are also becoming more readily available and can also be used to detect FMD viral antigens. The ELISA has replaced complement fixation (CF) in most laboratories as it is more specific and sensitive and it is not affected by pro- or anti-complement factors. If the sample is inadequate or the diagnosis remains uncertain, sample materials can be tested by reverse transcription polymerase chain reaction (RT-PCR) and/or virus isolation using susceptible cell cultures or 2–7-day old unweaned mice to amplify any nucleic acid or live virus that may be present. The cultures should preferably be of primary bovine (calf) thyroid, but pig, lamb or calf kidney cells, or cell lines of comparable sensitivity may be used. Once a cytopathic effect (CPE) is complete in the cultures, the fluids can be tested for FMDV using ELISA, CF or RT-PCR. Similar tests can be performed on homogenised suspensions of the dissected musculo-skeletal tissues of any mice that die.

Serological tests: The demonstration of specific antibodies to structural proteins in nonvaccinated animals is indicative of prior infection with FMDV. This is particularly useful in mild cases or where epithelial tissue cannot be collected. Tests for antibodies to some NSPs of FMDV are useful in providing evidence of previous or current viral replication in the host, irrespective of vaccination status. NSPs, unlike structural proteins, are highly conserved and therefore are not serotype specific and as a consequence, the detection of these antibodies is not serotype restricted.
Virus neutralisation tests (VNTs) and ELISAs for antibodies to structural proteins are used as serotype-specific serological tests. VNTs depend on tissue cultures and are therefore more prone to variability than ELISAs; they are also slower and subject to contamination. ELISAs for detection of antibodies have the advantage of being faster, and are not dependent on cell cultures. The ELISA can be performed with inactivated antigens, thus requiring less restrictive biocontainment facilities.

Requirements for vaccines and diagnostic biologicals: Inactivated virus vaccines of varying composition are available commercially. Typically, virus is used to infect a suspension or monolayer cell culture and the resulting preparation is clarified, inactivated with ethyleneimine and blended with adjuvant. Many FMD vaccines are multivalent to provide protection against the different serotypes likely to be encountered in a given field situation.

The finished vaccine must be shown to be free from residual live virus. This is most effectively done using in-vitro tests on concentrated inactivated virus preparations prior to formulation of the vaccine and freedom from live virus is subsequently confirmed during in-vivo and/or in-vitro tests on the finished product. Challenge tests are also conducted in vaccinated cattle to establish a PD$_{50}$ (50% protective dose) value or protection against generalised foot infection (PGP), although a serological test is considered to be satisfactory where a valid correlation between the observed protection, and the specific antibody response has been established.

FMD vaccine production facilities should also meet the OIE requirements for Containment Group 4 pathogens.

Diagnostic and reference reagents are available from the OIE Reference Laboratories for FMD or the FAO (Food and Agriculture Organization of the United Nations) World Reference Laboratory for FMD. The Institute for Animal Health Pirbright Laboratory has dual designations as both the FAO World Reference Laboratory and as an OIE Reference Laboratory for FMD.

A. INTRODUCTION

Foot and mouth disease (FMD) is caused by a virus of the genus Aphthovirus, family Picornaviridae. There are seven serotypes of FMD virus (FMDV), namely O, A, C, SAT 1, SAT 2, SAT 3, and Asia 1, that infect cloven-hoofed animals. Infection with any one serotype does not confer immunity against another. Within serotypes, many strains can be identified by biochemical and immunological tests.

Of the domesticated species, cattle, pigs, sheep, goats and water buffalo (Bubalus bubalis) are susceptible to FMD (Food and Agricultural Organization of the United Nations [FAO]; 1984). Many species of cloven-hoofed wildlife may become infected, and the virus has occasionally been recovered from other species as well. Amongst the camelidae, Bactrian camels and new world camelids have been shown to be susceptible (Larska et al., 2009). In Africa, SAT serotypes of FMD viruses are often maintained by African buffalo (Syncerus caffer). There is periodic spillover of infection into livestock or sympatric cloven-hoofed wildlife. Elsewhere in the world cattle are usually the main reservoir for FMD viruses, although in some instances the viruses involved appear to be specifically adapted to pigs. The pig-adapted Cathay strain of type O FMDV apparently does not infect large ruminants in the field or experimentally and requires cells of porcine origin for primary isolation. Small ruminants can play an important role in the spread of FMDV, but it is not clear whether the virus can be maintained in these species for long periods in the absence of infection of cattle. Strains of FMDV that infect cattle have been isolated from wild pigs, antelope and deer. The evidence indicates that, in the past, infection of deer was derived from contact, direct or indirect, with infected domestic animals, and that apart from African buffalo, wildlife has not, so far, been shown to be able to maintain FMD viruses independently for more than a few months.

Infection of susceptible animals with FMDV can lead to the appearance of vesicles on the feet, in and around the oral cavity, and on the mammary glands of females. The vesicles rupture and then heal whilst coronary band lesions may give rise to growth arrest lines that grow down the side of the hoof. The age of lesions can be estimated from these changes as they provide an indicator of the time since infection occurred (UK Ministry of Agriculture, Fisheries and Food; 1986). Mastitis is a common sequel of FMD in dairy cattle. Vesicles can also occur at other sites, such as inside the nostrils and at pressure points on the limbs – especially in pigs. The severity of clinical signs varies with the strain of virus, the exposure dose, the age and breed of animal, the host species and the immunity of the animal. The signs can range from a mild or apparently infection to one that is severe. Death may result in some cases. Mortality from a multifocal myocarditis is most commonly seen in young animals: myositis may also occur in other sites.
Chapter 2.1.5. – Foot and mouth disease

On premises with a history of sudden death in young cloven-hoofed livestock, close examination of adult animals may often reveal the presence of vesicular lesions if FMD is involved. The presence of vesicles in fatal cases is variable.

In animals with a history of vesicular disease, the detection of FMDV in samples of vesicular fluid, epithelial tissue, oesophageal–pharyngeal (OP) sample, milk, or blood is sufficient to establish a diagnosis. Diagnosis may also be established by the detection of FMDV in the blood, heart or other organs of fatal cases. A myocarditis may be seen macroscopically (the so-called "tiger heart") in a proportion of fatal cases.

FMD viruses may occur in all the secretions and excretions of acutely infected animals, including expired air. Transmission is generally effected by direct contact between infected and susceptible animals or, more rarely, indirect exposure of susceptible animals to the excretions and secretions of acutely infected animals or uncooked meat products. Following recovery from the acute stage of infection, infectious virus disappears with the exception of low levels that may persist in the oropharynx of some ruminants. Live virus or viral RNA may continue to be recovered from oropharyngeal fluids and cells collected with a probang cup. FMD virus has also been shown to persist in a nonreplicative form in lymph nodes (Juleff et al., 2008). Animals in which the virus persists in the oropharynx for more than 28 days after infection are referred to as carriers. Pigs do not become carriers. Circumstantial evidence indicates, particularly in the African buffalo, that carriers are able, on rare occasions, to transmit the infection to susceptible domestic animals with which they come in close contact: the mechanism involved is unknown. The carrier state in cattle usually does not persist for more than 6 months, although in a small proportion, it may last up to 3 years. In African buffalo, individual animals have been shown to harbour the virus for at least 5 years, but it is probably not a lifelong phenomenon. Within a herd of buffalo, the virus may be maintained for 24 years or longer. Sheep and goats do not usually carry FMD viruses for more than a few months, whilst there is little information on the duration of the carrier state in Asian buffalo species and subspecies.

Because of the highly contagious nature and economic importance of FMD, the laboratory diagnosis and serotype identification of the virus should be done in a facility that meets the requirements for Containment Group 4 pathogens as outlined in Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities. Countries lacking access to such a specialised national or regional laboratory should send specimens to an OIE FMD Reference Laboratory. Vaccine production facilities should also meet the requirements for Containment Group 4 pathogens.

Diagnostic and standard reagents are available in kit form or as individual items from OIE Reference Laboratories for FMD. The use of inactivated antigens in the enzyme-linked immunosorbent assay (ELISA), as controls in the antigen-detection test or to react with test sera in the liquid-phase blocking or solid-phase competitive ELISA, reduces the disease security risk involved compared with the use of live virus. Reagents are supplied freeze-dried –30°C and –5°C and –90°C and –50°C, respectively, for many years. The International Atomic Energy Agency has produced a manual that includes a recommended test and quality control protocols. There are a number of commercially available diagnostic test kits, for the detection of virus antigens or antibodies.

B. DIAGNOSTIC TECHNIQUES

For laboratory diagnosis, the tissue of choice is epithelium or vesicular fluid. Ideally, at least 1 g of epithelial tissue should be collected from an unruptured or recently ruptured vesicle, usually from the tongue, buccal mucosa or feet. To avoid injury to personnel collecting the samples, as well as for animal welfare reasons, it is recommended that animals be sedated before any samples are obtained.

Epithelial samples should be placed in a transport medium composed of equal amounts of glycerol and 0.04 M phosphate buffer, pH 7.2–7.6, preferably with added antibiotics (penicillin [1000 International Units (IU)], neomycin sulphate [100 IU], polymyxin B sulphate [50 IU], mycostatin [100 IU]). If 0.04 M phosphate buffer is not available, tissue culture medium or phosphate-buffered saline (PBS) can be used instead, but it is important that the final pH of the glycerol/buffer mixture be in the range pH 7.2–7.6. FMDV is extremely labile in low pH and buffering of the transport media is critical for successful sample collection. Samples should be kept refrigerated or on ice until received by the laboratory.

Where epithelial tissue is not available from ruminant animals, for example in advanced or convalescent cases, or where infection is suspected in the absence of clinical signs, samples of OP fluid can be collected by means of a probang (sputum) cup (or in pigs by swabbing the throat) for submission to a laboratory for virus isolation or reverse-transcription polymerase chain reaction (RT-PCR). Viraemia may also be detected by examining serum samples by means of RT-PCR or virus isolation. For the collection of throat swabs from pigs, the animal should be held on its back in a wooden cradle with the neck extended. Holding a swab in a suitable instrument, such as an artery forceps, the swab is pushed to the back of the mouth and into the pharynx.

OIE Terrestrial Manual 2012 147
Before the collection of OP samples from cattle or large ruminants (e.g. buffalo), 2 ml transport fluid (composed of 0.08 M phosphate buffer containing 0.01% bovine serum albumin, 0.002% phenol red, antibiotics [1000 units/ml penicillin, 100 units/ml mycostatin, 100 units/ml neomycin, and 50 units/ml polymyxin], and adjusted to pH 7.2) should be added to a container of around 5 ml capacity capable of withstanding freezing above dry ice (solid carbon dioxide) or liquid nitrogen (Kitching & Donaldson, 1987).

An OP sample is collected by inserting a probang over the tongue into the oro-pharyngeal area and then passing it vigorously backwards and forwards 5–10 times between the first portion of the oesophagus and the back of the pharynx. The purpose is to collect oro-pharyngeal fluid and especially superficial epithelial cells from these areas, including the proximal part of the oesophagus, the walls of the pharynx, the tonsillar crypts and the surfaces of the soft palate. If the sample does not contain adequate cellular debris the actions may be repeated.

After collection of OP fluid by probang, the contents of the cup should be poured into a wide-necked transparent bottle of around 20 ml capacity. The fluid is examined, and should contain some visible cellular material. This fluid is then added to an approximately equal volume of transport fluid, ensuring that cellular material is transferred; the mixture is shaken gently and should have a final pH of around pH 7.6. Samples contaminated with ruminal contents may be unsuitable for culture. Samples seen to contain blood are not entirely satisfactory. Repeat sampling can be done after the mouth and throat of the animal have been rinsed with water or PBS. Where several animals are to be sampled, the probang must be cleaned and disinfected between each animal. This is done by washing the probang in tap water, immersing it in a suitable disinfectant (e.g. 0.5% [w/v] citric acid in tap water) and then rinsing off all the disinfectant with water before sampling the next animal.

OP samples from small ruminants are collected by putting 2 ml of transport fluid into a wide-necked bottle of about 20 ml capacity and, after collection, rinsing the probang cup in this transport fluid to discharge the OP sample. This is then transferred to a container of about 5 ml capacity for transport. The small container should be capable of withstanding freezing above dry ice or liquid nitrogen (Kitching & Donaldson, 1987).

Samples of OP fluid should be refrigerated or frozen immediately after collection. If they are to remain in transit for more than a few hours, they should preferably be frozen by being placed either above dry ice or liquid nitrogen. Before freezing, the containers should be carefully sealed using airtight screw caps or silicone. This is particularly important when using dry ice, as introduction of CO₂ into the OP sample will lower its pH, inactivating any FMDV that may be in the samples. Glass containers should not be used because there is a risk that they will explode on defrosting in the event of liquid nitrogen leaking into them. Samples should reach the laboratory in a frozen state or, if this is not feasible, maintained under reliable cold chain conditions during transit.

Special precautions are required when sending perishable suspect FMD material both within and between countries. The International Air Transport Association (IATA), Dangerous Goods Regulations (DGR) has explicit requirements for packaging and shipment of diagnostic specimens by all commercial means of transport. These are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens. Forms and guidance on sample submission and specifications for manufacture of probang cups can be found on the website of the Pirbright OIE Reference Laboratory at http://www.wrlfmd.org/. Procedures for collection and shipment of field samples for the diagnosis of vesicular diseases and its differential diagnosis can be found at the Pan-American FMD OIE Reference Laboratory at http://www.panaftosa.org.br

1. Identification of the agent

A range of sample types, including epithelium, OP samples and serum, may be examined by virus isolation or RT-PCR. By contrast, ELISA CF and the lateral flow device are suited to the examination of epithelial suspensions, vesicular fluids or cell culture supernatants, but are insufficiently sensitive for the direct examination of OP samples or serum.

a) Virus isolation

The epithelium sample should be taken from the PBS/glycerol, blotted dry on absorbent paper to reduce the glycerol content, which is toxic for cell cultures, and weighed. A suspension should be prepared by grinding the sample in sterile sand in a sterile pestle and mortar with a small volume of tissue culture medium and antibiotics. Further medium should be added until a final volume of nine times that of the epithelial sample has been added, giving a 10% suspension. This is clarified on a bench centrifuge at 2000 g for 10 minutes. Once clarified, such suspensions of field samples suspected to contain FMDV are inoculated onto cell cultures or into unweaned mice. Sensitive cell culture systems include primary bovine (calf) thyroid cells and primary pig, calf or lamb kidney cells. Established cell lines, such as BHK-21 (baby hamster kidney) and IB-RS-2 cells, may also be used but are generally less sensitive than primary cells for detecting low amounts of infectivity. The sensitivity of any cells used should be tested with standard preparations of FMDV. The use of IB-RS-2 cells aids the differentiation of swine vesicular disease virus (SVDV) from FMDV (as SVDV will only grow in cells of porcine origin) and is often essential for the isolation of porcinephilic strains, such as O Cathay. The cell cultures should be examined for cytopathic effect (CPE) for 48 hours. If no CPE is
detected, the cells should be frozen and thawed, used to inoculate fresh cultures and examined for CPE for another 48 hours. Unweaned mice are an alternative to cell cultures and should be 2–7 days old and of selected inbred strains. Some field viruses may require several passages before they become adapted to mice (Skinner, 1960). In the case of OP fluids, pretreatment with an equal volume of chloro-fluoro-carbons may improve the rate of virus detection by releasing virus from immune complexes.

b) **Immunological methods**

- **Enzyme-linked immunosorbent assay**

  The preferred procedure for the detection of FMD viral antigen and identification of viral serotype is the ELISA (Ferris & Donaldson, 1992; Roeder & Le Blanc Smith, 1987). This is an indirect sandwich test in which different rows in multiwell plates are coated with rabbit antisera to each of the seven serotypes of FMDV. These are the ‘capture’ sera. Test sample suspensions are added to each of the rows, and appropriate controls are also included. Guinea-pig antisera to each of the serotypes of FMDV are added next, followed by rabbit anti-guinea-pig serum conjugated to an enzyme. Extensive washing is carried out between each stage to remove unbound reagents. A colour reaction on the addition of enzyme substrate and chromogen indicates a positive reaction. With strong positive reactions, this will be evident to the naked eye, but results can also be read spectrophotometrically at an appropriate wavelength. In this case, an absorbance reading greater than 0.1 above background indicates a positive reaction; the serotype of FMDV can also be identified. Values close to 0.1 should be confirmed by retesting or by amplification of the antigen by tissue culture passage and testing the supernatant once a CPE has developed. A suitable protocol is given below. Other protocols are available with slightly different formats and interpretation criteria (Alonso et al., 1993).

  Depending on the species affected and the geographical origin of samples, it may be appropriate to simultaneously test for SVDV or vesicular stomatitis virus (VSV). Ideally a complete differential diagnosis should be undertaken in all vesicular conditions.

  Rabbit antiserum to the 146S antigen of each of the seven serotypes of FMDV (plus SVDV or VSV if required) is used as a trapping antibody at a predetermined optimal concentration in carbonate/bicarbonate buffer, pH 9.6.

  Control antigens are prepared from selected strains of each of the seven types of FMDV (plus SVDV or VSV if appropriate) grown on monolayer cultures of BHK-21 cells (IB-RS-2 cells for SVD or VSV). The unpurified supernatants are used and pretitrated on ELISA plates. The final dilution chosen is that which gives an absorbance at the top of the linear region of the titration curve (optical density approximately 2.0), so that the five-fold dilutions of the control antigens used in the test give two additional lower optical density readings from which the titration curve can be derived. PBS containing 0.05% Tween 20 and phenol red indicator is used as a diluent (PBST).

  Guinea-pig antisera prepared by inoculating guinea-pigs with 146S antigen of one of the seven serotypes of FMDV (plus SVDV if required) and preblocked with normal bovine serum (NBS) is used as the detecting antibody. Predetermined optimal concentrations are prepared in PBS containing 0.05% Tween 20, and 5% dried, nonfat skimmed milk (PBSTM).

  Rabbit (or sheep) anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase and preblocked with NBS is used at a predetermined optimum concentration in PBSTM. As an alternative to guinea-pig or rabbit antisera, suitable monoclonal antibodies (MAbs) can be used coated to the ELISA plates as capture antibody or peroxidase-conjugated as detecting antibody.

- **Test procedure**

  i) ELISA plates are coated with 50 µl/well rabbit antiviral sera in 0.05 M carbonate/bicarbonate buffer, pH 9.6. Rows A to H receive, respectively, antisera to serotypes O, A, C, SAT 1, SAT 2, SAT 3, Asia 1 and SVDV or VSV (optional).

  ii) Leave overnight at 4°C in a stationary position or place on an orbital shaker set at 100–120 revolutions per minute in a 37°C incubator for 1 hour.

  iii) Prepare test sample suspension (10% original sample suspension or undiluted clarified cell culture supernatant fluid).

  iv) The ELISA plates are washed five times in PBS.

  v) On each plate, load wells of columns 4, 8 and 12 with 50 µl PBST. Additionally, add 50 µl of PBST to wells 1, 2 and 3 of rows A to H on plate 1. To well 1 of row A of plate 1 add 12.5 µl of control antigen type O, to well 1 of row B add 12.5 µl of control antigen type A; continue in this manner for control antigen of types C, SAT 1, SAT 2, SAT 3, Asia 1 and SVDV or VS (if appropriate) in order to well 1,
rows C to H. Mix diluent in well 1 of rows A to H and transfer 12.5 µl from well 1 to 2 (rows A to H), mix and transfer 12.5 µl from well 2 to 3, mix and discard 12.5 µl from well 3 (rows A to H) (this gives a five-fold dilution series of each control antigen). It is only necessary to change pipette tips on the micropipette between antigens. The remainder of the plate can be loaded with the test sample(s). Add 50 µl of sample one to wells 5, 6 and 7 of rows A to H, the second sample is placed similarly in columns 9, 10 and 11, rows A to H.

If more than two samples are to be tested at the same time, the other ELISA plates should be used as follows:

Dispense 50 µl of the PBST to the wells (rows A to H) of columns 4, 8 and 12 (buffer control columns). Note that the control antigens are not required on these plates. These test samples may be added in 50 µl volumes in rows A to H to columns 1, 2, 3; 5, 6, 7; 9, 10, 11, respectively.

vi) Cover with lids and place on an orbital shaker at 37°C for 1 hour.

vii) Wash the plates by flooding with PBS – wash three times as before and empty residual wash fluid. Blot the plates dry.

viii) Transfer 50 µl volumes of each guinea-pig serum dilution to each plate well in the appropriate order, e.g. rows A to H receive, respectively, antisera to serotypes O, A, C, SAT 1, SAT 2, SAT 3, Asia 1 and SVDV or VSV (optional).

ix) Cover the plates with lids and replace on the orbital shaker. Incubate at 37°C for 1 hour.

x) The plates are washed again three times, and 50 µl of rabbit anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase is added to each well. The plates are incubated at 37°C for 1 hour on a rotary shaker.

xi) The plates are washed again three times, and 50 µl of substrate solution, containing 0.05% % H$_2$O$_2$ plus orthophenylene diamine or a suitable alternative chromogen, is added to each well.

xii) The reaction is stopped after 15 minutes by the addition of 50 µl of 1.25 M sulphuric acid. The plates are read at 492 nm on a spectrophotometer linked to a computer.

• Lateral flow device test

There are commercially available lateral flow devices (LFD) (Ferris et al., 2009), but the OIE has not yet received a validation dossier for these tests. As soon as a dossier is received, the manufacturer could apply for inclusion on the OIE test register.

• Complement fixation test (an alternative test for international trade)

In general, the ELISA is preferable to the complement fixation test (CFT) because it is more sensitive and it is not affected by pro- or anti-complementary factors (Ferris & Dawson, 1988). If ELISA reagents are not available, or if subtyping is pursued, the CFT may be performed as follows:

The CF50% protocol in tubes used widely in South America for typing, subtyping and for establishing serological relationships (r values) is performed as follows: 0.2 ml antiserum to each FMD serotype diluted at a predetermined optimal dilution in veronal buffer diluent (VBD) or borate-saline solution (BSS) is placed in separate tubes. To these, 0.2 ml of test sample suspension is added, followed by 0.2 ml of a complement dilution containing 4 units of complement. The test system is incubated at 37°C for 30 minutes prior to the addition of 0.4 ml 2% standardised sheep red blood cells (SRBC) in VBD or BSS sensitised with rabbit anti-SRBC. The reagents are incubated at 37°C for further 30 minutes and the tubes are subsequently centrifuged and read. Samples with less than 50% haemolysis are considered positive (3B).

Other protocols performed in microplates are available and are performed as follows: antisera to each of the seven types of FMDV are diluted in VBD in 1.5-fold dilution steps from an initial 1/16 dilution to leave 25 µl of successive antiserum dilutions in U-shaped wells across a microtitre plate. To these are added 50 µl of 3 units of complement, followed by 25 µl of test sample suspension(s). The test system is incubated at 37°C for 1 hour prior to the addition of 25 µl of 1.4% SRBC in VBD sensitised with 5 units of rabbit anti-SRBC. The reagents are incubated at 37°C for a further 30 minutes and the plates are subsequently centrifuged and read. Appropriate controls for the test suspension(s), antisera, cells and complement are included. CF titres are expressed as the reciprocal of the serum dilution producing 50% haemolysis. A CF titre $\geq$36 is considered to be a positive reaction. Titre values of 24 should be confirmed by retesting an antigen that has been amplified through tissue culture passage.
c) Nucleic acid recognition methods

RT-PCR can be used to amplify genome fragments of FMDV in diagnostic materials including epithelium, milk, serum and OP samples. RT combined with real-time PCR has a sensitivity comparable to that of virus isolation and automated procedures enhance sample throughput (Reid et al., 2003). Serotyping primers have also been developed (Vangrysperre & De Clercq, 1996). Simplified RT-PCR systems for potential field-use are under development (Callahan et al., 2002).

- Agarose gel-based RT-PCR assay

The procedure used at the OIE Reference Laboratory at Pirbright is described (Reid et al., 2000). The RT-PCR assay consists of the three successive procedures of (i) extraction of template RNA from the test or control sample followed by (ii) RT of the extracted RNA, (iii) PCR amplification of the RT product and (iv) detection of the PCR products by agarose gel electrophoresis.

- Test procedure

i) Add 200 µl of test sample to 1 ml of TRIzol® Reagent in a sterile tube. Store at –70°C until required for RNA extraction.

ii) Transfer 1 ml of the solution from i) into a fresh, sterile tube containing 200 µl of chloroform. Vortex mix for about 10–15 seconds and leave at room temperature for 3 minutes.

iii) Centrifuge for 15 minutes at 20,000 g.

iv) Transfer 500 µl of the aqueous phase into a fresh, sterile tube containing 1 µl of glycogen (20 mg/ml) and add 500 µl of iso-propyl-alcohol (propan-2-ol). Vortex mix for a few seconds.

v) Leave at room temperature for 10 minutes then centrifuge for 10 minutes at 20,000 g.

vi) Discard the supernatant fluid from each tube and add 1 ml of 70% ethanol. Vortex mix for a few seconds.

vii) Centrifuge for 10 minutes at 20,000 g.

viii) Carefully remove the supernatant fluid from each tube taking care not to dislodge or lose any pellet at the bottom of the tube.

ix) Air dry each tube at room temperature for 2–3 minutes.

x) Resuspend each pellet by adding 20 µl of nuclease-free water to the tube.

xi) Keep the extracted RNA samples on ice if the RT step is about to be performed. Otherwise store at –70°C.

xii) For each sample to be assayed, add 2 µl of random hexamers (20 µg/ml) and 5 µl of nuclease-free water into a sterile 0.5 ml microcentrifuge tube. It is recommended to prepare the dilution in bulk for the total number of samples to be assayed but allowing for one extra sample.

xiii) Add 5 µl of RNA from the extraction procedure described above to give a volume of 12 µl in each tube. Mix by gently pipetting up and down.

xiv) Incubate at 70°C for 5 minutes.

xv) Cool at room temperature for 10 minutes.

xvi) During the 10-minute incubation period, prepare the RT reaction mixture described below for each sample. Prepare the reaction mixture in bulk in a sterile 1.5 ml microcentrifuge tube for the number of samples to be assayed plus one extra sample.

First strand buffer, 5× conc. (4 µl); bovine serum albumin (acetylated), 1 mg/ml (2 µl); dNTPs, 10 mM mixture each of dATP, dCTP, dGTP, dTTP (1 µl); DTT, 1 M (0.2 µl); Moloney Murine Reverse Transcriptase, 200 U/µl (1 µl).

xvii) Add 8 µl reaction mix to the 12 µl of random primer/RNA mix. Mix by gently pipetting.

xviii) Incubate at 37°C for 45 minutes.

xix) Keep the RT products on ice if the PCR amplification step is about to be performed, otherwise store at –20°C.

xx) Prepare the PCR mix described below for each sample. It is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample.

Nuclease-free water (35 µl); PCR reaction buffer, 10× conc (5 µl); MgCl₂, 50 mM (1.5 µl); dNTPs, 10 mM mixture each of dATP, dCTP, dGTP, dTTP (1 µl); primer 1, 10 pmol/µl (1 µl); primer 2, 10 pmol/µl (1 µl); Taq Polymerase, 5 units/µl (0.5 µl).
Chapter 2.1.5. – Foot and mouth disease

xxi) Add 45 µl of PCR reaction mix to a well of a PCR plate or to a microcentrifuge tube for each sample to be assayed followed by 5 µl of the RT product to give a final reaction volume of 50 µl.

xxii) Spin the plate or tubes for 1 minute in a suitable centrifuge to mix the contents of each well.

xxiii) Place the plate in a thermal cycler for PCR amplification and run the following programme:

- 94°C for 5 minutes: 1 cycle;
- 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes: 30 cycles;
- 72°C for 7 minutes: 1 cycle.

xxiv) Mix a 20 µl aliquot of each PCR reaction product with 4 µl of staining solution and load onto a 1.5% agarose gel. After electrophoresis a positive result is indicated by the presence of a 328 bp band corresponding to FMDV sequence in the 5' untranslated region of the genome.

- Stock solutions
  i) Nuclease-free water, TRizol® Reagent, chloroform, glycogen, iso-propyl-alcohol (propan-2-ol), ethanol, random hexanucleotide primers, First strand buffer, BSA (acetylated), dNTPs, DTT, Moloney Murine Reverse Transcriptase, PCR reaction buffer (10×), MgCl₂ and Taq Polymerase are commercially available.
  ii) Primers at a concentration of 10 pmol/µl: Primer 1 sequence 5'-GCCTG-GTCTT-TCCAG-GTCT-3' (positive strand); Primer 2 sequence 5'-CCAGT-CCCCT-TCTCA-GATC-3' (negative strand).

- Real-time RT-PCR assay
  The real-time RT-PCR assay can use the same procedures of extraction of total RNA from the test or control sample followed by RT of the extracted RNA as for the conventional agarose gel-based procedure. Automated extraction of total nucleic acid from samples followed by automated pipetting programmes for the RT and PCR steps (Reid et al., 2003) can be used as an alternative to the manual procedures described above. PCR amplification of the RT product is performed by a different procedure. A one-step method for combining the RT and PCR steps has also been described (Shaw et al., 2007). Detection of the PCR products in agarose gels is not required following real-time amplification.

  i) Take the RT products from step xix (see above).
  ii) Prepare the PCR mix described below for each sample. Again it is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample: nuclease-free water (6 µl); PCR reaction master mix, 2× conc. (12.5 µl); real-time PCR forward primer, 10 pmol/µl (2.25 µl); real-time PCR reverse primer, 10 pmol/µl (2.25 µl); TaqMan® probe, 5 pmol/µl (1 µl).
  iii) Add 24 µl PCR reaction mix to a well of a real-time PCR plate for each sample to be assayed followed by 1 µl of the RT product to give a final reaction volume of 25 µl.
  iv) Spin the plate for 1 minute in a suitable centrifuge to mix the contents of each well.
  v) Place the plate in a real-time PCR machine for PCR amplification and run the following programme:

- 50°C for 2 minutes: 1 cycle;
- 95°C for 10 minutes: 1 cycle;
- 95°C for 15 seconds, 60°C for 1 minute: 50 cycles.

  vi) Reading the results: Assign a threshold cycle (CT) value to each PCR reaction from the amplification plots (a plot of the fluorescence signal versus cycle number; different cut-off values may be appropriate for different sample types; Parida et al., 2007). The CT values used to assign samples as either FMDV positive or negative should be defined by individual laboratories using appropriate reference material. For example at the OIE Reference Laboratory at Pirbright, negative test samples and negative controls should have a CT value at >50.0. Positive test samples and positive control samples should have a CT value <40. Samples with CT values falling within the range 40–50 are designated “borderline” and can be retested. Strong positive FMD samples have a CT value below 20.0 (Reid et al., 2001).

- Stock solutions for real-time PCR assay
  i) Nuclease-free water and real-time PCR reaction master mixes are available from commercial suppliers.
  ii) Either of the two following primers and probe sets can be used for real-time PCR of FMDV:
5'UTR (Reid et al., 2001) Forward primer: CACYT YAAGR TGACA YTGRT ACTGG TAC; Reverse primer: CAGAT YCCRA GTGCC ICIGT TTA and TaqMan® probe: CCTCG GGGTA CCTGA AGGGC ATCC.

3D (Callahan et al., 2002) Forward primer: ACTGG GTTTT ACAAA CCTGT GA; Reverse primer: GCGAG TCCTG CCACG GA and TaqMan® probe: TCCTT TGCAC GCCGT GGGAC.

- Molecular epidemiology

The molecular epidemiology of FMD is based on the comparison of genetic differences between viruses. Dendrograms showing the genomic relationship between vaccine and field strains for all seven serotypes based on sequences derived from the 1D gene (encoding the VP1 viral protein) have been published (Knowles & Samuel, 2003; see also http://www.wrlfmd.org/). Comparison of whole genome sequences can provide further discrimination between closely related viruses and help to recreate the transmission pathways between farms within outbreaks (Cottam et al., 2008). RT-PCR amplification of FMDV RNA, followed by nucleotide sequencing, is the current preferred option for generating the sequence data to perform these comparisons. Many laboratories have developed techniques for performing these studies, and reference laboratories hold databases containing over 3000 partial sequences.

The recommended method for VP1 analysis is to:

i) Extract FMDV RNA directly from epithelial suspensions or from a low cell culture passage.
ii) Perform an RT-PCR of the complete 1D gene (or if only part of the 1D gene, then the 3' end of the gene is more useful).
iii) Determine the nucleotide sequence of the PCR product (or at least 170 nucleotides [preferably 420 for the SAT types] at the 3' end of the gene).

A protocol, complete with primer sequences, is available from the OIE Reference Laboratories on request or can be downloaded from the following World Wide Web URLs:

http://www.wrlfmd.org/

http://bvs.panaftosa.org.br/

2. Serological tests

Serological tests for FMD are performed in support of four main purposes namely: 1) to certify individual animals prior to import or export (i.e. for trade); 2) to confirm suspected cases of FMD; 3) to substantiate absence of infection; 4) to demonstrate the efficacy of vaccination. For substantiating freedom from infection, different approaches are required according to whether the population has been vaccinated or not and if vaccination has been used, whether this has been applied as an emergency application or as part of an ongoing programme of vaccination. Different tests and different interpretations of test results will be appropriate according to the above-mentioned purposes and the validation of the selected procedure must take account of the purpose. For example, test cut-offs may be set at a different threshold for herd-based serosurveillance than is appropriate for certifying freedom from infection for individual animals for the purposes of international trade.

Serological tests for FMD are of two types; those that detect antibodies to viral structural proteins (SP) and those that detect antibodies to viral nonstructural proteins (NSPs).

The SP tests are serotype-specific and detect antibodies elicited by vaccination and infection; examples are the virus neutralisation test (VNT) (Golding et al., 1976), the solid-phase competition ELISA (SPCE; Brocchi et al., 1990; Chenard et al., 2003; Mackay et al., 2001; Paiba et al., 2004) and the liquid-phase blocking ELISA (LPBE; Hamblin et al., 1986; 1987). These tests are serotype-specific and are highly sensitive, providing that the virus or antigen used in the test is closely matched to the strain circulating in the field. They are the prescribed tests for trade and are appropriate for confirming previous or ongoing infection in nonvaccinated animals as well as for monitoring the immunity conferred by vaccination in the field. The VNT requires cell culture facilities, the use of live virus and takes 2–3 days to provide results. The ELISAs are blocking- or competition-based assays that use serotype-specific polyclonal antibodies (PAbs) or MAbs, are quicker to perform and are not dependent on tissue culture systems and the use of live viruses. Low titre false-positive reactions can be expected in a small proportion of the sera in either ELISA. An approach combining screening by ELISA and confirming the positives by the VNT minimises the occurrence of false-positive results. Reference sera to standardise FMD SP serological tests for some serotypes and subtypes are available from the Reference Laboratory at Pirbright.

The detection of antibody to the NSPs of FMDV can be used to identify past or present infection with any of the seven serotypes of the virus, whether or not the animal has also been vaccinated. Therefore the tests can be
used to confirm suspected cases of FMD and to detect viral activity or to substantiate freedom from infection on a population basis. For certifying animals for trade, the tests have the advantage over SP methods that the serotype of virus does not have to be known. However, there is experimental evidence that some cattle, vaccinated and subsequently challenged with live virus and confirmed persistently infected, may not be detected in some anti-NSP tests, causing false-negative results (Brocchi et al., 2006). These assays measure antibody to NSPs using antigens produced by recombinant techniques in a variety of in-vitro expression systems. Antibody to the polyproteins 3AB or 3ABC are generally considered to be the most reliable indicators of infection (Mackay et al., 1997). In animals seropositive for antibody to 3AB or 3ABC, antibody to one or more of the other NSPs can aid in the final interpretation of the test (Bergmann et al., 2000; Mackay et al., 1997). However, lack of vaccine purity may affect diagnostic specificity as the presence of NSPs in some vaccine preparations may result in misclassification in animals that have been repeatedly vaccinated. Procedures for evaluating vaccine purity are covered in Section D of this chapter.

International standard sera for testing of cattle have been developed and are available from the OIE Reference Laboratories in Brazil and UK (Campos et al., 2008). In the future, standard sera will also be made available for sheep and pigs. Bovine serum panels have also been established to compare the sensitivity of NSP tests (Parida et al., 2007).

a) Virus neutralisation test (a prescribed test for international trade)

The quantitative VN microtest for FMD antibody is performed with IB-RS-2, BHK-21, lamb or pig kidney cells in flat-bottomed tissue-culture grade microtitre plates.

Stock virus is grown in cell monolayers and stored at –20°C after the addition of 50% glycerol. (Virus has been found to be stable under these conditions for at least 1 year.) The sera are inactivated at 56°C for 30 minutes before testing. The control standard serum is 21-day convalescent or post-vaccination serum. A suitable medium is Eagle’s complete medium/LYH (Hank’s balanced salt solution with yeast lactalbumin hydrolysate) with hepes buffer and antibiotics.

The test is an equal volume test in 50 µl amounts.

- Test procedure
  i) Starting from a 1/4 dilution, sera are diluted in a twofold, dilution series across the plate, using at least two rows of wells per serum, preferably four rows, and a volume of 50 µl.
  ii) Previously titrated virus is added; each 50 µl unit volume of virus suspension should contain about 100 TCID₅₀ (50% tissue culture infective dose) within an accepted range (e.g. 32–320 TCID₅₀).
  iii) Controls include a standard antiserum of known titre, a cell control, a medium control, and a virus titration used to calculate the actual virus titre used in the test.
  iv) Incubate at 37°C for 1 hour with the plates covered.
  v) A cell suspension at 10⁶ cells/ml is made up in medium containing 10% bovine serum (specific antibody negative) for cell growth. A volume of 50 µl of cell suspension is added to each well.
  vi) Plates are sealed with pressure-sensitive tape and incubated at 37°C for 2–3 days. Alternatively, the plates may be covered with loosely fitting lids and incubated in an atmosphere of 3–5% carbon dioxide at 37°C for 2–3 days.
  vii) Microscope readings may be feasible after 48 hours. The plates are finally fixed and stained routinely on the third day. Fixation is effected with 10% formalin/saline for 30 minutes. For staining, the plates are immersed in 0.05% methylene blue in 10% formalin for 30 minutes. An alternative fixative/stain solution is naphthalene blue black solution (0.4% [w/v] naphthalene blue black, 8% [w/v] citric acid in saline). The plates are rinsed in tap water.
  viii) Positive wells (where the virus has been neutralised and the cells remain intact) are seen to contain blue-stained cells sheets; the negative wells (where virus has not been neutralised) are empty. Titres are expressed as the final dilution of serum present in the serum/virus mixture where 50% of wells are protected (Kärber, 1931). The test is considered to be valid when the amount of virus used per well is in the range log₁₀ 1.5–2.5 TCID₅₀, and the positive standard serum is within twofold of its expected titre.
  ix) Interpretation of tests can vary between laboratories in regard to the negative/positive cut-off threshold. Laboratories should establish their own criteria by reference to standard reagents that can be obtained from the OIE Reference Laboratory at Pirbright. In general, a titre of 1/45 or more of the final serum dilution in the serum/virus mixture is regarded as positive. A titre of less than 1/16 is considered to be negative. For certification of individual animals for the purposes of international trade, titres of 1/16 to 1/32 are considered to be doubtful, and further serum samples may be requested for testing; results
are considered to be positive if the second sample has a titre of 1/16 or greater. For the purposes of herd-based serosurveillance as part of a statistically valid serological survey, a cut-off of 1/45 may be appropriate. Cut-off titres for evaluating immunological protection afforded by vaccination have to be established from experience of potency test results with the relevant vaccine and target species.

b) Solid-phase competition enzyme-linked immunosorbent assay (a prescribed test for international trade)

The method described (Paiba et al., 2004) can be used for the detection of antibodies against each of the seven serotypes of FMDV. As an alternative to guinea-pig or rabbit antisera, suitable MAb’s can be used coated to the ELISA plates as capture antibody or peroxidase-conjugated as detecting antibody (Brocchi et al., 1990). A commercial kit is available for serotype O with a different format but similar performance characteristics (Chenard et al., 2003).

Rabbit antiserum to the 146S antigen of one of the seven types of FMDV is used as the trapping antibody at a predetermined optimal concentration in carbonate/bicarbonate buffer, pH 9.6.

Antigens are prepared by inactivating viruses propagated in cell culture with ethyleneimine using the procedures described for vaccine manufacture. The final dilution chosen is that which, after addition of an equal volume of diluent, gives an absorbance on the upper part of the linear region of the titration curve (optical density approximately 1.5). PBS containing 0.05% Tween 20, 10% NBS and 5% normal rabbit serum and phenol red indicator is used as a diluent (blocking buffer).

Guinea-pig antisera, prepared by inoculating guinea-pigs with 146S antigen of one of the seven serotypes and preblocking with NBS, is used as the detecting antibody. Predetermined optimal concentrations are prepared in blocking buffer PBS containing 0.05% Tween 20, and 5% dried, nonfat skimmed milk (PBSTM).

Rabbit (or sheep) anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase and preblocked with NBS is used as conjugate at a predetermined optimum concentration in PBSTM blocking buffer.

Test sera are diluted in PBST blocking buffer.

The solid-phase competitive ELISA is more specific but as sensitive as the liquid-phase blocking ELISA (Mackay et al., 2001; Paiba et al., 2004). Methods have been described for the development of secondary and working standard sera (Goris & De Clercq, 2005a) and for charting assay performance (Goris & De Clercq, 2005b).

• Test procedure

i) ELISA plates are coated with 50 µl/well rabbit antiserum homologous to the antigen being used, diluted in carbonate/bicarbonate buffer, pH 9.6, and left overnight in a humid chamber at 4°C.

ii) The ELISA plates are washed three times with PBS.

iii) Then 50 µl of the FMDV antigen diluted in blocking buffer is added to each well of the ELISA plates. (Blocking buffer: 0.05% [w/v] Tween 20, 10% [v/v] NBS, 5% [v/v] normal rabbit serum.) The plates are covered and placed on an orbital shaker at 37°C for 1 hour, with continuous shaking.

iv) After washing three times with PBS, 40 µl of blocking buffer is added to each well, followed by 10 µl of test sera (or control sera), giving an initial serum dilution of 1/10.

v) Immediately 50 µl of guinea-pig anti-FMDV antiserum diluted in blocking buffer is added, giving a final serum dilution of 1/10.

vi) The plates are covered and incubated on an orbital shaker at 37°C for 1 hour.

vii) After washing three times with PBS, 50 µl of anti-guinea-pig Immunoglobulin conjugate (preblocked by incubation for 1 hour at room temperature with an equal volume of NBS) diluted in blocking buffer is added. The plates are covered and incubated for 1 hour at 37°C on an orbital shaker.

viii) After washing three times with PBS, 50 µl of substrate solution, containing 0.05% H₂O₂ plus orthophenylene diamine or a suitable alternative chromogen, is added to each well.

ix) The reaction is stopped after 10 minutes by the addition of 50 µl of 1 M sulphuric acid. The plates are read at 492 nm on a spectrophotometer linked to a computer.

x) Controls: On each plate two wells are used for conjugate control (no guinea-pig serum), four wells each for strong and weak positive sera, two wells for negative sera, and four wells for 0% competition (no test sera).

xi) Interpretation of the results: A percentage of inhibition is calculated for each well, either manually or using a suitable computer programme (100 – [optical density of each test or control value/mean optical density of the 0% competition] × 100%), representing the competition between the test sera and the
guinea-pig anti-FMDV antisera for the FMDV antigen on the ELISA plate. Laboratories should validate the assay in terms of the cut-off value above which sera should be considered positive in relation to (i) the particular serotypes and strains of virus under investigation (ii) the purpose of testing (iii) the population under test, using the methods described in Chapter 1.1.5 Principles and methods of validation of diagnostic assays for infectious diseases. At the OIE Reference Laboratory at Pirbright, for serotype O, for all species, for the purposes of demonstrating freedom from infection in a naïve population, greater than 60% inhibition is considered positive (Paiba et al., 2004). For maximum sensitivity, for example when certifying individual animals for international trade, an inconclusive range may be set between 40 and 60%.

c) Liquid-phase blocking enzyme-linked immunosorbent assay (a prescribed test for international trade)

Antigens are prepared from selected strains of FMDV grown on monolayers of BHK-21 cells. The unpurified supernatants are used and pretitrated in a twofold dilution series but without serum. The final dilution chosen is that which, after addition of an equal volume of diluent (see below), gives an absorbance on the upper part of the linear region of the titration curve (optical density approximately 1.5). PBS containing 0.05% Tween 20 and phenol red indicator is used as a diluent (PBST). The other reagents used in the test are the same as those in the solid-phase blocking ELISA. An example of the test procedure is described below. Temperature and incubation times can vary depending on the protocol.

- **Test procedure**
  
  i) ELISA plates are coated with 50 µl/well rabbit antiserum homologous to the antigen being used and left overnight in a humid chamber at room temperature.

  ii) The ELISA plates are washed three times with PBS.

  iii) In U-bottomed multwell plates (carrier plates) 50 µl of a duplicate, twofold series of each test serum is prepared, starting at 1/8. To each well, 50 µl of a constant dose of viral antigen that is homologous to the rabbit antiseras used to coat the plates is added and the mixtures are left overnight at 4°C, or incubated at 37°C for 1 hour. The addition of the antigen increases the final serum dilution to 1/16.

  iv) Then 50 µl of serum/antigen mixtures is transferred from the carrier plates to the rabbit-serum coated ELISA plates and the plates are incubated at 37°C for 1 hour on a rotary shaker.

  v) After washing, 50 µl of guinea-pig antiserum homologous to the viral antigen used in the previous step (iv) (preblocked with NBS and diluted in PBST containing 5% skimmed milk powder) is added to each well. The plates are then incubated at 37°C for 1 hour on a rotary shaker.

  vi) The plates are washed and 50 µl of rabbit anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase (preblocked with NBS and diluted in PBST containing 5% skimmed milk powder) is added to each well. The plates are incubated at 37°C for 1 hour on a rotary shaker.

  vii) The plates are washed again three times and 50 µl of substrate solution, containing 0.05% H₂O₂ plus orthophenylene diamine or a suitable alternative chromogen, is added to each well.

  viii) The reaction is stopped after 15 minutes by the addition of 50 µl of 1 M sulphuric acid. The plates are read at 492 nm on a spectrophotometer linked to a computer.

  ix) Controls: A minimum of four wells each of strong positive, weak positive and negative bovine reference sera at a final dilution of 1/32 should be included on each plate together with an equivalent number of reaction (antigen) control wells containing antigen in diluent alone without serum. For end-point titration tests, duplicate twofold dilution series of positive and negative homologous bovine reference sera should be included on at least one plate of every run.

  x) **Interpretation of the results:** Antibody titres are expressed as the 50% end-point titre, i.e. the dilution at which the reaction of the test sera results in an optical density equal to 50% inhibition of the median optical density of the reaction (antigen) control wells (Kärber, 1931). The median is calculated as the mean of two mid-values of the reaction control wells, eliminating from the calculation the highest and lowest values (alternatively, the mean value can be used after setting suitable tolerance limits to control for inter-well variation). In general sera with titres greater than or equal to 1/90 are considered to be positive. A titre of less than 1/40 is considered to be negative. For certification of individual animals for the purposes of international trade, titres of greater than 1/40, but less than 1/90 are considered to be doubtful, and further serum samples may be requested for testing; results are considered to be positive if the second sample has a titre of 1/40 or greater. For the purposes of herd-based serosurveillance as part of a statistically valid serological survey, a cut-off of 1/90 may be appropriate. Cut-off titres for evaluating immunological protection afforded by vaccination have to be established from experience of potency test results with the relevant vaccine and target species.
d) Nonstructural protein (NSP) antibody tests

Antibody to expressed recombinant FMDV NSPs (e.g. 3A, 3B, 2B, 2C, 3ABC) can be measured by different ELISA formats or immunoblotting. These ELISAs either use purified antigens absorbed directly to microplates or use PAbs or MAbs to trap specific antigens from semi-purified preparations (Bergmann et al., 2000; De Diego et al., 1997; Mackay et al., 1997; Sorensen et al., 1998). The index screening method used in Panaftosa is described in detail below. Other indirect and competitive ELISAs detecting bovine antibodies to 3ABC have been shown to have equivalent diagnostic performance characteristics (Brocchi et al., 2006). This same study corroborates preliminary data from Panaftosa that suggests that the diagnostic performance characteristics of these tests are similar in cattle, sheep and pigs.

- Indirect enzyme-linked immunosorbent assay
  - Preparation of recombinant antigens (see Section B.2.d Enzyme-linked immunoelectrotransfer blot assay below)
  - Test procedure
    i) Microplates are coated overnight at 4°C with 1 µg/ml of the fusion antigen 3ABC in carbonate/bicarbonate buffer, pH 9.6 (100 µl per well). Antigen 3ABC was expressed and purified as indicated for the EITB (enzyme-linked immunoelectrotransfer blot) tests (Neizert et al., 1991).
    ii) The plates are washed six times with PBS, pH 7.2, supplemented with 0.05% Tween 20 (PBST).
    iii) Test sera (100 µl per well) are added in a 1/20 dilution in blocking buffer consisting of PBS, 0.05% Tween 20, 5% nonfat dry milk, 10% equine sera and 0.1% *Escherichia coli* lysate. Each plate includes a set of strong and weak positive and negative controls calibrated against the International Standard Sera described below.
    iv) The plates are incubated for 30 minutes at 37°C and washed six times in PBST.
    v) Horseradish-peroxidase-conjugated rabbit anti-species IgG is diluted optimally in the blocking buffer, added at 100 µl per well and the plates are incubated for 30 minutes at 37°C.
    vi) After six washings, each well is filled with 100 µl of 3′3′, 5′5′-tetramethylbenzidine plus 0.004% (w/v) H₂O₂ in phosphate/citrate buffer, pH 5.5.
    vii) The reaction is stopped after 15 minutes of incubation at room temperature by adding 100 µl of 0.5 M H₂SO₄. Absorbance is read at 450 nm and at 620 nm for background correction.
  - Interpreting the results: Test results are expressed as per cent positivity relative to the strong positive control [(optical density of test or control wells/optical density of strong positive control) × 100] or alternatively as a test to control (T/C) index relative to a cut-off (i.e. threshold positive) control. Profiling the NSP antibody reactivity levels in herds along with age/vaccination stratification aids interpretation of herd infection status in vaccinated populations (Bergmann et al., 2003). Test cut-off values, with or without suspicious zones, need to be determined with consideration to the purpose of testing and the intended target population. Inconclusive results may be followed up using confirmatory tests, retesting with EITB or a second NSP ELISA (taking account of the conditional dependence of the two tests). The overall test system sensitivity and specificity must be taken into account when designing the sera surveillance programme. Although not a prescribed test for trade, NSP ELISAs may be a valuable adjunct in circumstances where the serotype or subtype of virus in the originating country is not known.

- Enzyme-linked immunoelectrotransfer blot assay (EITB)

The EITB assay has been widely applied in South America as a confirmatory test for the above-described index screening method. Further information is available from the OIE Reference Laboratory, Panaftosa, PAHO/WHO.

  - Preparation of test strips containing the recombinant antigens
    i) The five bioengineered FMDV NSPs 3A, 3B, 2C, 3D and 3ABC are expressed in *E. coli* C600 by thermo-induction. The 3D polypeptide is expressed in its complete form (McCullough et al., 1992) whereas the rest of the proteins are obtained as fusions to the N-terminal part of the MS-2 polymerase gene (Strebel et al., 1986).
    ii) The expressed polymerase is purified over phosphocellulose, followed by poly(U) Sepharose columns. The fused proteins 3A, 3B, 2C and 3ABC are purified by sequential extraction of the bacterial extracts with increasing concentrations of urea. The 7M fraction containing the fusion proteins is further purified on a preparative 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). The fusion protein band is excised from the gel and electroeluted (McCullough et al., 1992).
iii) A mixture containing 20 ng/ml of each one of the purified recombinant polypeptides is separated on 12.5% SDS-PAGE and electrophoretically transferred to nitrocellulose (McCullough et al., 1992).

- **Test procedure**
  
  i) The required amount of test strips should be assessed, taking into account that for each nitrocellulose sheet, which defines one transferred gel, a positive, a weakly positive, a cut-off and a negative control serum should be assayed. In general, 24 nitrocellulose strips, each 3 mm wide, should result from a gel.
  
  ii) A volume of 0.8 ml of saturation buffer (50 mM Tris/HCl, pH 7.5; 150 mM NaCl; 0.2% Tween 20; 5% nonfat dry milk; and 0.05% bacterial *E. coli* lysate) is added to each well. The antigen-coated strips are blocked by placing the trays on a rocker and agitating for 30 minutes at room temperature (20–22°C).
  
  iii) A dilution of 1/200 of test sera and of each of the controls is added to the appropriate trough. The strips must be completely submerged and facing upwards, and maintained in that position during the whole process.
  
  iv) Strips are incubated for 60 minutes on a rocker at room temperature.
  
  v) Liquid is removed from the trays, and each test strip is washed three times with washing solution (50 mM Tris/HCl, pH 7.5; 150 mM NaCl; and 0.2% Tween 20) by agitation for 5 minutes.
  
  vi) The alkaline-phosphatase-conjugated rabbit anti-bovine solution is added to each test well, and the strips are incubated with shaking for 60 minutes at room temperature.
  
  vii) The liquid is removed from the trays and each test strip is washed three times with washing solution as above.
  
  viii) Substrate solution (0.015% bromochloroindolyl phosphate/0.03% nitroblue tetrazolium) is prepared in substrate buffer (100 mM NaCl; 5 mM MgCl₂; and 100 mM Tris/HCl, pH 9.3), and is added to each test well.
  
  ix) Strips are incubated by placing the test tray on the orbital mixer and agitating until the cut-off control shows five distinct, discernible bands. Strips are washed with running deionised water and air-dried.
  
  x) **Interpreting the results:** The EITB may be scanned with a densitometer but visual reading, although more subjective, is considered suitable as well. Individual control sera are tested that exhibit minimal but consistent staining for each of the five antigens. A test sample is considered positive if antigens 3ABC, 3A, 3B and 3D (±2C) demonstrate staining densities equal to or higher than that of their appropriate controls. A sample is considered negative if two or more antigens demonstrate densities below their control sera. Test samples not fitting either profile are considered indeterminate.

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C. **REQUIREMENTS FOR VACCINES**

The control of FMD is usually a national responsibility and, in many countries, the vaccine may be used only under the control of the Competent Authority.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements. Varying requirements relating to quality, safety and efficacy apply in particular countries or regions in order for manufacturers to obtain an authorisation or licence for a veterinary vaccine. Where possible, manufacturers should seek to obtain such a license or authorisation for their FMD vaccines as independent verification of the quality of their product.

If virulent FMDV is used to produce FMD vaccine, the FMD vaccine production facility should operate under the appropriate biosecurity procedures and practices. The facility should meet the requirements for Containment outlined in chapter 1.1.3 of this *Terrestrial Manual*.

Routine vaccination against FMD is used in many countries or zones recognised as free from foot and mouth disease with vaccination and in countries where the disease is endemic. In contrast, a number of disease-free countries have never vaccinated their livestock but have preferred the use of strict movement controls and culling of infected and contact animals when outbreaks have occurred. Nevertheless, many disease-free countries maintain the option to vaccinate and have their own strategic reserves of highly concentrated inactivated virus preparations. Such antigen reserves offer the potential of supplying formulated vaccine in an ‘emergency’ at short notice (Doel et al., 1994). (See also Chapter 1.1.10 *International standards for vaccine banks.*)
Traditional FMD vaccines may be defined as a fixed formulation containing defined amounts (limits) of one or more chemically inactivated cell-culture-derived preparations of a seed virus strain blended with a suitable adjuvant/s and excipients. See chapter 1.1.6 for biotechnology-derived vaccines such as recombinant or peptide vaccines.

Antigen banks may be defined as stockpiles of antigen components, registered or licensed according to the finished vaccine, and which can be stored under ultra-low temperatures for a very long time for subsequent formulation into vaccine as and when required.

The vaccines are formulated for their specific purpose and in the case of vaccines destined for use in cattle, both aluminium hydroxide saponin adjuvanted and oil adjuvanted vaccines may be used. For use in swine, double oil emulsions are preferred due to their efficacy.

FMD vaccines may be classified as either ‘standard’ or ‘higher’ potency vaccines. Standard potency vaccines are formulated to contain sufficient antigen and appropriate adjuvant to ensure that they meet the minimum potency level required (recommended at Section D.4.b as 3 PD50 [50% protective dose]) for the duration of the shelf life claimed by the manufacturer. This kind of vaccine is usually suitable for use in routine vaccination campaigns. For vaccination in naïve populations to control FMD outbreaks, higher potency vaccines (e.g. > 6 PD50 for the duration of the shelf life claimed by the manufacturer) are recommended for their wider spectrum of immunity as well as their rapid onset of protection.

Conventional live FMD vaccines are not acceptable due to the danger of reversion to virulence and as their use would prevent the detection of infection in vaccinated animals.

Because of the presence of multiple serotypes of the virus it is common practice to prepare vaccines from two or more different virus serotypes. In certain areas, it may be advisable to include more than one virus strain per serotype to ensure broad antigenic coverage against prevailing viruses.

1. Seed virus management

a) Characteristics of the seed virus

Selection of master seed viruses (MSVs) should ideally be based on their ease of growth in cell culture, virus yield, stability and broad antigenic spectrum (Samuel et al., 1990). Isolates to prepare MSVs should be characterised and distributed, preferably by the OIE FMD Reference laboratories; they should be selected in accordance with the epidemiological importance of each variant.

The exact source of the isolate should be recorded and should include details such as the location, species and the type of material from which the virus was derived. Unique nomenclature should be used to identify the FMDV strain. The *in-vitro* passage history of the virus and details of the ingredients should be recorded in accordance with chapter 1.1.6.

b) Method of culture

Methods of culture shall comply with the chapter 1.1.6. Where no suitable established vaccine strain exists, new vaccine strains are derived through the establishment of MSVs from local field isolates by adapting them to growth in suspension or monolayer cells by serial passage. In order to remove the risk of contaminating lipid-enveloped viruses, it is recommended that putative MSVs undergo a validated organic solvent treatment prior to, or during, adaptation.

c) Validation as a vaccine strain

MSVs must be, well characterised and proven to be pure and free from all extraneous agents in accordance with Chapter 1.1.7 Tests for sterility and freedom from contamination of biological materials and those listed by the appropriate licensing authorities. Homology should be established with the original candidate isolates and effectiveness against the circulating strains from which they were developed should be proven. This often encompasses a number of methods, the most reliable being *in-vivo* protection assays. Alternatively, *in-vitro* tests (preferably virus neutralisation) can also be used, which require the availability of post-vaccination sera against these master seeds (see Section D of this chapter).

Seed viruses may be stored at low temperature (e.g. −70°C) or freeze-dried. Working seed viruses may be expanded in one or a few more passages from the master seed stock and used to infect the final cell culture.
Consideration should also be given to minimising the risk of transmission of transmissible spongiform encephalopathy agents (TSEs) by ensuring that TSE risk materials are not used as the source of the virus or in any of the media used in virus propagation.

d) Emergency procedures for provisional acceptance of new MSV, and subsequent release of formulated vaccines

In the case of incursion in a region of a new strain that is antigenically distinct from existing vaccine strains, it may be necessary to develop a new vaccine strain from a representative field isolate. Before the new MSV can be accepted, full compliance should be demonstrated with the relevant guidelines to demonstrate freedom from all extraneous agents listed by the appropriate licensing authorities using both general and specific tests, and to establish homology to the original candidate isolates. The time taken to raise the specific antisera necessary to neutralise the new strain for use in the general tests for detection of extraneous agents and to conduct other specific tests that require specialised techniques may be lengthy. Therefore, in emergency situations where there is insufficient time to complete full testing of the MSV, provisional acceptance of the new strain should be based on a risk analysis of the possibility of contamination of the antigen produced from the new MSV with extraneous agents. This risk assessment should take into account that a validated procedure to inactivate enveloped viruses must be used when establishing the MSV and that the virus is inactivated using a chemical inactivant with first order kinetics. Further assurance is provided by the requirement for the kinetics of inactivation to be monitored and recorded for each production batch.

2. Method of manufacture

The recommended method of virus propagation for antigen production is the growth of FMDV in large-scale suspension cultures or monolayers using cell lines under sterile conditions.

Cattle tongue epithelium in surviving conditions in medium with salts but without products of biological origin, may be acceptable for vaccine production but only if the method of production is entirely compliant with the standard requirements referred to in chapter 1.1.6. In addition, in order to remove the risk of contaminating lipid-enveloped viruses, the harvested virus suspension must undergo a validated organic solvent treatment prior to BEI/EI inactivation. A validated procedure is applied to ensure inactivation of all possible extraneous agents and each batch is independently tested in an official laboratory for absence of extraneous agents. Adequate in-process and final products tests are in place to ensure consistency and safety of the final product. Consideration should also be given to minimising the risk of transmission of transmissible spongiform encephalopathy agents (i.e. BSE) by ensuring safe sourcing of the epithelium.

A suitable strain of the virus is used to infect a suspension or monolayers of an established cell line, such as BHK. Such cell cultures should be proven to be free from contaminating microorganisms.

It is common practice to keep stocks of BHK cells over liquid nitrogen and revive as necessary. On revival, they are expanded in nutrient medium to a volume and cell density appropriate to seeding the main culture.

When the virus is expected to have reached its maximum yield, the culture is clarified, often by chloroform treatment followed by centrifugation and/or filtration. The virus is subsequently inactivated by addition of an inactivant of first order, usually ethyleneimine (EI) in the form of binary ethyleneimine (BEI) (Bahnemann, 1975; 1990). It is important that the necessary safety precautions for working with BEI/EI are fully observed.

The BEI is added to a virus suspension, to give a predetermined final concentration. Inactivation must be duly validated and documented to show the inactivation kinetic and the results of the inactivation controls. The time period for BEI treatment and temperature used for inactivation must be validated for the actual conditions and equipment used.

To decrease the likelihood of live virus failing to contact the inactivant, e.g. EI/BEI, it is essential to transfer the vessel contents immediately to a second sterile vessel where inactivation is allowed to go to completion according to the validated inactivation kinetic and taking into account possible regulatory requirements for additional waiting times.

During inactivation, the virus titre is monitored by a sensitive and reproducible technique. The inactivation procedure is not satisfactory unless the decrease in virus titre, plotted logarithmically, is linear and extrapolation indicates that there is less than 1 infectious virus unit per $10^4$ litres of liquid preparation at the end of inactivation.

After inactivation any residual EI/BEI in the harvest can be removed, or neutralised, for example by adding excess sodium thiosulphate solution to a final concentration of 2%.
The inactivated virus may be concentrated/purified by procedures such as ultrafiltration, polyethylene glycol precipitation or polyethylene oxide adsorption (Adamowicz et al., 1974; Wagner et al., 1970). Concentrated inactivated virus may be purified further by procedures such as chromatography. These concentrated and purified antigens can be formulated into vaccines or stored at low temperatures for many years, and made into vaccine when required by dilution in a suitable buffer and addition of adjuvants (Doel & Pullen, 1990).

Conventional FMD vaccines are usually formulated as oil adjuvanted or aqueous. Oil-adjuvanted vaccines are usually formulated as water-in-oil emulsion using mineral oils, such as Marcol and Drakeol. The mineral oil is usually premixed with an emulsifying agent before the addition of a proportion, or all, of the aqueous phase of the vaccine, and emulsified by use of a colloid mill or continuous mechanical or flow ultrasonic emulsifier.

More complex double emulsions (water/oil/water) may be produced by emulsifying once more in an aqueous phase containing a small amount of detergent such as Tween 80 (Barnett et al., 1996, Doel et al., 1994; Herbert, 1965).

The aqueous vaccine is prepared by adsorbing the virus on to aluminium hydroxide gel, one of the adjuvant constituents of the final vaccine blend.

The final blend of the vaccine may include other components, such as antifoam, phenol red dye, lactalbumin hydrolysate, tryptose phosphate broth, amino acids, vitamins, buffer salts and other substances. An adjuvant, such as saponins, may also be incorporated, as well as preservatives.

Preservatives may be used as long as their usefulness as a preservative and absence of interference with FMDV antigen has been properly.

When using novel components, including adjuvants or preservatives, in any vaccine it is important to take into account that its status with regard to residues in products derived from food-producing species must be assessed to ensure that adequate assurance can be giving to licensing authorities in relation to safety for consumers. This requirement limits considerably the choice of adjuvants and preservatives for use in food-producing species.

3. In-process control

In general, virus titres reach optimum levels within about 24 hours of the cell culture being infected. The time chosen to harvest the culture may be based on a number of assays; for instance cell death. Virus concentration may be assessed by an infectivity test, sucrose density gradient (Bartelling & Meloen, 1974; Fayet et al., 1971) or serological techniques. It is preferable to use a method for measuring antigenic mass, such as sucrose density gradient analysis, as well as one that measures infectivity, as the two properties do not necessarily coincide and the different methods may complement one another.

a) Inactivation kinetics

During inactivation of the virus, timed samples should be taken at regular intervals for the purpose of monitoring the rate and linearity of the inactivation process. Virus titres in the samples are determined by inoculation of cell cultures proven to be highly susceptible to FMDV, e.g. BHK. Such cultures permit the testing of statistically meaningful samples under reproducible conditions. The log10 infectivities of the timed samples are plotted against time, and the inactivation procedure is not considered to be satisfactory unless at least the latter part of the slope of the line is linear and extrapolation indicates that there would be less than one infectious particle per 10^4 litres of liquid preparation at the end of the inactivation period.

b) Inactivation control

The test for innocuity is an in-process test that should be carried out for every batch of antigen. Cells used to test for absence of residual live virus are not suitable if use of an amount of virus corresponding to 1 µg of 146S antigen gives a titre of less than 10^6 TCID50 (European Pharmacopoeia, 2008). Following inactivation, a sample of each batch of inactivated antigen representing at least 200 doses should be tested for freedom from infectious virus by inoculation of sensitive monolayer cell cultures, preferably of the same origin as those used for the production of antigen. It may be preferable to concentrate the antigen to do this, in which case it must be shown that the concentrated material does not interfere with the sensitivity or reading of the assay. The cell sheets are examined daily over a period of 2–3 days, after which the spent medium is transferred to fresh monolayers and the original monolayers are replenished with fresh medium. Using this method, traces of live virus can be amplified by the passage procedure and detected on the basis of CPE observed. Two to three passages of the original virus preparation are commonly used. A variant on this method is to freeze–thaw the old monolayers to release intracellular virus, which can be detected by further passage.
4. Final product batch tests

a) Sterility

The bulk inactivated antigen, concentrated antigen and the final formulated product should undergo sterility testing. The preferred method is to collect any contaminating microorganisms by membrane filtration of the material to be examined and to detect any organisms present by incubation of the membranes with culture media. This procedure allows the removal of preservatives, etc., which may inhibit the detection of microorganisms. Guidelines on techniques and culture media, which allow the detection of a wide range of organisms, are described in the European Pharmacopoeia (2008) (see also chapter 1.1.7).

b) Identity testing

The bulk inactivated antigen, concentrated antigen and the final formulated product should undergo identity testing to demonstrate that the relevant strains are present. No other FMD virus serotype registered on the manufacturing site should be present in the vaccine, to be assured by adequate tests.

c) Viral nonstructural protein testing

Nonstructural proteins refer to proteins not present in the FMD viral capsid. Only products claiming to be purified from NSPs have to demonstrate their level of purification. Unless consistency of purification is demonstrated and approved in the registration dossier, and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.6, NSP lack of reactivity has to be demonstrated in the final product (see Section C.5. Requirements for registration of vaccine).

Confirmation of vaccine purity may be shown by testing sera from animals vaccinated at least twice with the batch for absence of antibodies to nonstructural proteins.

d) Safety

Unless consistent safety of the product is demonstrated and approved in the registration dossier and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.6, batch safety testing is to be performed.

This final product batch safety test is conducted to detect any abnormal local or systemic adverse reactions, For the purposes of batch release, each of at least two healthy sero-negative target animals is inoculated by the recommended route of administration with the recommended dose of vaccine. The animals are observed for local and systemic reactions to vaccination for no fewer than 14 days. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the batch. If the potency test is performed in the target species, observation of the safety during this test can also be considered as an alternative to the batch safety test described here.

e) Potency

Potency is examined on the final formulated product, or alternatively for antigen banks on a representative batch of vaccine prepared from the same bulk inactivated antigen.

The potency testing standard is the vaccination challenge test. However, for batch release indirect tests can also be used for practicability and animal welfare considerations, as long as correlation has been validated to percentage of protection in the target animal. Frequently indirect potency tests include antibody titration after vaccination of target species. Alternative methods could be used if suitably validated.

Ideally, indirect tests are carried out for each strain for one species and each formulation of vaccine to establish correlation between the indirect test results and the vaccine efficacy.

i) Expected percentage of protection (EPP) (Maradei et al., 2008; Periolo et al., 1993)

The EPP estimates the likelihood that cattle would be protected against a challenge of 10,000 bovine infective doses after a single vaccination.

- Individual sera collected 30–60 days post-vaccination using a full dose of the vaccine are required from a group of either 16 or 30 18–24 month-old cattle.
- This panel of sera and sera of two control cattle are tested for antibody titres to the homologous FMD vaccine strain in a strongly correlated LPB-ELISA (see Sections B.2.a and B.2.c).
- The antigens used in the ELISA may be inactivated using BEI.
The EPP is determined by reference to predetermined tables of correlation between serological titres and clinical protection (Maradei et al., 2008; Periolo et al., 1993).

Batches with at least 75% EPP (with 16 vaccinated cattle) or at least 70% EPP (with 30 vaccinated cattle) are satisfactory for potency.

The presence of more than one serotype in a vaccine does not diminish the induction of antibodies against another serotype or the correlation of antibody titre with protection.

Other tests were published using different ELISA methods and VNT methods to indirectly evaluate the protection given by vaccines. Their results could be accepted only if a strong correlation with protection in relation to the vaccine strain being tested and the serological method being used has been scientifically demonstrated and published in a peer-reviewed journal (Ahl et al., 1990).

5. Requirements for registration of vaccine

a) Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.1–4) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

b) Safety

For the purposes of gaining regulatory approval, a trial batch of vaccine should be tested for local and systemic toxicity by each recommended route of administration in an in-vivo test in eight animals of each target species (European Pharmacopoeia, 2008). Single dose and repeat dose tests using vaccines formulated to contain the maximum permitted payload and number of antigens should be conducted. The repeat dose test should correspond to the primary vaccination schedule (e.g. two injections) plus the first revaccination (i.e. a total of three injections). The animals are observed for local and systemic reactions to vaccination for no fewer than 14 days after each injection. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the vaccine.

c) Efficacy

Vaccine efficacy is estimated in vaccinated animals directly, by evaluating their resistance to live virus challenge. The uncertainty of measurement in this test should be taken into account when interpreting its significance (Goris et al., 2007; 2008). Vaccine efficacy should be established for every strain to be authorised for use in the vaccine.

Live reference FMD viruses corresponding to the main vaccine virus strains used in the region are available under certain conditions from OIE Reference Laboratories for FMD in the region. These reference viruses are stored at ultralow temperatures; they are already titrated for immediate use in challenge tests in cattle and are sent, in strict accordance with shipping regulations, with instructions for use at a predetermined dilution in PD50 and PGP challenge tests, described hereafter.

Each challenge virus is prepared at an OIE Reference Laboratory for FMD as follows: tongue tissue infected with FMDV should be obtained from the original FMD field case, which has been sent to the Reference Laboratory in glycerol buffer as described in Section B of this chapter.

The preparation of cattle challenge virus follows the process described in Section B.1.a Virus isolation, with the aim of obtaining a sterile 10% suspension in Eagle’s minimal essential medium with 10% sterile fetal bovine serum.

The stock of challenge virus to be aliquoted is prepared from lesions collected from two cattle above 6 months of age that have been recognised as free from FMDV antibodies. These animals are tranquillised, for example using Xylazine 100 mg/ml (following the instructions for use), then inoculated intradermally in the tongue with the suspension in about 20 sites of 0.1 ml each. The vesiculated tongue tissue is harvested at the peak of the lesions, approximately 2 days later.

A 2% suspension is prepared as above, filtered through a 0.2 µm filter, aliquoted and frozen in the gas phase of liquid nitrogen; this constitutes the challenge virus stock. The infective titres of this stock are determined both in cell culture (TCID50) and in two cattle (BID50). Two tranquillised cattle are injected intradermally in the tongue with tenfold dilutions (1/10 through 1/10,000), using four sites per dilution
Vaccine manufacturers may wish to exploit this potential by including a claim that their vaccines do not induce antibody to one or more NSPs and can be used in conjunction with an appropriate diagnostic test. In a majority of NSPs so that they induce little, if any, NSP-specific antibody. Under these circumstances, detection of NSP antibodies can provide evidence that vaccinated animals have been exposed to FMDV. Consequently, FMD antigens used to formulate vaccines that may be used in these circumstances should be purified to reduce the NSP content. With current manufacturing techniques it is possible to exclude the majority of NSPs so that they induce little, if any, NSP-specific antibody. Under these circumstances, detection of NSP antibodies can provide evidence that vaccinated animals have been exposed to FMDV.

**PD₅₀ test:**

The number of protective doses in a vaccine is estimated from the resistance to live virus challenge of animal groups receiving different amounts of vaccine. Cattle of at least 6 months of age, obtained from areas free from FMD that have not previously been vaccinated against FMD and are free from antibodies to FMDV should be used. Three groups of no fewer than five cattle per group should be vaccinated by the route recommended by the manufacturer. The vaccine should be administered at different doses per group by injecting different volumes of the vaccine. For example, if the label states that the injection of 2 ml corresponds to the administration of 1 dose of vaccine, a 1/4 dose of vaccine would be obtained by injecting 0.5 ml, and a 1/10 dose would be obtained by injecting 0.2 ml. These animals and a control group of two nonvaccinated animals are challenged either 3 weeks (aqueous) or up to 4 weeks (oil) after vaccination with a suspension of bovine virus that is fully virulent and appropriate to the virus types in the vaccine under test by inoculating the equivalent of a total of 10,000 BID₅₀ (50% bovine infectious dose) intradermally into two sites on the upper surface of the tongue (0.1 ml per site). Animals are observed for at least 8 days. Unprotected animals show lesions at sites other than the tongue. Control animals must develop lesions on at least three feet. From the number of animals protected in each group, the PD₅₀ content of the vaccine is calculated. There are a variety of methods for calculating PD₅₀ (FAO, 1997), but procedures based on the Kärber (1931) method are generally preferred when interpreting PD₅₀ estimates calculated in this way. The vaccine should contain at least 3 PD₅₀ per dose for cattle.

**PGP test (protection against generalised foot infection)**

For this method, a group of 16 FMD-seronegative cattle of at least 6 months of age, with the same characteristics described for the PD₅₀ test, are vaccinated with a bovine dose by the route and in the volume recommended by the manufacturer. These animals and a control group of two nonvaccinated animals are challenged 4 weeks or more after vaccination with the challenge strain, which is a suspension of bovine virus that is fully virulent and appropriate to the virus types in the vaccine under test by inoculating a total of 10,000 BID₅₀ intradermally into at least two sites on the upper surface of the tongue. Unprotected animals show lesions on the feet within 7 days after inoculation. Control animals must develop lesions on at least three feet; for routine prophylactic use, the vaccine should protect at least 12 animals out of 16 vaccinated. Animals are observed at 7–8 days after challenge (Viana Filho et al., 1993). This test does not provide an estimate of how many protective doses are in a single vaccine dose but gives a certain measure of the protection following the injection of single commercial bovine doses of vaccine in a limited cattle population (Maradei et al., 2008; Periolo et al., 1993).

**Efficacy in other species**

Efficacy tests in other target species, such as sheep, goats, pigs or buffalo are either different or not yet standardised. In general, a successful test in cattle is considered to be sufficient evidence of the quality of a vaccine to endorse its use in other species. Under circumstances where a vaccine is produced for use primarily in a species other than cattle, it may be more appropriate to potency test the vaccine in that same species. With respect to sheep, goats and African (Syncerus caffer) or Asiatic buffalo (Bubalus bubalis), due to the often inapparent nature of the disease in these species, potency results from a cattle test may be a more reliable indicator of vaccine quality than attempting a potency test reliant on the detection of clinical signs in these other species.

d) **Purity: testing for antibody against nonstructural proteins**

The OIE Terrestrial Animal Health Code stipulates that a criterion for regaining FMD free status following an outbreak, if vaccine is used, is to test the vaccinated animals for antibody against NSP. Likewise, countries wishing to be recognised as FMD free with vaccination must demonstrate the absence of virus circulation by showing that vaccinated animals are free from antibody to NSPs arising as a result of infection. Consequently, FMD antigens used to formulate vaccines that may be used in these circumstances should be purified to reduce the NSP content. With current manufacturing techniques it is possible to exclude the majority of NSPs so that they induce little, if any, NSP-specific antibody. Under these circumstances, detection of NSP antibodies can provide evidence that vaccinated animals have been exposed to FMDV. Vaccine manufacturers may wish to exploit this potential by including a claim that their vaccines do not induce antibody to one or more NSPs and can be used in conjunction with an appropriate diagnostic test. In addition to providing supporting documentation on the processes involved in such purification, manufacturers should demonstrate lack of immunogenicity against NSPs as part of the licensing procedure in order to make such a claim on their product literature. A recommended test method that can be used is to vaccinate not less than 8 naïve cattle with a trial blend of the vaccine containing the maximum number of
strains and amounts of antigen permitted on the authorisation. Cattle should be vaccinated at least three times at 21- to 30-day intervals and then tested before each revaccination and 30–60 days after the last vaccination for the presence of antibody to NSPs using the tests described in Section B.2.d of this chapter. Negative results in NSP assays may support claims that the vaccine does not induce antibody to NSPs for the number of injections tested. These cattle may be the same as those used for the safety test described in Section C.5.b of this chapter.

e) Duration of immunity

The duration of immunity (D.O.I) of an FMD vaccine will depend on the efficacy (formulation and antigen payload). As part of the authorisation procedure the manufacturer should be required to demonstrate the D.O.I. of a given vaccine by either challenge or the use of a validated alternative test, such as serology at the end of the claimed period of protection, in compliance with Section 5.c. D.O.I. studies should be conducted in each species for which the vaccine is indicated or the manufacturer should indicate that the D.O.I. for that species is not known. Likewise, the manufacturer should demonstrate the effectiveness of the recommended booster regime in line with these guidelines, usually by measuring the magnitude and kinetics of the serological response observed.

In endemic or outbreak situations, vaccine is usually given as a primary course consisting of one or two doses of vaccine 3–4 weeks apart (based on animal population immunological status, vaccine potency, virus-vaccine matching, virus challenge levels, and other factors), followed by revaccination every 6–12 months. The frequency of revaccination will depend on the epidemiological situation and the type and quality of vaccine used.

For target animals born to vaccinated dams, vaccination should be delayed to allow decline of maternally derived antibodies. Primary vaccination of offspring to nonvaccinated dams can occur as early as 1 week of age (Auge De Mello et al., 1989).

Information should be provided by manufacturers to indicate the appropriate vaccination programme(s) to minimise interference with maternally derived antibodies in target species.

f) Stability

The stability of all vaccines, including oil emulsion vaccines, should be demonstrated as part of the shelf-life determination studies for authorisation.

The shelf life of conventional FMD vaccines is usually 1–2 years at 2–8°C. Vaccines should never be frozen or stored above the target temperature.

g) Precautions (hazards)

Current FMD vaccines are innocuous and present no toxic hazard to the vaccinators. Manufacturers should provide adequate warnings that medical advice should be sought in case of self-injection of an oil-emulsion vaccine.

6. Storage and monitoring of concentrated antigens

Chapter 1.1.10 provides international standards for vaccine banks.

The process of storing concentrated antigens at ultra-low temperature for later formulation into FMD vaccine as described in Section C.2, is a well-established procedure for building stocks of immunogenic material ready to be formulated into vaccines in case of need. It not only forms the basis for the storage of antigens in a strategic reserve for emergency purposes, but allows the manufacturer immediate access to many different antigen strains that can be rapidly formulated and dispatched to the customer (Lombard & Fussel, 2007). Such stockpiling minimises delays subsequent to an order, particularly where a multivalent vaccine is requested. Another advantage of this procedure is that much of the quality testing can be completed well in advance of shipment. It is necessary to state that the concentrated antigens have to be controlled using standards indicated in Sections C.1–4.

a) Storage conditions

- Facilities

It is important that all aspects of the storage of concentrated antigens conform fully to internationally accepted requirements such as those referred to in chapter 1.1.6. Housing, facilities and procedures should ensure the security of the stored antigen and prevent tampering, contamination or damage.
• Containment of stored antigens

The dose numbers or volumes stored are an important consideration, particularly where a reserve is shared between OIE Members and there is variation in number of doses perceived to be needed by each Member in an emergency. Where the requirement is for a large stockpile of a particular vaccine strain that can only be produced from several separate production runs, vaccine bank managers must consider the need to either formulate each lot into a representative final blend for testing purposes or mixing the individual batches, at some convenient point, for ease of formulating and/or testing.

The type of container used to hold antigen concentrate is important. Under ultra-low temperature conditions it is important to use containers made from materials that do not become brittle or fragile at a temperature range allowing for heat sterilisation and cold storage.

• Labelling of stored antigens

The concentrated antigens do not need to be labelled according to final or finished vaccine requirements and may be labelled as “in process” materials. Under ultra-low temperature conditions, the method of labelling must be of a durable nature. From experience, wire tagging bottles is the most preferred option using a metal/plastic tag sizeable enough to allow the necessary detail. Such detail should include the antigen/vaccine strain, batch number, date received and should also include an individual container or stock number. This information should be clear to read and marked on the tag using an indelible marker pen. Storage records and positions of containers should be carefully maintained.

b) Monitoring of stored concentrated antigens

It is vitally important that antigen concentrates are optimally maintained and routinely monitored in order to have some assurance that they will be efficacious when needed. Therefore arrangements should be made to monitor these antigen concentrates on a routine basis and to include where necessary, and at appropriate time intervals, a testing regime to ensure integrity of the antigen component or acceptable potency of the final product.

146S quantification, vaccination serology or vaccination challenge studies can be used for monitoring FMD antigen banks (Barnett & Statham, 1998; Bartelling & Meloen, 1974; Doel & Pullen, 1990; Fayet et al., 1971). It is recommended to carry out these tests on receipt (year 0) and every 5 years thereafter.

To support these testing requirements for depositories of antigen, concentrates should include a number of small samples that are representative of the larger stock. Small aliquots stocks of FMD antigen have usually consisted of a volume representing approximately one milligram of antigen. These aliquots should be stored side by side with the bulk antigen.

7. Emergency release of vaccines prepared from concentrated antigens

In situations of extreme urgency and subject to agreement by the competent authority, a batch of vaccine may be released before completion of the tests and the determination of potency if a test for sterility has been carried out on the bulk inactivated antigen and all other components of the vaccine and if the test for safety and the determination of potency have been carried out on a representative batch of vaccine prepared from the same bulk inactivated antigen. In this context, a batch is not considered to be representative unless it has been prepared with not more than the amount of antigen or antigens and with the same formulation as the batch to be released (European Pharmacopoeia, 2008).

D. VACCINE MATCHING TESTS

1. Introduction

Appropriate vaccine strain selection is an important element in the control of FMD and is necessary for the application of vaccination programmes in FMD-affected regions as well as for the establishment and maintenance of vaccine antigen reserves to be used in the event of new FMD incursions.

Vaccination against one serotype of FMDV does not cross-protect against other serotypes and may also fail to protect fully or at all against other strains of the same serotype. The most direct and reliable method to measure cross-protection is to vaccinate relevant target species and then to challenge them by exposure to the virus isolate against which protection is required. This will take account of both potency and cross-reactivity.
However, such an approach requires the use of live FMDV and appropriate biosecurity procedures and practices must be used. The facility should meet the requirements for Containment Group 4 pathogens as outlined in chapter 1.1.3. In addition to the safety concerns, this procedure is slow and expensive and requires specific expertise that is best available in OIE Reference laboratories. The use of animals for such studies should be avoided wherever possible by the use of in vitro alternatives.

A variety of in vitro serological methods can be used to quantify antigenic differences between FMDV strains and thereby estimate the likely cross-protection between a vaccine strain and a field isolate. Genetic characterisation and antigenic profiling can also reveal the emergence of new strains for which vaccine matching may be required and, conversely, may indicate that an isolate is similar to one for which vaccine matching information is already available. Such tests should be carried out in laboratories that work according to the standard specified in Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities and Chapter 1.1.4 Quality management in veterinary testing laboratories, preferably OIE Reference Laboratories for FMD.

Shipping of samples should be in accordance with chapter 1.1.3 Sections H and I and chapter 1.1.1.

Vaccine potency also contributes to the range of antigenic cover provided by a vaccine. A highly potent vaccine that stimulates a strong immune response may give greater protection against a heterologous virus than an equally cross-reactive vaccine that stimulates a weaker immune response (Brehm et al., 2008). Furthermore, booster doses of vaccine can increase efficacy and the subsequent breadth of antigenic cover provided by a given vaccine, although the onset of full protection may be delayed (Pay, 1984).

2. Selection of field viruses for vaccine matching

For vaccine matching, preferably, more than one representative isolate should be evaluated from any outbreak.

Viruses should be selected based on epidemiological information, for instance isolation at different stages of an outbreak, from different geographical locations, or from different hosts (Alonso et al., 1987). Field evidence for a suspected lack of vaccine efficacy, as shown by reduced apparent protection, is an important criterion for vaccine matching.

The serotype of the field isolate is usually determined by ELISA or CFT using type-specific serological reagents, although methods based on MAbs or genetic typing may also be used. If the number of viruses exceeds the capacity of the laboratory to carry out methods described in Section D.4 Vaccine matching tests, a pre-selection of isolates should be done.

In order to minimise the risk of missing a relevant sample, the pre-selection should be carried out using all the isolates received by the laboratory. The recommended approach is to engage in serological validated antigenic profiling methods using ELISA. The performance of VP1 sequencing could be used to verify the homogeneity of the virus isolate population.

Only isolates showing important differences with vaccine strains are selected for vaccine matching tests.

3. Selection of vaccine strains to be matched

The serotype of the virus, the region of origin and any information on the characteristics of the field isolate and, as appropriate, the vaccine strain used in the region of origin, may give indications as to the vaccine strains to be selected for vaccine matching tests. The availability of reagents for matching to particular vaccine strains may limit the extent of testing that is possible. Antigenic characterisation has two purposes; first, to choose the most effective vaccine strain for use in a particular circumstance and, second, to monitor, on an ongoing basis, the suitability of vaccine strains maintained in strategic antigen reserves.

4. Vaccine matching test

The serological relationship between a field isolate and a vaccine virus (‘r’ value) can be determined by VNT or ELISA (Kitching et al., 1988; Mattrion et al., 2009; Pereira, 1977). One way testing is recommended (r₁) with a vaccine antiserum, rather than two way testing (r₂), which also requires an antiserum against the field isolate to be matched. VNT using chequer-board titration method will give more accuracy to the results obtained. In-vitro neutralisation may be more relevant to predict in-vivo protection by the vaccine than other measures of virus-antibody interaction.

VNT is the method of choice (Mattrion et al., 2009) compared with the ELISA, which can be used only as a screening method for vaccine matching.
For either VNT or ELISA, post-vaccination sera should be derived from at least five cattle 21–30 days after inoculation. The titre of antibody to the vaccine strain is established for each serum. Sera are used individually or pooled, after excluding low responders.

a) Relationship between the field isolate and the vaccine strain

The recommended standard test is the VNT. The ELISA can be used as a screening method.

i) Vaccine matching by two-dimensional (chequerboard) neutralisation test

This test uses antiserum raised against a vaccine strain. The titres of this serum against 100 TCID₅₀ of the homologous vaccine strain and the same dose of a field isolate are compared to determine how antigenically ‘similar’ the field virus is to the vaccine strain.

Test procedure

The procedure is similar to that of the VNT (see Section B.2.a).

Additional biological reagents are: monovalent 21–30 day post-vaccination bovine sera (inactivated at 56°C for 45–60 minutes), the homologous vaccine strain; and the test virus, a field isolate of the same serotype as the vaccine strain.

a) Field isolates are passaged on cell cultures until adapted to give 100% CPE in 24 hours. Passages should be kept to a minimum. When adapted, determine the virus titre \( \log_{10} \text{TCID}_5 \text{/ml} \) by end-point titration.

b) For each test and vaccine virus a chequerboard titration is performed of virus against vaccine serum along with a back-titration of virus. Cells are added and incubated at 37°C for 48–72 hours after which time CPE is assessed.

c) Antibody titres of the vaccine serum against the vaccine strain and field isolate for each virus dose used are calculated using the Spearman–Kärber method. The titre of the vaccine serum against 100 TCID₅₀ of each virus can then be estimated by regression. The relationship between the field isolate and the vaccine strain is then expressed as an ‘r’ value as:

\[
r = \frac{\text{reciprocal arithmetic titre of reference serum against field virus}}{\text{reciprocal arithmetic titre of reference serum against vaccine virus}}
\]

Interpretation

Interpretation of the results: It is generally accepted that in the case of neutralisation, \( r \) values greater than 0.3 indicate that the field isolate is sufficiently similar to the vaccine strain that use of a vaccine based on this strain is likely to confer protection against challenge with the field isolate (Rweyemamu, 1984).

Conversely, values less than 0.3 suggest that the field isolate is sufficiently different from the vaccine strains tested that a vaccine based on these strains is less likely to protect. In this case, either the field isolate should be examined against other vaccine strains or the field isolate could be tested against existing vaccines in a heterologous cross protection challenge test. Alternatively, a suitable field isolate could be adapted to become a new vaccine strain.

Tests should always be repeated more than once. The confidence with which ‘r’ values can be taken to indicate differences between strains is related to the number of times that the examination is repeated (Rweyemamu & Hingley, 1984). In practice, a minimum of at least three repetitions is advised.

ii) Vaccine matching by ELISA

The use of the Liquid-phase blocking ELISA for vaccine matching has been reported (Ferris & Donaldson, 1992).

b) Testing the fitness for purpose of a vaccine

Only when the r-value suggests an insufficient match of a certain vaccine strain, the suitability of a vaccine based on such a vaccine strain could be demonstrated by a heterologous cross-protection challenge test carried out as described in Section C.4.b. in animals vaccinated with a known vaccine and challenged with the (heterologous) field virus. If the r value is under 0.3, the following differences in the previously described test are recommended respecting the instructions for vaccination. Vaccinate at least seven cattle without FMD antibodies, with a commercial dose of the current vaccine to be used in the region. Between 28 and 30 days later, boost all these animals with a second commercial dose in the same conditions and vaccinate a second group of at least seven animals with the same vaccine dosage and same route. Challenge the two groups and two control animals (not vaccinated) 30 days later with the equivalent of a
total of 10,000 BID 50% (50% bovine infective dose) of the new field strain duly titrated. The results are valid if each of the two control animal shows podal lesions on at least three feet. Final results are reported either as the number of protected animals (without podal lesion) over the total number of animal per group, or by percentage of protection where 100% is the total number of animals used per group. If results in the group of once vaccinated cattle indicate a protection level under 75%, and in the group of twice vaccinated cattle, protection under 100%, the change for a more appropriate vaccine strain is recommended (Henderson, 1949).

The use of the Expected Percentage of Protection (EPP) method (Alonso et al., 1987) is not recommended under heterologous conditions. This method measures the reactivity of a panel of post-vaccination antisera using either VNT or ELISA and relates the serological titres to the probability of protection, established through correlation tables associating antibody titres with protection against the homologous vaccine strain. Consequently the correlation from the panels of antisera and accompanying challenge tests cannot be extrapolated to any other strain (Robiolo et al., 2010).

REFERENCES


Control of Veterinary Vaccines for use in Developing Countries, Mowat N. & Rweyemamu M., eds. FAO, Rome, Italy, 395–409.


Chapter 2.1.5. – Foot and mouth disease


Chapter 2.1.5. – Foot and mouth disease


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NB: There are OIE Reference Laboratories for foot and mouth disease (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the OIE Reference Laboratories for any further information on FMD diagnostic tests, reagents and vaccines.
CHAPTER 2.1.6.

HEARTWATER

SUMMARY

Heartwater (also known as cowdriosis) is an acute, fatal, non-contagious, infectious, tick-borne rickettsial disease of ruminants caused by Ehrlichia ruminantium (formerly Cowdria ruminantium) and transmitted by Amblyomma ticks. It occurs in nearly all sub-Saharan countries of Africa, in its neighbouring islands, and also in the Caribbean, threatening the American mainland. The disease can cause high mortality (up to 90%) in susceptible domestic ruminants. Goats and sheep are more susceptible than cattle, and European breeds are generally more susceptible than indigenous African breeds.

Clinically, the disease most commonly presents as an acute form characterised by a sudden high fever, depressed demeanour, nervous signs and a high mortality. Hydropericardium and hydrothorax and lung oedema are commonly associated post-mortem signs. Acute and peracute clinical forms of the disease occur: In the former, there are high death rates without many clinical manifestations and in the latter, there is a higher recovery rate.

Recovered animals become carriers of infection. Certain wild animals can play a role as reservoir; Rusa deer, white tailed deer, and springbok are susceptible to this infection and can experience high mortality.

Identification of the agent: The specific diagnosis of heartwater is based on the observation of colonies of E. ruminantium in capillary endothelial cells of the brain. In the absence of adequate tools, a piece of cerebellum can easily be removed with a curette through the foramen magnum after cutting off the head, while a sample of cerebral cortex can be obtained through a hole made in the skull with a hammer and a large nail. Brain smears are prepared by crushing to a paste and spreading thinly a small piece of cerebral or cerebellar cortex between two microscope slides. The capillaries are spread out in a single cell layer by drawing one slide across the other. The smears are air-dried, fixed with methanol and stained with Giemsa. With fast stains, the smears can be fixed and stained in less than 1 minute. The colonies (clusters) are reddish-purple to blue, and very often close to the nucleus of the infected endothelial cell. They can be scanty and difficult to find, particularly in peracute cases, but they are always present in the brain of a ruminant that died from heartwater, if not treated with drugs. Colonies are not likely to be detected in animals that were treated with antibiotics. The colonies are still visible 2 days after death in a brain that has been stored at room temperature (20–25°C) and up to 34 days in a brain that has been stored in a refrigerator at 4°C.

Fresh whole blood collected from suspect animals can be inoculated intravenously into a susceptible sheep or goat. The development of clinical signs and the demonstration of E. ruminantium in the brain of the inoculated animal during the febrile reaction are diagnostic for heartwater. Due to animal welfare concerns, this method should be avoided.

Ehrlichia ruminantium can be isolated from the blood of an infected host using cultivation on ruminant endothelial cells. When a cytopathic effect consisting of plaques of cell lysis appears, the presence of characteristic morulae is confirmed by staining the cell monolayer with eosin–methylene blue or by immunofluorescence or immunoperoxidase techniques using a specific antiserum.

DNA probes and especially the more sensitive polymerase chain reaction (PCR) techniques are available to reveal the presence of E. ruminantium in the blood of animals with clinical signs, and in the tick vectors, to a lesser extent in the blood or bone marrow of carrier animals. Apart from
diagnosis, PCR is widely used for research on the E. ruminantium genome and for epidemiological studies.

**Serological tests:** Serological tests available include indirect fluorescent antibody tests, enzyme-linked immunosorbent assays (ELISAs) and Western blot. However, when the whole E. ruminantium is used as antigen, cross-reactions with Ehrlichia spp. occur in all of these tests. Serology has limited diagnostic applications.

One recently developed ELISA uses a recombinant antigen expressed as a partial fragment of the recombinant major antigenic protein 1 (MAP1) antigens –the MAP1-B ELISA. This test has shown a dramatic improvement in specificity compared with previous tests. Although this test is more specific, it still detects cross-reacting antibodies to other Ehrlichial organisms. Hence, definitive proof of heartwater must rely on epidemiological evidence and additional molecular testing indicating the presence of the organism. This ELISA has made the interpretation of serological results more reliable in regions where Ehrlichia infections occur in ruminants. It can help to monitor experimental infections and to measure the immune response of immunised animals, whose pre-immunisation serological history is known. Serology has very limited diagnostic use as clinically infected animals remain seronegative during the febrile reaction and seroconvert after they recover from the infection.

Serology is also not an effective import test. Prior to importation of animals from a heartwater endemic region, it is important to study the epidemiological data to try to establish that the herd and the resident ticks are not infected; in addition repeated PCR testing should be carried out to demonstrate that the pathogenic agent is not present in the herd.

**Requirements for vaccines and diagnostic biologicals:** The immunisation against heartwater by the ‘infection and treatment’ method using infected blood is still in use in some countries. A first-generation vaccine consisting of inactivated purified elementary bodies of E. ruminantium emulsified in Montanide ISA 50 adjuvant has given promising results in experimentally controlled conditions and has demonstrated significant protection in the field. An additional isolate, Welgevonden, has been attenuated and shown to confer good protection, and significant protection has also been obtained using DNA vaccination. However, none of these new experimental vaccines has been fully validated under field conditions. Field trials have revealed that antigenic diversity is important in formulating effective vaccines, and further investigations are critical for the delivery of any vaccine in the field.

A. INTRODUCTION

Heartwater (cowdriosis) is a rickettsial disease of domestic and wild ruminants caused by *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*) and transmitted by *Amblyomma* ticks (Bekker *et al.*, 2002; Dumler *et al.*, 2001; Peter *et al.*, 2002). It is also known by the synonyms malkopsiekte (Afrikaans), péritcard exsudative infectieuse (French), hidrocarditis infectiosa (Portuguese), idropericardite dei ruminanti (Italian), and a variety of names in different African languages (Camus & Barre, 1988). *Ehrlichia ruminantium* is classified in the order Rickettsiales and in the family Anaplasmataceae, together with the genera *Anaplasma*. Although ruminants remain the primary target of the pathogen, in South Africa a possible canine *E. ruminantium* infection has been reported (Allsopp & Alsopp, 2001), and, more recently, *E. ruminantium* has been strongly suspected in several cases of rapidly fatal encephalitis in humans (Louw *et al.*, 2005). However, in all cases, evidence of *E. ruminantium* infection was based on molecular detection. Isolation and characterisation of the infectious agent is necessary before *E. ruminantium* can be considered an emerging pathogen in species other than ruminants and especially in humans.

Heartwater occurs in nearly all the sub-Saharan countries of Africa where *Amblyomma* ticks are present and in the surrounding islands: Madagascar, Reunion, Mauritius, Zanzibar, the Comoros Islands and Sao Tomé. The disease is also reported in the Caribbean (Guadeloupe, Marie-Galante and Antigua) (Perreau *et al.*, 1980), from where it threatens the American mainland. All domestic and wild ruminants can be infected, but the former appear to be the most susceptible. Indigenous domestic ruminants are usually more resistant to the disease. Wild animals could play a role as reservoir, but Rusa deer, white tailed deer, springbok, chital, timor deer, which are used in wildlife farming, seem to be the main wild ruminant species in which heartwater can have a significant economic impact (Peter *et al.*, 2002).

The average natural incubation period is 2–3 weeks, but can vary from 10 days to 1 month. In most cases, heartwater is an acute febrile disease, with a sudden rise in body temperature, which may exceed 41°C within
1–2 days after the onset of fever. It remains high for 4–5 weeks with small fluctuations and drops shortly before death.

Fever is followed by inappetence, sometimes listlessness, diarrhoea, particularly in cattle (Bezuidenhout et al., 1994), and dyspnoea indicative of lung oedema. Nervous signs develop gradually. The animal is restless, walks in circles, makes sucking movements and stands rigidly with tremors of the superficial muscles. Cattle may push their heads against a wall or present aggressive or anxious behaviour. Finally, the animal falls to the ground, pedalling and exhibiting opisthotonos, nystagmus and chewing movements. The animal usually dies during or following such an attack.

Subacute heartwater with less pronounced signs, and peracute heartwater with sudden death, can also occur, according to the breed of ruminant and the strain of *E. ruminantium* involved.

The most common macroscopic lesions are hydropericardium, hydrothorax, pulmonary oedema, intestinal congestion, oedema of the mediastinal and bronchial lymph nodes (Bezuidenhout et al., 1994), petechiae on the epicardium and endocardium, congestion of the brain, and moderate splenomegaly.

A tentative diagnosis of heartwater is based on the presence of *Amblyomma* vectors, nervous signs, and presence of transudates in the pericardium and thorax on post-mortem examination. When making a diagnosis based on clinical signs, the following other diseases should be considered: bovine cerebral babesiosis and theileriosis, anaplasmosis, botulism, haemonchosis in small ruminants, rabies and poisoning.

### B. DIAGNOSTIC TECHNIQUES

#### 1. Identification of the agent

During the febrile reaction, *E. ruminantium* can be readily isolated in culture from blood or plasma; however, it is difficult to detect these organisms in a blood smear. Typical colonies of *E. ruminantium* can be observed in brain smears made after death and this represents a definitive diagnosis for heartwater.

Opening the cranium is not necessary. An alternative method (Schreuder, 1980) is to cut off the head in front of the first cervical vertebra. Then, introduce a curette through the foramen magnum, between the medulla and the meninges. The curette is turned over towards the brain and removed with a piece of cerebellum. Another method consists of making a hole in the skull with a hammer and a large nail and aspirating a sample of brain cortex with a needle attached to a syringe. These methods also lessen the danger to the operator in cases where the nervous signs have been caused by rabies.

In the live animal, a brain biopsy may be obtained aseptically and harmlessly after local anaesthesia, although with difficulty; appropriate restraint must be used especially with large and horned animals. Colonies of *Ehrlichia* are observed during the febrile period. This method is useful for experimental studies, but not suitable for routine diagnosis.

Colonies of *E. ruminantium* are still present 48 hours after death in a brain that has been stored at room temperature (20–25°C) and for up to 34 days in a brain that has been stored in a refrigerator at 4°C (Camus & Barre, 1988).

A small fragment of grey matter (approximately the size of a match head) is placed on a microscope slide, crushed to a paste consistency by another slide and, while maintaining pressure, the slides are drawn over each other lengthwise to produce a single layer of cells. The slides are air-dried, fixed in methanol, stained with Giemsa diluted with Sörensen buffer (2.54 g KH₂PO₄; 8.55 g Na₂HPO₄·H₂O; q.s. to 5 litres with distilled water), pH 7.2, and washed with tap water. Fast Giemsa stains (DiffQuick, RAL555, Field’s stain, CAM’s Quick stain) give quicker results, but the colour contrast is usually poorer. Some ‘fast’ stains do provide excellent contrast, e.g. Hema 3 stain.

The slides are examined under a microscope at a low magnification (×10 objective) to locate the cerebral capillaries. An oil-immersion lens with a magnification of at least ×50 is useful for identifying the colonies of rickettsiae. Experience is required *E. ruminantium* colonies must be differentiated from other haemoparasites (*Babesia bovis*), certain blood cells (thrombocytes, granulocytes), normal subcellular structures (mitochondria, mast cell granules), or stain artefacts (stain precipitates), etc. The specificity of the reading can be improved by staining formalin-fixed brain sections using immunoperoxidase techniques.

*Ehrlichia ruminantium* colonies are formed from clusters of granules (0.2–0.5 µm), sometimes arranged in the shape of a ring or a horseshoe (1–3 µm), that are placed close to the nucleus inside the endothelial cell. The granules can be scanty, particularly in peracute cases, but they are always present in the brain of an animal that...
died from heartwater. However, if the animal has been treated with doxycycline or oxytetracycline 48 hours before, the granules of *Ehrlichia* tend to fuse making the diagnosis very difficult, and sometimes impossible.

Fresh whole blood collected from suspect animals can be inoculated intravenously into a susceptible sheep or goat. The development of clinical signs and the demonstration of *E. ruminantium* in the brain of the inoculated ruminant are diagnostic for heartwater.

Transmission electron microscopy has been used to demonstrate that the *E. ruminantium* organisms develop inside a vacuole-like structure, which is surrounded by a membrane in the endothelial cell’s cytoplasm (Pienaar, 1970). Each organism is enclosed by a double membrane. Within the vacuole-like structure, *E. ruminantium* electron-dense forms (elementary bodies), as well as intermediate reticulate forms, are identified.

**Isolation of *Ehrlichia ruminantium* using in-vitro culture**

Although numerous cell lines have been shown to support growth of *E. ruminantium*, isolation is not the first choice of test for a rapid diagnosis of cowdriosis as isolation is a labour intensive and time-consuming laboratory procedure. For a rapid diagnosis, polymerase chain reaction (PCR)/molecular diagnosis is preferable. However, *E. ruminantium* isolation should be encouraged for typing the strains present in a region for the purpose of vaccination programmes. *Ehrlichia ruminantium* can be isolated from the blood of reacting animals by cultivation on ruminant endothelial cells (Smith et al., 1998). Endothelial cells from umbilical cord, aorta, or the pulmonary artery of different ruminant species (cattle, goat, sheep) are used most often for isolation, although other endothelial cell types (brain capillaries, circulating endothelial cells, etc.) have been described for the routine culture of the microorganism. Endothelial cell lines from sable, eland, buffalo, kudo and bush pig can also be used to grow *E. ruminantium*. No standard cell line has yet been designated for isolation.

- **Isolation procedure**
  
  i) The blood of the reacting animal (optimal time for detection of the organism is the second or third day of febrile reaction) is collected in anticoagulant (heparin or sodium citrate, not ethylene diamine tetra-acetic acid) and diluted 1/2 in the culture medium consisting of Glasgow minimal essential medium (MEM) supplemented with 10% inactivated fetal bovine serum, 2.95 mg/ml tryptose phosphate broth, 200 mM L-glutamine, and antibiotics if necessary (penicillin 100 international units/ml, streptomycin 100 µg/ml).

  ii) The culture medium is poured off the endothelial cell monolayer, and infective blood (approximately 2 ml for a 25 cm² flask) is added. The flask is incubated at 37°C on a rocking platform for 2 hours.

  iii) After incubation, the blood is poured off and the monolayer is gently washed three times with culture medium prewarmed at 37°C. Fresh culture medium is added and the flask is incubated at 37°C. The medium is changed twice weekly.

  (The use of plasma instead of blood is more efficient when taken from an animal with a febrile reaction >41°C. In this case, steps ii and iii above may be replaced with the following:

  - Seed 4 ml of plasma (smaller inoculum can be used if there is a limited amount of plasma available) on to a susceptible endothelial cell culture and incubate for 1 hour at 37°C on a rocking platform.

  - Wash off plasma with growth medium and then add 5 ml of growth medium (per 25 cm² flask) and observe for development of cytopathic effect.)

  iv) The monolayer is inspected regularly for the appearance of small plaques of cell lysis. The first plaques generally appear after about 2 weeks. Passaging on uninfected cell monolayers is performed when the lysis reaches 80% of the cell layer. The remaining cells are stained with eosin/methylene blue or Giemsa or DiffQuik and examined microscopically for the presence of *E. ruminantium* morulae. Alternatively, cells can be stained by an indirect fluorescent antibody (IFA) test or an immunoperoxidase test using an *E. ruminantium*-specific antiserum; the immunoperoxidase test is not commonly used.

**Isolation of *Ehrlichia ruminantium* using in-vivo culture**

It is feasible to assess the presence of heartwater in a herd, a region or a country, or to isolate a strain of *E. ruminantium* by inoculating blood or tick homogenate into a susceptible animal. However, due to the animal welfare concerns, this method is not recommended. Blood from individual animals, or pooled blood, is injected slowly at a dose of 10–100 ml intravenously into a susceptible sheep or goat. Blood as an inoculum, to determine infection status of herd, will be infective if there are clinically infected donor animals present; however, the method will rarely detect infection in carrier/recovered animals. Another method consists of collecting and homogenising adult *Amblyomma* ticks, and after centrifuging the homogenate and then inoculating the resulting supernatant into susceptible hosts. This method can be more sensitive than
blood from suspect animals (especially if blood is from recovered animals) because the concentration of *E. ruminantium* is higher in the tick than in the blood. However, the tick infection rate in the field is variable and sometimes as low as 1% (Camus *et al.*, 1996). In this case, to detect an infection as many ticks as possible should be used; at least 100 ticks are needed. In both cases, the inoculum, to which 10% dimethyl sulphoxide (final concentration) has been added, can be stored in liquid nitrogen for several years. Note that inoculation of tick homogenates into susceptible animals may cause anaphylaxis, which can be prevented by the simultaneous administration of adrenaline. The development of clinical signs and the detection of circulating rickettsiae by molecular methods and/or the demonstration of *E. ruminantium* in the brain of the inoculated ruminant, on the second or third day of fever, are diagnostic for heartwater. In addition, confirmation could be accomplished by *in-vitro* isolation on endothelial cells using plasma from the inoculated animals.

2. Molecular methods

a) Detection of *Ehrlichia ruminantium* using DNA probes

A genomic DNA fragment pCS20 specific for *E. ruminantium* has been cloned and used as a nucleic acid probe (Mahan *et al.*, 1992; Waghela *et al.*, 1991). It recognises all strains of *E. ruminantium* tested to date. This probe, designated pCS20, readily detects infection in clinically ill animals and experimentally infected *Amblyomma* ticks (Mahan *et al.*, 1992; 1995b; 2000; Yunker *et al.*, 1993). However, it is not sufficiently sensitive to detect most carrier animals or low level infections in ticks (Peter *et al.*, 2000; 2002). The pCS20 probe proved nevertheless to be more sensitive than 16S and MAP1 (major antigenic protein 1) probes for the detection of *E. ruminantium* in ticks when hybridised on a PCR-amplified product of the homologous DNA fragment (Allsopp *et al.*, 1999).

b) Detection of *Ehrlichia ruminantium* using PCR and nested PCR

Two primers – AB128 (5'-ACT-AGT-AGA-AAT-TGC-ACA-ATC-TAT-3') and AB129 (5'-TGA-TAA-CTT-GGT-GCG-GGA-AAT-CCT-T-3') – have been designed from the DNA sequence of the pCS20 probe (Mahan *et al.*, 1992) for use in a PCR. These primers amplify a 279 base pair DNA fragment which is specific only for *E. ruminantium*. Hybridisation of the amplified PCR products to a labelled pCS20 probe, as an additional step, resulted in a 350-fold more sensitive assay than using the nucleic acid probe to detect *E. ruminantium* directly in DNA extracted from ticks. Low levels of infection in animals and in ticks fed on carrier animals are detected by PCR, while a hybridisation reaction with the pCS20 probe alone (without PCR first) usually remains negative (Peter *et al.*, 1995). Experimentally, the detection limit of the conventional PCR assay was found to be between 10 and 10^2 organisms, whereas it was between 1 and 10 organisms after PCR/hybridisation. The PCR/hybridisation has been shown to detect 37 strains from all endemic areas with a specificity of 98%. However, the sensitivity of the PCR assay is variable, ranging from 88 to 97% with tick samples containing 10^7 to 10^4 organisms, and dropping to 61% and 28% with samples containing 10^3 and 10^2 organisms, respectively (Peter *et al.*, 2000). Consequently, the rate of 86% of ticks testing positive when fed on a clinically reacting animal dropped to 21% when fed on carrier animals due to a lower rickettsemia in such animals. The PCR/hybridisation assay has been used widely to define the epidemiology of heartwater in southern Africa.

Two nested PCR assays have been developed to enhance detection of low levels of rickettsiemia and to do away with the hybridisation step (Martinez *et al.*, 2004; Semu *et al.*, 2001). Both use the pCS20 region as the target sequence. The Semu *et al.* assay uses two external primers U24 (5'-TTT-CCC-TAT-GAT-ACA-GAA-GTG-AAC-3') and L24 (5'-AAA-GCA-AGG-ATT-GTG-ATC-TGG-ACC-3') and then the AB 128 and AB 129 for the nested reaction. The sensitivity of detection of this assay is one gene copy of the pCS20 fragment or 1 organism. The other nested PCR assay (Martinez *et al.*, 2004) uses a pair of external primers comprises the AB128 sense primer together with an anti-sense primer called AB130. These amplify a 413 bp fragment used as a template in a second round PCR using AB128 and AB129 as internal primers. The use of AB128 and AB129 primers avoids the need to repeat a full evaluation of the test specificity. The nested PCR shows a hundred-fold improvement in sensitivity compared with a simple PCR, and an average detection limit of 6 organisms. The direct implication of this was an increase in the detection rate in wild ticks of from 1.7% to 36% in an epidemiological study in the Caribbean. The detection limit is comparable to that of the PCR/hybridisation method, which is nevertheless much more complex and time-consuming to perform. The pCS20 nested PCR allowed regular detection of *E. ruminantium* organisms from ticks, blood, brain and lungs from infected animals, whether the samples were processed fresh, or after freezing or preservation in 70% ethanol. One drawback of the nested PCR is that extreme care needs to be exercised to prevent introduction of contamination due to repeated opening of the tubes containing the first PCR reaction when conducting the nested reaction.

A nested PCR targeting the entire map1 polymorphic gene has been developed in parallel in order to type the strains by restriction fragment length polymorphism or sequencing of the amplification fragment directly from the pathological samples testing positive in the pCS20 nested PCR (Martinez *et al.*, 2004). An
additional nested PCR targets the polymorphic map1 gene and can be used to type circulating heartwater strains for vaccine selection and disease management. PCR amplicons are analysed by restriction fragment length polymorphisms or sequencing. A high genetic diversity of *E. ruminantium* is observed in the field may influence the formulation of vaccines and needs to be further investigated. The map1 nested PCR performs well although with a slightly lower sensitivity than the pCS20 nested PCR. Its detection limit was evaluated at around 60 organisms and only 91% of samples testing positive in the pCS20 nested PCR also tested positive in the map1 nested PCR; some positives of low intensity found using the pCS20 nested PCR were negative in the map1 PCR.

Primers 32F1 and 32R1 designed from the sequence of the MAP1 gene of *E. ruminantium* as well as additional primer sets designed to target the MAP1, MAP2, gitA, and 16S rDNA genes of *E. ruminantium* have been used in PCR to detect the pathogen in tick, blood and bone marrow of carrier sheep and wild African ungulates, but these methods has not been widely evaluated and used.

Although the PCR methods have proved highly effective in detecting infection in ticks or in animal samples during the clinical phase of the disease or after death, only limited studies have been done to evaluate their value in healthy carrier ruminants. *Ehrlichia ruminantium* can easily be demonstrated in the blood of infected animals just before the onset of the febrile period and for a few days after recovery (Mahan et al., 1992; Semu et al., 2001), but after that period, its detection is sporadic and appears to be dependent on the rickettsemia levels. In one study in Zimbabwe only between 3.3 and 26.7% of cattle, and 23.3% of goats were found to be positive, while data from ticks collected in the same area would suggest that given the age of the cattle or goats, they should have all been exposed or infected with *E. ruminantium* (Mahan et al., 1998b). A comparison of the indirect MAP1-B ELISA and the pCS20 PCR/hybridisation assay, to evaluate their respective detection sensitivity levels over a period of 8 weeks (tests performed every 2 weeks), was done on 15 cattle located in Zimbabwe on a heartwater-endemic farm where tick control was minimal and the infection pressure was high (Simbi et al., 2003). The *E. ruminantium* tick infection rate on this farm was between 10 and 12%. The data demonstrated that the pCS20-PCR assay was more reliable in detecting infection in blood of these cattle than detection of antibodies by the indirect MAP1-B ELISA. These cattle were not always PCR positive or positive for antibodies at every testing time and some cattle were negative by PCR throughout the study. These data suggest that the rickettsemia levels fluctuate from high to low, and that the PCR detects infection when the levels are high. Hence detecting carrier/recovered animals is less reliable than detecting clinically infected animals. This highlighted the fact, that for determination of infection status of sub-clinical animals, it is advisable to repeatedly test the blood of such animals for *E. ruminantium* by the pCS20-PCR assay. Whether the absence of detection in most carrier animals is due to an insufficient sensitivity of the PCR methods for detecting very low rickettsemia, or is due to an intermittent release of organisms in the circulation, is not fully understood. A useful technique for confirming the status of a suspected carrier animal, whose blood is PCR negative, is to feed batches of naive ticks on the animal and then test the ticks by a pCS20 semi-nested PCR. It is not known whether ticks act simply by concentrating circulating organisms, or also by amplifying their number or even by inducing release of micro-organisms in the circulation during feeding.

c) Detection of *Ehrlichia ruminantium* using the reverse line blot technique

The reverse line blot technique (RLB) has been used for the simultaneous detection and identification of Anaplasma and *Ehrlichia* species known to occur in ruminants on the basis of differences in the small subunit rRNA gene (Bekker et al., 2002). Primers 16S8FE and B-GA1B-new were designed from conserved domains and used to amplify a 492–498 bp fragment of the 16S rRNA gene spanning the variable V1 region. Species-specific oligonucleotide probes were designed in this V1 loop to allow species-specific detection of *E. ruminantium*, *E. ovina*, *E. sp. strain Omatjenne*, *Anaplasma marginale*, *A. centrale*, *A. bovis*, *A. ovis* and *A. phagocytophilum*. One oligonucleotide probe cross-reactive with all species (catch-all probe) was also designed to serve as control in case a PCR product does not hybridise to any of the species-specific probes. In the method, the species-specific probes are covalently linked to the hybridisation membrane, which is hybridised with the PCR product obtained using primers 16S8FE and B-GA1B-new. PCR products obtained from all above-mentioned microorganisms were shown to bind with specific oligonucleotide probes only. No PCR product was detected and no hybridisation occurred when the PCR-RLB was applied to *Theileria annulata*, Babesia bigemina or mammalian DNA. Similarly, negative control ticks were always negative in the RLB assay whereas it was possible to detect *Ehrlichia ruminantium* infection in 15–70% of ticks fed on experimentally infected or long-term carrier sheep. In Mozambique, *E. ruminantium* could also be detected in the blood of 12 sentinel small ruminants placed in the field with the infected animals; mixed infection was detected in five of the infected sentinel animals, thus demonstrating the usefulness of the method for detecting multiple infections. However, the sensitivity of the assay has not yet been determined and there is a need to further validate the technique in large epidemiological studies.

d) Detection of *Ehrlichia ruminantium* using real-time PCR

Two real-time PCR (QPCR) tests have been described for the detection and quantitative determination of *E. ruminantium* organisms. In a first test, a 182 bp fragment from the non-polymorphic map1-1 gene was
amplified and detection carried out using the SYBR Green method (Postigo et al., 2002). DNA from six different isolates was successfully amplified. The detection limit mentioned was higher than 0.1 organism/μl, but this finding was not subjected to an in-depth investigation. Counting *E. ruminantium* under the microscope after Giemsa staining does not give very precise results. The method does not significantly improve the detection sensitivity of a nested PCR, although it does allow *E. ruminantium* organisms to be quantified. In addition to limited laboratory validation, the QPCR method was used in only one study aimed at following the *E. ruminantium* kinetics in the blood of experimentally infected sheep. *Ehrlichia ruminantium* was detected only during the hyperthermia reaction period. QPCR thus does not improve detection of asymptomatic carriers compared with nonquantitative PCR.

A second SYBR Green-based real-time PCR has been described and fully validated for use in the characterisation of *E. ruminantium* replication and release kinetics in endothelial cell cultures and its subsequent use to control the mass production process in bio-reactors (Peixoto et al., 2005). The product is an 873 bp fragment from the map1 gene. The external standard for quantifying *E. ruminantium* is a pCI-neo plasmid containing one copy of the map1 target sequence, and is a more precise method of quantifying the organisms than the method described previously where the standard is based on the counting of *E. ruminantium* bodies under the microscope. The dynamic quantitative range allows accurate measurements to be taken in samples containing 10^2 to 10^8 gene copies. The method was successfully applied to four different isolates but has not been validated for use on diagnostic samples.

- **Reading the results**

  As *E. ruminantium* is an obligate intra-cellular bacteria that cannot be cultivated in acellular media and its isolation is complex and takes several weeks, molecular detection techniques are the best methods for the diagnosis of cowdriosis. PCR proves to be easier to perform and more sensitive than DNA probes. With all PCRs, however, care must be taken to ensure that no cross-contamination occurs between samples. Negative and positive controls must be included in each test. As heartwater serology has several limitations (see Section B.3), the PCR could be used to help confirm if seronegative animals, originating from an endemic area, are not infected, prior to translocating them to a heartwater-free area that has the risk of becoming infected, because of the presence of potential vectors. However, despite interesting experimental results in detecting subclinical carriers, there is not enough available information on the reliability of carrier detection by PCR; more extensive field studies need to be conducted to recommend the best protocol of detecting carrier animals. It is nevertheless clear from the Zimbabwe study in cattle that detection of infection in carrier hosts is going to be difficult and will require repeated testing to confirm status of infection (Simbi et al., 2003). The current results obtained with the PCR, the nested PCR, the RLB assay and more recently the QPCR, show that the direct detection of *E. ruminantium* in the blood is only reliable during and around the febrile phase of the disease. PCR-based methods appear to be more reliable in detecting infection in ticks, and this could have epidemiological value in determining the geographical distribution of *E. ruminantium*. In addition, when necessary in endemic areas, the inclusion of testing (originally naive) ticks fed on a suspect animal would greatly improve the sensitivity of carrier detection when serology and PCR on blood have failed. The procedure is nevertheless not convenient for routine diagnostic laboratories as it requires the maintenance of tick colonies and the capacity to experimentally infect animals.

### 3. Serological tests

Various serological tests for diagnosing heartwater have been described: an IFA test with *E.-ruminantium*-infected endothelial cell culture as antigen (CIFA test), indirect ELISA, a competitive ELISA (C-ELISA), and a Western blot. The IFA test using *E.-ruminantium*-infected mouse peritoneal macrophages (MIFA) (Du Plessis & Malan, 1987) is no longer used.

The major drawback of all of these tests is the detection of false-positive reactions due to common antigenic determinants between the *E. ruminantium* MAP1 (Jongejan & Thielemans, 1989) and similar proteins in several *Ehrlichia* species (Mahan et al., 1993; Semu et al., 2001). Almost all of these tests are no longer used to study the epidemiology or for diagnosis. The CIFA test is still used in some places, but care must be taken when interpreting the results because of the problem of false-positive reactions.

To minimise the problem of cross-reactions with *Ehrlichia*, two ELISAs based on a recombinant MAP1 antigen have been developed. The first is an indirect ELISA that uses an immunogenic region of the MAP1 protein (called MAP1-B) and gives far fewer cross-reactions with *Ehrlichia spp.* (MAP1-B ELISA) (Van Vliet et al., 1995). The second is a competitive ELISA that uses the MAP1 gene cloned in a baculovirus and monoclonal antibodies (MABs) raised against the MAP1 protein (MAP1 C-ELISA) (Katz et al., 1997). Both tests have dramatically improved specificity, but they still show some reactivity with high titre sera against *E. canis, E. chaffeensis* and an unclassified white-tailed deer agent. The MAP 1-B ELISA has been the most extensively used and will be described in detail. The MAP1-B ELISA does detect antibodies to *E. muris* (Mahan S.M., pers. comm.) an *Ehrlichial* agent that is very closely related to *E. ruminantium*; this agent is found in white tailed deer in Georgia USA and is transmitted by *Amblyomma americanum* ticks (Lofis et al., 2006). Hence serology as a diagnostic tool
for detecting of individual animals exposed specifically to *E. ruminantium* is unreliable. Serology should be considered at a herd level taking into consideration the epidemiological environment and, if necessary, be complemented by molecular techniques.

**a) Indirect fluorescent antibody test with infected endothelial cell tissue culture as antigen (CIFA test) (Martínez et al., 1990)**

To prepare the antigen, an *E. ruminantium* strain is cultivated in ruminant endothelial cell cultures. When most cells are lysed, the remaining adherent cells are scraped and mixed with the supernate. The cells are centrifuged three times with phosphate buffered saline (PBS) at 200 g for 10 minutes. Of the washed cell suspension, 10 µl are placed in every well of an immunofluorescence slide. The antigen slides are dried, fixed in acetone and stored at –20°C.

- **Test procedure**
  i) The sera to be tested are diluted 1/20 or a higher dilution in PBS, added to the antigen wells and incubated for 30 minutes in a humid chamber at 37°C.
  ii) The slides are then washed in PBS buffer for 15 minutes.
  iii) The appropriate anti-species conjugate, usually diluted 1/60, is added to cover the wells. The slides are incubated again for 30 minutes at 37°C.
  iv) After a second washing, the slides are mounted in glycerine buffer under a cover-slip and examined under a fluorescence microscope.
  v) Control positive and negative sera are included on each slide.

**b) MAP1-B enzyme-linked immunosorbent assay (Semu et al., 2001; Van Vliet et al., 1995)**

Using the vector pQE9, the PCR fragment MAP1-F2R2, which encodes the amino acids 47–152 of the MAP1 protein including the immunogenic region MAP1-B, is expressed in *Escherichia coli* M15[pREP4] as a fusion protein containing six additional histidine residues. The recombinant MAP1-B is purified using Ni²⁺-NTA agarose (nitrilotriacetic acid agarose) under denaturing conditions as described by the manufacturer. The antigen is preserved at 4°C and each batch is titrated.

The antigen is diluted at 0.5 µg/ml in 0.05 M sodium carbonate buffer, pH 9.6, and immobilised on to polystyrene plates by incubation for 1 hour at 37°C, and stored at 4°C until use. However, in initial trials, an antigen concentration of 2 µg/ml reduced background noise and improved specificity (data not shown: Semu et al., 2001).

- **Test procedure**
  i) Plates are blocked for 30 minutes by adding 100 µl per well of 0.1 M PBS, pH 7.2, supplemented with 0.1% Tween 20 and 3% non-fat dry milk (PBSTM).
  ii) The plates are washed three times with PBS supplemented with 0.1% Tween 20 (PBST) and twice with distilled water.
  iii) 100 µl of test serum diluted 1/100 in PBSTM is added in duplicate to wells, which are then incubated for 1 hour at 37°C.
  iv) Plates are washed three times in PBST and twice in distilled water.
  v) Horseradish-peroxidase-conjugated anti-species IgG optimally diluted in PBSTM is added at 100 µl per well and the plate is incubated for 1 hour at 37°C.
  vi) After washing as in step iv, each well is filled with 100 µl of 0.1 M citrate buffer, pH 5.5, containing 0.5 mg/ml orthophenylene-diamine and 3 µl/ml of 9% H₂O₂.
  vii) The reaction is stopped after 30 minutes of incubation at room temperature (20–25°C) by adding 50 µl of 2 N H₂SO₄. Absorbance is read at 495 nm. Positive and negative controls are included in each plate.

**c) MAP1 competitive enzyme-linked immunosorbent assay (Mondry et al., 1998)**

*Recombinant MAP1 antigen is prepared as follows:* 8-day-old *Trichoplusia ni* insect larvae are infected by a baculovirus expressing the *map1* gene and moribund larvae are homogenised (10% [w/v]) in PBS supplemented with 0.001% (v/v) Triton X-100.

*Anti-MAP1 MAb is prepared as follows:* spleen cells of BALB/C mice previously inoculated with larval homogenate are fused to SP2/0 cells. Supernatant fluids from hybridoma cell cultures are screened for
reactivity with MAP1 by immunoblotting and immunoperoxidase methods. A reactive cell culture is subcloned, isotyped and subsequently used for ascites production.

After a further 1/800 (v/v) dilution in PBS, the antigen is immobilised on to polystyrene plates (Nunc-Immuno Plates PolySorp) by incubation overnight at 4°C, and stored at –70°C.

- **Test procedure**
  
  i) Prior to use, the plates are blocked for 30 minutes by adding 100 µl per well of PBS, pH 7.2, supplemented with 0.05% Tween 20 and 5% nonfat dry milk.
  
  ii) Plates are washed three times with PBS/Tween, 50 µl/well of test serum diluted 1/50 in PBS supplemented with 0.05% Tween 20 and 1% nonfat dry milk (PBSTM) is added in duplicate and the plates are incubated for 30 minutes at 37°C.
  
  iii) Without an intervening washing step, 75 µl/well of the MAb diluted 1/4000 (v/v) in PBSTM is added and the plates are incubated for another 30 minutes at 37°C.
  
  iv) Plates are washed three times in PBS/Tween and horseradish-peroxidase-conjugated anti-mouse IgG optimally diluted in PBSTM is added at 50 µl per well. The plate is incubated for 1 hour at 37°C.
  
  v) After three washings as before, 100 µl of 0.1 M citrate buffer, pH 5.5, containing 0.5 mg/ml O-phenylene diamine and 3 µl/ml of 9% H₂O₂ are added to each well. After 30 minutes of incubation at room temperature in the dark, the reaction is stopped by adding 50 µl of 2 N H₂SO₄ and the absorbance is read at 495 nm. Positive and negative controls are included in each plate.

- **Reading the results**

All serological tests based on non-recombinant *E. ruminantium* antigens, such as CIFA, ELISAs, and Western blot, are still used for experimental studies but are no longer used for seroepidemiological studies. The tests have been compared and applied to known positive and negative sera to *E. ruminantium* (Du Plessis *et al.*, 1993). No false-positive reactions were observed with any of the tests against known negative sera. There is good correlation among tests, but the specificity of all five tests is low because cross-reactions occur with certain *Ehrlichia* spp.

The interpretation of results of the various tests applied to field surveys is thus difficult in areas where *E. ruminantium* infections occur in ruminants, which is probably the case in most of the heartwater-endemic regions of Africa. This situation has also been demonstrated in farms without *Amblyomma* but infected with tick species not known to be vectors of *E. ruminantium* (Kokono *et al.*, 2003; Van Vliet *et al.*, 1995).

Both the MAP1-B ELISA and the MAP1 C-ELISA have shown a high specificity after evaluation in 3000 ruminant sera (goat, sheep and cattle) collected from 14 *A. variegatum*-infested islands of the Lesser Antilles, among which only three are known to be infected by *E. ruminantium* (Mondry *et al.*, 1998). Overall specificity calculated from the 11 heartwater-free islands was 98.5% and 99.4% for the MAP1 C-ELISA and the MAP1-B ELISA, respectively. Although a few false-positive sera are still found, these tests are likely to solve much of the specificity problems of the earlier serological tests. However, high seroprevalence in vector-free areas of Zimbabwe or South Africa has also been reported although not explained (it may be caused by a cross-reacting agent not transmitted by *Amblyomma*) and should be kept in mind when interpreting the results.

Evaluating the sensitivity of the tests is more problematic as it would require knowledge of the exact status of a high number of animals sampled in the field. As mentioned before there is currently no simple technique available to confirm if an animal is infected. Experimentally, the sensitivity of the C-ELISA in goats was reported to be 91.6–95.4% for the MAP1-B ELISA, and 96.3–96.9% for the MAP1 C-ELISA (Mondry *et al.*, 1998). However, in another study the sensitivity averaged 95% for cut-off values set at 31% and 26.6% of the positive control serum for sheep and goat sera, respectively (Mboloi *et al.*, 1999). Indeed, calculations are based on a limited number of experimentally inoculated animals in a period of time soon after inoculation, when almost all the animals are still positive. Sensitivity in cattle is even lower and several reports show that after infection most of the animals become seronegative again in less than 6 months and some animals never seroconvert (Mahan *et al.*, 1998c; Semu *et al.*, 2001). This observation is in line with the difference in antibody prevalence observed between small ruminants and cattle in epidemiological surveys that cannot be explained by a lower risk of infection of the latter. For example, in Zimbabwean farms situated in endemic areas, more than 90% of goats presented antibodies in their serum compared with only 33% of cattle maintained in the same conditions (Mahan *et al.*, 1998c). Similar observations were made in the Caribbean. In addition, some areas of Zimbabwe, which was labelled heartwater-free, had a large number of goats positive for MAP1-B antibodies; this further complicates the serodiagnosis of heartwater (Kokono *et al.*, 2003).
Serological tests are useful for the assessment of heartwater antibody response in vaccinated animals. The tests should not be used to screen animals for importation into heartwater-free areas. Antibodies are maintained at detectable levels in naturally infected domestic ruminants for a few months only and circulating antibodies disappear more rapidly in cattle than in small ruminants. It is thus possible that serologically negative animals may be carriers of infection. Serology should therefore only be regarded as a diagnostic method to be applied at the herd level and not at the individual animal level (Peter et al., 2001). When interpreting diagnostic serology results, other epidemiological parameters must be considered.

Molecular methods, such as PCR assay, could potentially help in detecting carrier animals, but this approach has still significant drawbacks (see Section B.2 Molecular methods).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No commercial vaccines are available at present. The only method of immunisation against heartwater remains the ‘infection and treatment’ method using infected blood followed by treatment of reacting animals with tetracycline (Bezuidenhout et al., 1994). This method is still in use in several areas, but it is likely to be replaced soon by preparations using attenuated or inactivated organisms, which have given promising research results.

1. Inactivated vaccine preparations

Vaccination with preparations of inactivated E. ruminantium elementary bodies emulsified in oil adjuvants was shown to be possible following the demonstration that susceptible goats can be protected by inactivated Ehrlichia in Freund’s adjuvant (Martinez et al., 1994). This vaccine also protected against challenge in sheep (Mahan et al., 1995a) using different strains of E. ruminantium (Mahan et al., 1998a), and in cattle (Totte et al., 1997) using the same strain as in goats. A first generation vaccine preparation of inactivated Ehrlichia in Montanide ISA 50 oil adjuvant was shown to be similarly effective to the Freund’s adjuvant preparation on laboratory challenge of immunised goats and sheep (Martinez et al., 1996).

In initial vaccine trials, animals were immunised with two subcutaneous injections of 250–1000 µg of antigen (depending on trial) emulsified (50/50) in Montanide ISA 50 adjuvant in a volume of 2 ml. It has recently been shown in goats in experimental conditions that the vaccine dose can be lowered to 35 µg of antigen without decreasing the effect on protection (Vachiery et al., 2006). From the initial description, this represents a 28-fold reduction in the dose of vaccine from 1 mg to 35 µg of E. ruminantium without any modification to the protective effect. The process for the mass production of E. ruminantium has been developed in parallel (Marcelino et al., 2006). Critical parameters have been determined and optimised for the production of E. ruminantium in endothelial cells in stirred-tank bioreactors. Using serum-free medium in such bioreactors, E. ruminantium production yields reached a 6.5-fold increase compared with conventional methods. Using 2-litre bioreactors and the estimated efficient 30 µg vaccine dose, the cost estimation for one vaccine dose was around 0.11 euro, which makes it affordable in countries with limited resources. Efficacy trials conducted with vaccine preparations entirely produced using the mass production and purification process followed by preservation of the product in various solutions (NaCl versus PBS) and at different temperatures (−20°C, 4°C) have demonstrated that the efficacy of the vaccine is maintained after the entire mass production and preservation process (Marcelino et al., 2007).

In Zimbabwe, field trials of the inactivated vaccine emulsified in ISA 50 adjuvant have also demonstrated protection of sheep against natural tick challenge (Mahan et al., 1998a). In larger field trials conducted in East and South Africa, a significant reduction in mortality has been achieved in cattle, goats and sheep using either a prototype strain from Zimbabwe (Mbizi strain) or a local strain from the experimental sites (Mahan et al., 2001). However, in three out of four sites, the vaccine prepared from the local isolate was less effective than the prototype Mbizi vaccine, strongly suggesting an inadequate coverage of the antigenic repertoire of isolates present in each site. Lack of cross-protection between E. ruminantium isolates due to disparities of antigenic composition is well established, but the complexity of the E. ruminantium population structure in the field has been underestimated. It has recently been demonstrated in large field evaluation trials carried out in several farming systems in West Africa that, in limited geographical areas, more than 10 genotypes with differing cross-protection capacities can be present and have a significant influence on protection with inactive vaccine preparations (unpublished data).

The Mbizi strain inactivated vaccine is being developed commercially by Onderstepoort Biological Products in South Africa (Mahan S.M., pers. comm.). These inactivated vaccines do not prevent infection but do prevent or reduce death of vaccinated animals when exposed to live virulent challenge. The advantage however is that several field strains can be incorporated to make the vaccine more widely cross-protective.

A major challenge remains the characterization of the extent of strain diversity in a region to be covered by an appropriate formulation of the vaccine. This knowledge will also be essential for new generation vaccines that will be developed in the future.
2. Attenuated vaccine preparations

Infection of ruminants with live *E. ruminantium* strains induces a strong long-lasting protection against an homologous isolate. This is the basis for infection and treatment using virulent isolates. Isolates of attenuated virulence that do not necessitate treatment of animals would be ideal but a limited number of such attenuated isolates are available. An attenuated Senegal isolate has been obtained and shown to confer 100% protection against an homologous lethal challenge, but very poor protection against a heterologous challenge. The Gardel isolate, which gives a significant level of cross-protection with several isolates (although far from complete), has also been attenuated. Recently, a third isolate named Welgevonden from South Africa has been attenuated and shown to confer complete protection against four heterologous isolates under experimental conditions (Sweygarth et al., 2005). The main drawback of attenuated vaccines is their extreme lability, which necessitates their storage in liquid nitrogen and their distribution in frozen conditions. In addition, they have to be administered intravenously.

3. Recombinant vaccine preparations

Several reports show partial protection of mice using *map1* DNA vaccination and an improvement of protection by vaccination following a prime (plasmid) – boost (recombinant MAP1) protocol (Nyika et al., 2002). However, protection of ruminants has never been demonstrated using this strategy. In opposition, significant protection of sheep was reported against homologous and heterologous experimental challenge following plasmid vaccination using a cocktail of four ORFs (open reading frames) from the 1H12 locus in the *E. ruminantium* genome (Collins et al., 2003). No further results have been described since then. Recombinant vaccines will probably not be available in the near future.

REFERENCES


* * *

NB: There is an OIE Reference Laboratory for Heartwater (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Heartwater.
CHAPTER 2.1.7.

JAPANESE ENCEPHALITIS

SUMMARY

Definition of the disease: Japanese encephalitis virus (JEV) is a member of the genus Flavivirus in the family Flaviviridae and causes encephalitis, principally in horses and humans. JEV also infects pigs where it causes abortions and stillbirths. JEV is maintained in nature between mosquitoes and pigs. The major vector of JEV is a culicine mosquito, Culex tritaeniorhynchus. Pigs act as important amplifiers of the virus, and birds can also be involved in its amplification and spread in the environment. The disease has been observed in large parts of Asia and recently in the western Pacific region.

Description of disease: In horses, the infection is usually inapparent. Affected horses show clinical signs that include pyrexia, depression, muscle tremors, and ataxia. In pigs, abortions and stillbirths can occur when pregnant sows are infected with JEV for the first time. Infected pregnant sows usually show no clinical signs.

Identification of the agent: For virus isolation, brain material is collected from sick or dead horses that have demonstrated the clinical signs of encephalitis. Isolation procedures include the inoculation of mice and cell cultures. A suspension of brain tissue is inoculated intracerebrally into 2–4-day-old mice. If the mice show neurological signs followed by death within 14 days, then virus identification can be carried out by cell culture. The virus can also be isolated in primary cell cultures made from chicken embryos, porcine or hamster kidney cells and established cell lines such as African green monkey kidney (Vero), baby hamster kidney (BHK-21), or mosquito (C6/36) cells. Identification of the virus isolated in mice or in tissue cultures is confirmed by serological or nucleic acid detection methods such as reverse-transcription polymerase chain reaction assay.

Serological tests: Antibody assay is a useful technique for determining the prevalence of infection in a horse population, and also for diagnosing Japanese encephalitis in diseased individuals. The assay methods include virus neutralisation (VN), haemagglutination inhibition (HI), complement fixation (CF), and enzyme-linked immunosorbent assay (ELISA). There is serological cross-reactivity with other flaviviruses, such as West Nile virus, which can confuse the diagnosis. The plaque reduction VN test is the most specific and can be used to differentiate JEV infection from other flavivirus infections. Because of the cross-neutralisation within the Japanese encephalitis serocomplex, diagnosis by serology should be confirmed, preferably by virus isolation.

Requirements for vaccines: Two types of vaccines are commercially available in several Asian countries for humans and animals. For horses, inactivated vaccines prepared from infected mouse brains or in cell cultures have been used. For pigs, inactivated and live-attenuated vaccines are available.

A. INTRODUCTION

Japanese encephalitis (JE) is a disease caused by a mosquito-borne flavivirus that elicits clinical signs of encephalitis in infected humans and horses and can be fatal (Fenner et al., 1992; Hoke Jr & Gingrich, 1994). However infections in humans and horses usually result in subclinical infection. JE virus (JEV) also causes stillbirths and abortions in pigs, though infected pregnant sows usually demonstrate no clinical signs and the infection does not affect the future pregnancies.

JEV is maintained in nature among mosquitoes, wild birds and pigs. Pigs act as important amplifiers of the virus, and birds can also be involved in its amplification and spread. The principal vector of JEV is Culex tritaeniorhynchus in most parts of Asia. Other culicine mosquitoes also play a role as vectors. Because of low
titres and short duration of viraemia, humans and horses do not transmit viruses to biting mosquitoes and are considered as dead-end hosts. JEV is widespread in eastern, south-eastern and southern Asian countries and has recently spread to western India and to the western Pacific region including the eastern Indonesian archipelago, Papua New Guinea and Northern Australia (Mackenzie, 2005).

JEV belongs to the genus Flavivirus in the family Flaviviridae. JEV constitutes Japanese encephalitis serocomplex along with several important zoonotic viruses that include West Nile virus (see chapter 2.1.20), St Louis encephalitis virus, Murray Valley encephalitis virus and Kunjin virus. Only a single serotype of JEV has been identified, although antigenic and genetic differences among JEV strains have been demonstrated by several techniques including complement fixation, haemagglutination inhibition, neutralisation tests using polyclonal or monoclonal antibodies (Ali & Igarashi 1997; Banerjee, 1986; Hale & Lee, 1954; Hasegawa et al., 1994; Kimura-Kuroda & Yasui, 1986) and oligonucleotide fingerprints of viral RNA (Banerjee & Ranadive, 1989; Hori et al., 1986). Envelope (E) gene analysis was shown to be a good representative of the phylogenetic analysis of JEV. To date, five genotypes of JEV have been described based on phylogenetic analysis of the viral E gene (Solomon et al., 2003; Uchil & Sachidanandam, 2001; Williams et al., 2000).

**B. DIAGNOSTIC TECHNIQUES**

The definitive diagnosis of Japanese encephalitis in horses depends on the isolation of the causal virus. The isolation rate of virus from diseased or dead horses is usually very low, which may be due to the instability of the virus under certain environmental conditions, and also to the presence of antibody in infected animals. Clinical, serological and pathological findings are of assistance in diagnosis. Diagnosis is also possible by the detection of specific IgM and IgG antibodies in cerebrospinal fluid by enzyme-linked immunosorbent assay (ELISA) methods (Burke et al., 1982). Viral nucleic acid has been detected in the brain of infected horses by reverse transcription polymerase chain reaction (RT-PCR) (Lian et al., 2002).

The specimens collected for virus isolation are portions of the corpus striatum, cortex or thalamus of the brain of affected horses. The virus can also be isolated from blood and spinal cord samples. All materials should be refrigerated immediately after collection and frozen to –80°C if specimens are to be stored for more than 48 hours. Any potentially infected materials must be handled following containment level 3 procedures (see Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities), to prevent the risk of human infection. Humans may be infected by direct contact of infectious material with broken skin or mucous membranes, accidental parenteral inoculation or aerosol. Diagnosticians collecting samples should also take the appropriate precautions. A human vaccine is available and at risk field veterinarians and laboratory workers should be vaccinated.

1. Identification of the agent

Samples of brain and spinal cord are homogenised in a 10% suspension in buffered saline, pH 7.4, containing calf serum (2%) or bovine serum albumin (0.75%), streptomycin (100 µg/ml) and penicillin (100 units/ml). The calf serum should be free from Japanese encephalitis antibodies. The suspension is centrifuged at 1500 g for 15 minutes, and the supernatant fluid is removed for inoculation: 0.02 ml is inoculated intracerebrally into 2- to 4-day-old mice. The inoculated mice are kept under clinical observation for 14 days. No clear clinical signs may develop, but anorexia becomes evident by the disappearance of the white milk spot on the abdomen. The skin then changes colour from pinkish to dark red, and convulsions develop immediately before the mice die. Severely ill mice should be euthanised. Brains of dead and euthanised mice are collected and stored at –80°C for a further passage.

To identify the virus, sucrose/acetone-extracted antigen is prepared from the infected mouse brains of a second passage in mice as described in Section B.2.b.1. This antigen is checked for its ability to agglutinate the red blood cells (RBCs) of 1-day-old chickens or of geese at different pH values between pH 6.0 and 7.0, at intervals of pH 0.2, according to the method described (Clarke & Casals, 1958). Briefly, RBC suspensions of 1/24 dilution are prepared in the diluent with different pH values. In a 96-well plate with a U-shaped bottom, 25 µl volumes of the extracted antigen are diluted serially. Then, 25 µl of the diluted RBCs is added to each well. The plate is incubated at 37°C for 1 hour, and the haemagglutination result is read. If the antigen is able to haemagglutinate red blood cells, it is used in a haemagglutination inhibition (HI) test using a Japanese encephalitis antisera.

Primary cultures of chicken embryo, African green monkey kidney (Vero), baby hamster kidney (BHK) cells, or the C6/36 mosquito cell line (a cloned cell line from Aedes albopictus) may be used for virus isolation. The specimens, such as brain and blood taken from animals suspected of being infected, and the brain suspension from mice after inoculation, are inoculated onto the cell cultures. Monoclonal antibodies specific to flavivirus and Japanese encephalitis virus are used to identify the virus in the indirect fluorescent antibody test (Lian et al., 2002). RT-PCR assay can also be used for identification of JEV in clinical specimens or cell culture fluid using appropriate primers specific for JEV (Chung et al., 1996; Jan et al., 2000; Lian et al., 2002; Tanaka, 1993). Recently a new nucleic acid detection method, reverse transcription loop-mediated isothermal amplification (RT-
2. Serological tests

Serological tests are useful to determine the prevalence of infection in an animal population, the geographical distribution of the virus, and the degree of antibody production in vaccinated horses. If serology is to be used for the diagnosis of the disease in individual horses, it should be remembered that horses in an endemic area may have been inapparently infected with the virus or may have been immunised with a vaccine. Antibody assay is a useful technique for determining the prevalence of infection in a horse population, and also for diagnosing JE in diseased individuals. The assay methods include virus neutralisation (VN), haemagglutination inhibition (HI), complement fixation (CF), and enzyme-linked immunosorbent assay (ELISA). Diagnosis requires a significant rise in antibody titre in paired sera collected during the acute and convalescent phases. The specificity of each serological test should also be considered. A latex agglutination test to detect swine antibodies to Japanese encephalitis has recently been described (Xinglin et al., 2002). An ELISA for antibodies to a nonstructural protein (NS1) of JEV can be used to differentiate antibodies following natural infection from those induced by inactivated vaccines.

In some regions of the world, there is a need to carry out additional tests for related viruses before an unequivocal diagnosis of Japanese encephalitis can be made. For example, in Australia antigenically closely related viruses of Murray Valley encephalitis and Kunjin virus occur. Recent expansion of the distribution of West Nile virus in North America, where St Louis encephalitis virus was known to be endemic, further illustrates the flexibility of flaviviruses to adapt to new environments. The presence of antibody to these other flaviviruses can make serological diagnosis of Japanese encephalitis difficult. There is some cross reactivity with other flaviviruses on all the tests; the plaque reduction VN test is the most specific, especially if a 90% neutralisation threshold is used.

a) Virus neutralisation

The plaque reduction test using chicken embryo primary cultures, African green monkey kidney (Vero) cells or baby hamster kidney (BHK) cells is sensitive and the most specific serological procedure available. The cross reaction with other flaviviruses is minimal; however, if an animal has a high titre to another flavivirus, such as West Nile virus, there may be a low cross-neutralising antibody titre to JEV.

Japanese encephalitis virus (Nakayama strain or JaGAr-01 strain) is propagated in cell culture. The supernatant of virus-infected cell culture is stored in aliquots at –80°C and is used for virus neutralisation test.

- Test procedure
  i) Dilute the test sera in 1/10 dilution in culture medium and then inactivate sera for 30 minutes in a water bath at 56°C.
  ii) Make twofold serial dilutions of the inactivated sera in cell culture medium in test tubes.
  iii) Dilute stock virus in cell culture medium to make 200 plaque-forming units (PFU)/0.2 ml.
  iv) Mix one volume of each diluted serum with an equal volume of diluted virus in test tubes. Positive and negative control sera are included in each test. Culture medium is also included for the virus control.
  v) Incubate for 90 minutes in a water bath at 37°C.
  vi) Add 200 µl of the virus/serum mixture to each well of a preformed monolayer of BHK-21 or Vero cells in 6-well culture plates.
  vii) Incubate the plates in a CO₂ atmosphere for 90 minutes at 37°C.
  viii) Remove the inoculum and add 1 ml of overlay medium (1.5% carboxymethyl cellulose, 1% fetal calf serum in Eagle’s medium).
  ix) Incubate the plates in a CO₂ atmosphere for 4–6 days at 37°C.
  x) After removing the culture fluid, fix the plates in a solution containing 2.5% potassium dichromate, 5% glacial acetic acid and 5% formalin for 30 minutes at room temperature. Wear rubber gloves when handling the fixing solution.
  xi) Stain the plate in 0.1% crystal violet solution for 30 minutes at room temperature.
  xii) Discard the stain and rinse the cells with tap water.
  xiii) Air dry the cells and count the plaques.
xiv) Estimate the serum dilution that reduces the number of plaques by 50% or more of the control without serum. The validity of the test is confirmed when the mean number of plaques in the control wells without serum is within 50 to 150 plaques.

b) **Haemagglutination inhibition**

The HI test is widely used for the diagnosis of Japanese encephalitis, but has cross-reactivity with other flaviviruses. For this test, the sera must first be treated with acetone or kaolin, and then adsorbed with homotypic RBCs to remove any nonspecific haemagglutinins in the test sera. The RBCs of geese or of 1-day-old chickens are used at the optimum pH (see the table below). The optimal pH is dependent on the JEV strain used. The test should be conducted with the treated sera and 8 units of standard antigen; this is commercially available in some countries.

- **Haemagglutination (HA)**

- **Preparation of virus antigen**

  1. **Sucrose–acetone extraction of antigen from infected suckling mouse brains (SMB)**
     i) Homogenise infected SMB with 4 volumes of 8.5% sucrose.
     ii) Add the homogenate drop-wise to 20 times its volume of cold acetone.
     iii) Centrifuge (500 g for 5 minutes), then remove the supernatant.
     iv) Resuspend the sediment with the same volume as above of cold acetone, and keep in an ice bath for 1 hour.
     v) Centrifuge (500 g for 5 minutes), then remove the supernatant.
     vi) Pool the sediment with cold acetone in a single tube.
     vii) Centrifuge (500 g for 5 minutes), then remove the supernatant.
     viii) Spread the sediment inside the tube and vacuum dry for 1–2 hours.
     ix) Dissolve the dry sediment with saline: 0.4 volume of original homogenate.
     x) Centrifuge (8000 g for 1 hour, 4°C). The supernatant is ready for use.

  2. **Infected fluid of Aedes albopictus, clone C6/36, cell line**
     i) Harvest the infected fluid after incubation of the infected cultures at 28°C for 1 week.
     ii) Centrifuge (1000 g for 15 minutes). The supernatant is ready for use.

- **Preparation of goose red blood cells**

  1. **Solutions**

     *Acid-citrate-dextrose (ACD):* 11.26 g sodium citrate (Na₃C₆H₅O₇·2H₂O); 4.0 g citric acid (H₃C₆H₅O₇·H₂O); 11.0 g dextrose (C₆H₁₂O₆); distilled water to a final volume of 500 ml. Autoclave at 10 lb (1.7 unit pressure) for 10 minutes.

     *Dextrose-gelatine-veronal (DGV):* 0.58 g veronal (Barbital); 0.60 g gelatine; 0.38 g sodium veronal (sodium barbital); 0.02 g (0.026 g) CaCl₂ (for CaCl₂·2H₂O); 0.12 g MgSO₄·7H₂O; 8.50 g NaCl; 10.0 g dextrose; distilled water to a final volume of 1000 ml. Autoclave at 10 lb (1.7 unit pressure) for 10 minutes (five times stock volume is easier to prepare).

  2. **Blood collection**

     1.5 ml of ACD + 8.5 ml of blood (0.5 ml of ACD + 2.8 ml of blood).

  3. **Washing (sterile)**

     i) Total blood + 2.5 volume of DGV. Centrifuge (500 g for 15 minutes), then remove the supernatant.
     ii) Resuspend the sedimented RBCs in three volumes (total blood) of DGV.
     iii) Centrifuge (500 g for 15 minutes), then remove the supernatant. Repeat steps 2 and 3 twice more (total four spin cycles).
     iv) Transfer the final RBC suspension to a flask with aluminium foil cover.
4. Adjusting the RBC concentration
   i) 0.2 ml of the RBC suspension + 7.8 ml of 0.9% NaCl (1/40 dilution).
   ii) Read the optical density (OD)$_{490}$ in a spectrophotometer with 10 mm tube.
   iii) Adjust the RBC stock so that 1/40 dilution gives 0.450 of OD$_{490}$. (Final volume = Initial volume $\times$ absorbance OD$_{490}$/0.450.)
   iv) Store the RBC stock in a refrigerator for up to 3 weeks.
   v) Before use, resuspend the RBCs gently and dilute 1/24 in virus-adjusting diluent (VAD).

- Antigen dilution
  1. Stock solutions (should be kept at 4°C): 1.5 M NaCl: 87.7 g NaCl and distilled water to a final volume of 1000 ml; 0.5 M boric acid: 30.92 g H$_3$BO$_3$ and hot distilled water to a final volume of 700 ml (dissolve boric acid and cool down); 1 N NaOH: 40.0 g NaOH and distilled water to a final volume of 1000 ml; borate saline (BS), pH 9.0: 80 ml 1.5 M NaCl, 100 ml 0.5 M H$_3$BO$_3$, 24 ml 1.0 N NaOH, and distilled water to a final volume of 1000 ml; 4% bovine albumin: 4 g bovine albumin fraction V (Armour), 90 ml BS, pH 9.0, adjust pH to 9.0 with 1 N NaOH, and BS, pH 9.0, to make a final volume of 1000 ml.

  2. Antigen diluent: 0.4% bovine albumin/borate saline (BABS): 10 ml 4% bovine albumin, pH 9.0, and 90 ml BS, pH 9.0.

  3. Twofold serial dilution of antigen with BABS on U-bottom microtitre plate.

- Addition of goose red blood cells
  1. Stock solutions
     1.5 M NaCl
     0.5 M Na$_2$HPO$_4$: 70.99 g Na$_2$HPO$_4$ (for Na$_2$HPO$_4$, 12 H$_2$O: 179.08 g), and distilled water to a final volume of 1000 ml.
     1.0 M NaH$_2$PO$_4$: 138.01 g NaH$_2$PO$_4$.H$_2$O (for Na$_2$PO$_4$, 2H$_2$O: 156.01 g), and distilled water to a final volume of 1000 ml.

  2. Working solution: virus adjusting diluent (VAD)

<table>
<thead>
<tr>
<th>VAD (pH)</th>
<th>1.5 M NaCl</th>
<th>0.5 M Na$_2$HPO$_4$</th>
<th>1.0 M NaH$_2$PO$_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>100</td>
<td>32</td>
<td>184</td>
</tr>
<tr>
<td>6.2</td>
<td>100</td>
<td>62</td>
<td>160</td>
</tr>
<tr>
<td>6.4</td>
<td>100</td>
<td>112</td>
<td>144</td>
</tr>
<tr>
<td>6.6</td>
<td>100</td>
<td>160</td>
<td>120</td>
</tr>
<tr>
<td>6.8</td>
<td>100</td>
<td>192</td>
<td>104</td>
</tr>
<tr>
<td>7.0</td>
<td>100</td>
<td>240</td>
<td>80</td>
</tr>
</tbody>
</table>

Values of VADs are not the pH of each VAD, but the pH after each VAD is mixed with an equal volume of BABS, pH 9.0.

3. Procedures
   i) 1 volume of stock goose RBCs + 23 volumes of VAD (1/24 dilution).
   ii) Add 25 µl of diluted RBCs to each well on microtitre plate containing diluted antigen (25 µl/well).
   iii) Incubate at 37°C for 1 hour, then read the result.
      ++ Complete agglutination (uniformly thin pellicle of RBCs following the curvature of the well bottom)
      + Partial agglutination (a ring associated with a rough or thinner pellicle)
      ± Minimal agglutination (a button on a thin or scattered pellicle)
      – Negative agglutination (clearly defined button with no RBC film)

   End point is the last dilution (highest dilution) in which ++ or + is observed.
   Titre: the reciprocal of the end point dilution.
• **Haemagglutination inhibition**

• **Preparation of test sera**

1. **Blood collection and separation of the sera**
   i) Incubate blood specimen at 37°C for 1 hour and then at 4°C overnight. If the test must be performed immediately, incubating the sample for 2–3 hours at 37°C can replace the overnight incubation.
   ii) Centrifuge (2000 g for 15 minutes) to separate the serum from the clot.
   iii) Heat inactivate at 56°C for 30 minutes.
   iv) Store at ~20°C if not processed immediately.

2. **2-mercaptoethanol treatment (perform this step when IgM antibody titres should be determined)**
   i) Place 50 µl of the sera into two small test tubes.
   ii) Add 150 µl of 0.13 M 2-mercaptoethanol in PBS into one test tube, and 15 µl PBS into another tube.
   iii) Incubate at 37°C for 1 hour, then cool in an ice bath.

3. **Acetone extraction**
   i) Add 2.5 ml of cold acetone to serum in a test tube. Cap with rubber stoppers, mix well and extract for 5 minutes in an ice bath.
   ii) Centrifuge cold (1500 g for 5 minutes), then remove the supernatant.
   iii) Repeat steps i and ii once more.
   iv) Spread the sediment inside tubes and vacuum dry at room temperature for 1 hour.
   v) Add 0.5 ml of BS, pH 9.0, to each tube. Apply rubber stoppers. Dissolve the sediment overnight at 4°C to make 1/10 dilution of the sera.

4. **Kaolin extraction as an alternative to acetone extraction**
   i) 25% acid-washed kaolin in BS, pH 9.0.
   ii) 1 volume of sera + 4 volumes of BS + 5 volumes of 25 % kaolin.
   iii) Extract at room temperature for 20 minutes with occasional shaking.
   iv) Centrifuge (1000 g for 30 minutes). The supernatant is 1/10 dilution of the sera.

5. **Adsorption with goose RBCs**
   i) To each treated serum add 1/50 volume of packed goose RBCs.
   ii) Adsorb for 20 minutes in an ice bath.
   iii) Centrifuge (800 g for 10 minutes). The supernatant is ready for the HI test (1/10 dilution).

• **Haemagglutination inhibition test**

1. **Primary haemagglutination titration of antigen**
   Dilute the antigen to make 8 units/50 µl.

2. **Serial twofold dilution of test sera on microtitre plate**

   **Serum–antigen reaction**
   Add 25 µl of diluted antigen into each well containing diluted test sera. Place the remainder of the antigen in empty wells and incubate at 4°C overnight.

3. **Secondary haemagglutination titration of the antigen**
   i) Serially dilute the prepared antigen (8 units/50 µl) twofold in a 25 µl system.
   ii) Add 25 µl of BABS to each well to make 50 µl/well.
Chapter 2.1.7. – Japanese encephalitis

4. **Addition of goose RBCs**
   
i) Dilute RBC stock (1/24) in VAD.
   ii) Distribute 50 µl into each well containing 50 µl of serum antigen mixture or secondary titration of antigen.
   iii) Incubate at 37°C for 1 hour then read the result.

   Serum HI titre: the reciprocal of the highest dilution of the test sera showing complete inhibition of HA.

5. **Interpretation of the results**

   Four-fold difference between the titre in the acute and convalescent sera is considered to be a significant rise or fall and is diagnostic of infection with a virus antigenically related to that used in the test.

   **c) Complement fixation**

   Complement fixation (CF) is sometimes used for serological diagnosis. The antigen for this test is extracted with acetone/ether from the brains of inoculated mice.

   **Antigen preparation**
   
i) Extract and weigh the brains of the inoculated dead mice.
   ii) Add to the brains 20 volumes of cold acetone, kept at –20°C, and homogenise.
   iii) Centrifuge the suspension at 5000 g for 5 minutes at 4°C, and remove the supernatant.
   iv) Add to the pellet the same volume of cold acetone as used in step ii above, and mix well.
   v) Extract with acetone by keeping the pellet at –20°C for 20 minutes, and repeat the centrifugation described in step iii above.
   vi) Repeat steps iv and v.
   vii) Repeat steps iv and v, but this time use cold acetone/ether (equal volume mixture).
   viii) Repeat steps iv and v twice using cold ether.
   ix) Remove the supernatant by aspirator and spread the pellet over the centrifuge tube.
   x) Vacuum dry for 1–2 hours.
   xi) Dissolve the pellet in cold saline (2 ml/g of brain) and keep at 4°C overnight.
   xii) Centrifuge at 5000 g for 1 hour. The supernatant is the antigen.

   **Test procedure**
   
i) Heat-inactivate the test sera at 1/4 dilution in gelatin–veronal buffer.
   ii) Serially dilute the sera twofold in a 96-well microtitre plate (25 µl).
   iii) Add 25 µl of 4 units of antigen and mix by vibration.
   iv) Add 50 µl of 2 units of complement (pooled fresh guinea-pig serum).
   v) Mix by vibration and incubate at 4°C for 18 hours.
   vi) Leave the microtray at room temperature for 15 minutes.
   vii) Add 25 µl of sensitised sheep RBCs to each well.
   viii) Mix by vibration and incubate at 37°C for 30 minutes, then read the result.
   ix) The highest dilution of test sera showing no haemolysis is the titre of the sera by CF test. A rise or drop of four-fold or more in the titre is considered to be significant.

   **d) Enzyme-linked immunosorbent assay**

   An indirect ELISA for prevalence studies of JE antibody in pigs has been described (Yang Dongkun *et al.*, 2006). Conventional serological methods cannot differentiate antibodies induced by natural infection from those induced by vaccination. To detect antibodies induced by natural infections but not those induced by inactivated vaccines, an ELISA method that detects antibodies against non-structural 1 (NS1) protein of JEV, which is induced only by infection, has been developed (Konishi *et al.*, 2004). In human medicine, virus-specific IgM assays have proved useful for diagnosis.
C. REQUIREMENTS FOR VACCINES

Two types of vaccines are commercially available in several Asian countries for humans and animals. For humans, inactivated vaccines prepared from infected mouse brains have been used for many years. An inactivated vaccine derived from Vero cell culture was licensed in 2009 in Japan. A live attenuated vaccine prepared in cell cultures has been used mainly in China (People’s Rep. of).

The vaccine for Japanese encephalitis in horses is prepared by formalin-inactivation of a virus suspension derived from infected mouse brains or cell cultures. For pigs, both inactivated and live-attenuated vaccines derived from cell cultures are used in Japan.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Background
   a) Rationale and intended use of the product

   Inactivated vaccines have been used to protect horses from encephalitis and possible subsequent death caused by JEV infection. In pigs, both inactivated and live attenuated vaccines have been used to protect pregnant sows from stillbirth.

2. Outline of production and minimum requirements for conventional vaccines
   a) Characteristics of the seed

   i) Biological characteristics

   Beijing-1 strain of JEV is used for vaccine production for humans in Japan. Other strains of JEV are also used for horses and pigs. The virus strains of inactivated vaccines must be lethal for 3-week-old mice when inoculated intraperitoneally, and must be able to grow in a primary culture of porcine kidney or susceptible cell lines. The virus strain for live attenuated vaccine must be lethal for 2-day-old mice when inoculated intracerebrally but shows no viraemia when inoculated in 1-month-old piglets and does not infect fetuses when inoculated in pregnant sows of the first month of gestation. The viruses have the capacity to haemagglutinate the RBCs of geese, 1-day-old chickens or pigeons. The viruses must be able to be neutralised by a standard antiserum to JEV.

   ii) Quality criteria (sterility, purity, freedom from extraneous agents)

   The seed virus must be free of contaminating bacteria, fungi, mycoplasmas and viruses. Tests for sterility and freedom from contamination of biological materials are found in chapter 1.1.7.

   b) Method of manufacture

   i) Procedure

   The virus is grown in the brains of 3–4-week-old mice or in a monolayer cultures. The cultures should be tested to confirm that they do not contain adventitious agents (see chapter 1.1.7). The seed virus is inoculated intracerebrally into mice. The brains of those mice that show severe clinical signs of encephalitis are collected. These brains are homogenised in PBS, centrifuged at 1500 \( g \) for 30 minutes, and the supernatant fluid is processed as the virus suspension.

   The seed virus is inoculated into cell cultures and the fluids are later harvested separately from each batch when virus replication is at its maximum. This fluid is filtered, or centrifuged at 1500 \( g \) for 30 minutes, and the supernatant fluid is processed as the virus suspension.

   For inactivated vaccine, formalin (0.5%) is added to the suspension to inactivate any live virus; this is considered to be the ‘undiluted virus suspension’. Adjuvant may be added to enhance its immunogenicity.

   The passage levels should not exceed three more than the original virus and two more than the seed virus. It is recommended that the original and seed viruses be maintained below –70°C, or below 5°C after lyophilisation.

   ii) Requirements for substrates and media

   Primary cell cultures for vaccine production must be obtained from healthy animals. Primary and line cells must be tested for and free from extraneous bacteria, fungi, mycoplasmas and viruses. Culture media, fetal bovine serum and supplements must be tested to confirm sterility.
iii) In-process control

The virus suspension should be examined for bacterial and fungal contamination by culture techniques and for virus infectivity by intracerebral mouse inoculation or inoculation into cell cultures. The inactivated undiluted virus suspension should be re-examined for contamination by cell culture and by microscopy after staining, and should be checked by intracerebral mouse inoculation to ensure complete inactivation of the virus by the formalin.

a) Sterility

iv) Final product batch tests

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

To test the inactivity of the final product, ten 3-week-old mice are inoculated intracerebrally with 0.03 ml of the product and observed daily. All mice tested should survive and show no encephalitis after 14 days' observation to ensure the complete inactivation of live virus.

The final product of inactivated and live attenuated vaccine must be tested for immunogenicity.

Inactivated vaccine: The product is diluted 1/10 in PBS. Thirty mice aged 2–3 weeks are inoculated intraperitoneally with 0.1 ml of the diluted product twice at 3-day intervals. There should be an equivalent uninoculated control group. Ten mice of each group are challenged intraperitoneally with tenfold dilutions (1/10, 1/100 and 1/1000) of the appropriate virus such as Nakayama strain 8 days following the first inoculation, and observed for 14 days. The survival rate should be more than 40% in the immunised group and the mortality rate in the control group should be more than 90%. The titre of challenge virus should not be less than 10^3 LD_{50} (50% lethal dose) per 0.2 ml.

c) Requirements for authorisation

i) Safety requirements

The live attenuated vaccine shows no viraemia when inoculated in 1-month-old piglets and does not infect fetuses when inoculated in pregnant sows in the first month of gestation. For inactivated vaccine, ten 3-week-old mice are inoculated intracerebrally with 0.03 ml of the product and no death must be observed after 14 days.

ii) Efficacy requirements

As JEV is maintained among vector mosquitoes, pigs and wild birds, control and eradication of JEV using vaccines is difficult. Vaccines are used to protect horses from encephalitis and pregnant sows from stillbirths.

iii) Stability

The final product must be shown to be fully effective for 12 months when stored at 4°C.

3. Vaccines based on biotechnology

No biotechnology-based vaccine is currently available.

REFERENCES


Chapter 2.1.7. — Japanese encephalitis


* * *
CHAPTER 2.1.8.

LEISHMANIOSIS

SUMMARY

Leishmaniosis is not a single entity but comprises a variety of syndromes due primarily to at least 16 species and subspecies of Leishmania. Dogs are commonly affected by L. infantum and L. chagasi (now regarded as synonyms), but canine infections with L. tropica, L. major and L. braziliensis have also been reported. In humans, the clinical spectrum ranges from asymptomatic infections to those with high mortality, with three distinct forms being classically described: visceral (VL), cutaneous (CL) and mucocutaneous (MCL). The vectors of these diseases are phlebotomine sandflies belonging to the genera Phlebotomus and Lutzomyia.

Identification of the agent: When clinical signs and characteristic lesions are present in affected humans and animals, the demonstration of the parasites in stained smears of splenic, bone marrow and lymph node aspirates, of skin scrapings, and in tissue biopsies, gives a positive diagnosis. If the infection is low grade, detection of parasites is possible only by attempting in-vitro or in-vivo isolation or by polymerase chain reaction (PCR). As there are very few morphological differences among various species, any isolated Leishmania organism must be identified by molecular, biochemical and/or immunological methods. Several centres throughout the world are presently using isoenzyme, DNA and antigen characterisation to identify the agent.

Serological tests: Serology is the preferred method for diagnosis of canine leishmaniosis and VL, even during the early stages of the disease. In subclinical forms, seropositive cases are confirmed by parasitological diagnosis or PCR. Serology is of less value for CL and MCL. Of the several serological techniques available, the indirect fluorescent antibody test and the enzyme-linked immunosorbent assay are the most suitable. Serodiagnostic antigens need to be prepared in the laboratory, though some commercial products are under evaluation.

Delayed hypersensitivity test: The leishmanin skin test is useful for determining the distribution of human infections, distinguishing immune from nonimmune cases. The test is positive in CL, MCL and cured VL, but negative in active VL.

Requirements for vaccines and diagnostic biologicals: There is no effective vaccine available at present for use in dogs or humans. Leishmanin is no longer available commercially and needs to be standardised.

A. INTRODUCTION

Leishmaniosis is caused by the vector-borne protozoan parasite, Leishmania. Various forms of clinical manifestations of human leishmaniosis have been described and divided into three entities: visceral leishmaniosis (VL, kala azar), cutaneous leishmaniosis (CL, oriental sore, uta, pian boi, chiclero’s ulcer) and mucocutaneous leishmaniosis (MCL, espundia) (World Health Organization [WHO], 1990). In the New World1, leishmanioses are caused by L. braziliensis complex (MCL and CL), L. mexicana complex (CL), L. peruviana (CL) and L. infantum (VL and CL); in the Old World, the aetiological agents are L. donovani (VL), L. infantum (VL and CL), L. tropica (CL), L. major (CL) and L. aethiopica (CL). Leishmania infantum and L. chagasi have been found to be identical by biochemical genotyping and should be regarded as synonyms (Mauricio et al., 2000). The diseases are mainly zoonoses with two exceptions, that of CL due to L. tropica in urban areas of Near and Middle East, and that of VL due to L. donovani the Indian sub-continent (northern India, Nepal and Bangladesh). Canine leishmaniasis (CanL) is a chronic viscero-cutaneous disease caused by L. infantum (= L. chagasi), for which the dog acts as the source reservoir. In some instances, parasites belonging to L. braziliensis complex, L. major and L. tropica have been

1 In this chapter, the term ‘New World’ refers to the Americas, and the term ‘Old World’ refers to Europe, Africa and Asia.
isolated from this host (Mohebali et al., 2005; Ryan et al., 2003). The vectors of leishmanioses are phlebotomine sandflies belonging to the genera Lutzomyia (New World) and Phlebotomus (Old World).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Clinical examination of suspected cases, parasitological diagnosis and immunodiagnosis are the routine methods available for the diagnosis of leishmaniosis. However, the demonstration of the parasite is the only way to confirm the disease conclusively. In VL and CanL, isolation and identification of the parasite from biopsies (lymph node, bone marrow, and spleen aspirate) coupled with molecular and immunodiagnostic tests are recommended. Parasitological diagnosis is necessary for confirmation of CL (through lesion scraping or needle aspiration from the edge of the lesions) as neither clinical examination nor serology is adequate. Smears of biopsy material are stained with Giemsa stain and examined microscopically at ×600–1000 magnification. Material should also be cultured in appropriate media at 22–26°C.

Morphological characteristics of amastigotes (in humans and mammalian hosts) and promastigotes (in phlebotomine sandflies and in cultures) are the following:

- **Amastigote**: small intracellular rounded or oval body, 1.5–3 × 2.5–6.5 µm in size, found in vacuoles within the cytoplasm of the macrophages. There is no free flagellum. The organism has a relatively large nucleus and a kinetoplast consisting of a rod-like body and a dot-like basal body;

- **Promastigote**: elongated extracellular organism, body size 15–20 × 1.5–3.5 µm with a single flagellum 15–28 µm long, arising close to the kinetoplast at the anterior. The nucleus is situated centrally.

The choice of the isolation and culture methods will depend on the immediate circumstances and on the technical capability and experience of the laboratory staff (WHO, 1989). **In-vitro** isolation offers certain advantages over the **in-vivo** methods: cultures become positive more rapidly (5–30 days compared with months for lesions to appear on an animal) and the materials are less expensive. However, for **in-vitro** isolation, the techniques used should be carried out under strictly sterile conditions, which is rarely feasible in the field. Unfortunately, there is still no ‘universal’ culture medium in which all the different leishmanias will grow easily, and it is almost impossible to predict which medium will be best suited to the growth of a particular isolate of Leishmania. Individual laboratories have to find the most suitable medium among biphasic blood agar media and tissue culture media supplemented with fetal calf serum (Evans, 1987). When attempting primary isolation of unknown organisms, a blood agar-based medium should be used — preferably NNN medium (Novy, McNeil and Nicolle), otherwise brain–heart infusion (BHI) agar medium or EMTM (Evans’ modified Tobie’s medium) should be used. For bulk cultivation of established isolates, suitable media are reported in Section B.1.a (see Evans, 1987 for media composition). The organisms from patients with VL and MCL can be very difficult to cultivate. The parasites sometimes die when subcultured, even when the initial isolation is successful. This seems especially common when the initial isolation has been introduced into a rich medium. Often this can be overcome if subcultures are made into less nutritionally rich media, such as NNN, or one of the semisolid media such as ‘sloppy Evans’ or semisolid Locke blood agar.

Hamster (Mesocricetus auratus) is the most commonly used animal for **in-vivo** isolation. Tissue suspensions or aspirates are inoculated intradermally into the nose and/or feet in the case of detection of dermotropic parasites. When the material is suspected to be infected with parasites causing VL, the inoculation should preferably be made by the intraperitoneal route. The resulting infection becomes apparent, weeks or months later, by the development of a nodule or ulcer at the site of inoculation, and in case of visceralotropic parasites, the infection becomes apparent, some months later, by massive infection of internal organs. The examination of Giemsa-stained smears of hamster tissue suspension/aspirate will show amastigotes. BALB/c mice are commonly used for the diagnosis of L. major.

Several techniques are now being used in many centres to identify the different Leishmania species, subspecies or strains.

a) Isoenzyme characterisation, also known as MLEE (multi-locus enzyme electrophoresis), is the reference method for species identification (Hart, 1989; Rioux et al., 1990; WHO, 1989; 1990), although this technique requires cultivation of a large number of parasites (5 × 10⁹ – 1 × 10¹⁰). The principles of enzyme electrophoresis are as follows: soluble enzymes are extracted from the organisms grown in media for bulk cultivation (BHI medium, MEM/FCS/EBLB [minimal essential medium/fetal calf serum/Evans’ blood lysate broth] medium, Schneider’s Drosophila medium). A small amount of the extract is then placed in an inert supporting substance, the matrix, containing a buffer at a fixed pH. The matrix is usually starch gel, but it could equally well be absorbent cellulose acetate, acrylamide or agarose. The pH of the buffer in the matrix is usually chosen so that the isoenzymes are negatively charged. A direct current is passed through the matrix carried by the ions in the buffer. When electrophoresis is completed, most proteins will have moved in
the matrix towards the anode, depending on the amount of negative charge. If stained at this stage with a general protein stain, many bands will be seen. However, the high substrate and cofactor specificity of enzymes make it possible to stain only these proteins. Hence, the electrophoretic mobility of one particular enzyme can be compared among several organisms. The stained matrix with its collection of stained isoenzyme bands is known as a zymogram. Normally one or more extracts from reference organisms, in which the enzyme banding patterns are well documented, are included in the gel to aid the interpretation of results. Most enzymes used for characterisation purposes are stained by methods incorporating a dehydrogenase reaction. At least 12 enzymes should be examined; organisms showing identical zymograms are classified into zymodemes of a given species.

b) The monoclonal antibody (MAb) technique is applied to the analysis and classification of *Leishmania* species and subspecies (Grimaldi & McMahon-Pratt, 1996). For the production of the antibodies, BALB/c mice are immunised with membrane preparations from either promastigotes or amastigotes. Antibody-secreting hybridoma cultures are then selected and cloned by limiting dilutions. Specificity to *Leishmania* strains is assessed through immunofluorescence or immunoradiometric assays. This analysis should be quantitative, as the amount of the same surface antigen may vary among *Leishmania* species. Monoclonal antibodies have also been used in immunohistochemical techniques applied to tissue biopsies.

c) DNA hybridisation probes are a very specific tool the principle of which is to allow labelled, single-stranded nuclear or kinetoplast DNA sequences from well characterised standard strains to find and hybridise with homologous DNA sequences from or within unknown *Leishmania* isolates (Gramiccia et al., 1992; WHO, 1985a). Only complementary DNA sequences will form double-stranded DNA, which can be detected by autoradiography if the probe is radionlabelled, or by immunoenzymatic reaction. These techniques are sensitive enough to identify $10^2$–$10^3$ organisms spotted on to nylon filters. Much fewer parasites (<10) are required for identification through the *in situ* hybridisation technique.

d) Polymerase chain reaction (PCR)-based methods are available for diagnosis and/or identification of *Leishmania* from different types of human and canine samples. Essentially, techniques developed either to detect organisms from fresh or frozen, formalin-fixed and paraffin-embedded biopsies, or to identify established isolates of *Leishmania* include: (a) digestion of material with proteinase K and DNA extraction; (b) standard PCR amplification using oligonucleotide sequences (primers) selected from the small-subunit rRNA gene (Mathis & Deplazes, 1995), kinetoplast DNA minicircles (Maarten et al., 1992) or other highly repetitive genomic DNA sequences (Bulle et al., 2002; Piarroux et al., 1993); (c) analysis of amplification products by 1–2% agarose gel. To increase sensitivity, a nested or semi-nested PCR using internal primers from the above sequences can be performed. In human VL, PCR has a sensitivity comparable with that of culture-based methods, but gives results much faster. In CanL, the diagnostic efficacy of PCR as compared with serology depends on the natural course of the disease, the sensitivity being highest shortly after infection (Quinnell et al., 2001). In American CL and MCL, PCR appears to be consistently more sensitive than any previously recommended method of diagnosis (De Brujin et al., 1993). Different techniques have been described that improve both sensitivity and specificity of the method, such as the PCR-RFLP (restriction fragment length polymorphism) analysis in which the PCR products are digested by appropriate restrictions enzymes and the resulting restriction fragment pattern is analysed for species or strain identification (Minodier et al., 1997; Volpini et al., 2004). Real-time PCR methods, which allow the continuous monitoring of the accumulation of PCR products during amplification, have been described and are available commercially. They can be more sensitive than conventional PCR, and are mainly addressed to study the kinetics of infection and monitoring therapeutic response (Bell & Ranford-Cartwright, 2002; Bossolasco et al., 2003). In addition, real-time PCR has been reported to be useful for evaluating infections in less invasive samples such as blood (Francino et al., 2006).

### 2. Serological tests

Several serological tests are used for detecting anti-leishmanial antibodies. Sensitivity values reported below for each test, however, apply only to individuals who are not immunocompromised. A high percentage of patients with VL co-infected with human immunodeficiency virus (HIV) have been reported to be seronegative for anti-leishmanial antibodies (Gradoni et al., 1992).

a) **Indirect fluorescent antibody test**

The indirect fluorescent antibody (IFA) test is widely used because it is easy to perform. The test is genus specific, although significant cross-reactions have been reported in individuals infected with *Trypanosoma cruzi*. For these subjects, serological tests based on specific recombinant *Leishmania* antigens would be more appropriate (see Section B.2.b and d below). In Chagas disease-free areas, the IFA test for the diagnosis of clinical VL or CanL has a sensitivity of 96% and specificity of 98%, which is similar to the ELISA. Although amastigotes from frozen sections or smears of infected organs can be used as antigen, cultured promastigotes represent the commonest antigen source.
Antigen preparation

i) Harvest 3–4 ml of the liquid media of a 3-day-old culture showing flourishing promastigote growth (see Section B.1 for culture media).

ii) Wash the organisms three times with phosphate buffered saline (PBS), pH 7.2–7.4, by centrifugation at 350 g for 15 minutes at room temperature.

iii) Resuspend the final cell pellet in PBS and adjust the promastigote concentration to approximately 4 × 10⁶/ml with the aid of a haemocytometer.

iv) Distribute 30 μl of the promastigote suspension on to each circle of a multispot slide and allow to dry at room temperature.

v) Fix the promastigotes in cold acetone for 10 minutes, then put the slides into a plastic box and keep in a deep freezer (−35°C) for no longer than 2–3 months.

Test procedure

i) Wash the frozen antigen-coated slides in PBS and allow to dry at room temperature.

ii) Inactivate the sera for 30 minutes in a water bath at 56°C.

iii) Make doubling dilutions of test sera from 1/80 to 1/10,240 for human VL, and from 1/40 to 1/5120 for CanL. Positive and negative control sera, at dilutions of 1/80 and 1/160 for human VL, and of 1/40 and 1/80 for CanL, are also included in the test. No standard sera are available, but internal standards should be prepared and titrated.

iv) Distribute 30 μl of diluted serum samples on to each slide circle and incubate for 30 minutes at 37°C.

v) Remove the serum samples by vigorous washing in PBS, followed by immersion of the slides in PBS for 10 minutes. Allow the slides to dry.

vi) Distribute 30 μl of diluted fluorescein isothiocyanate (FITC)-conjugated anti-immunoglobulin on to each slide circle and incubate for 30 minutes at 37°C. FITC-conjugated anti-human and anti-dog immunoglobulins are commercially available. Follow the instructions for the appropriate dilution.

vii) Repeat step v and mount with a cover-slip in a few drops of PBS/glycerol (50% [v/v] of each).

viii) Read the slides under a fluorescent microscope. The highest dilution showing fluorescent promastigotes is taken to be the antibody titre. In human VL, the threshold titre usually ranges from 1/80 to 1/160, while in CanL it ranges from 1/40 to 1/160. As IFA test performance may vary in different laboratories, it is better for each laboratory to define its own threshold titre using defined positive and negative reference sera.

b) Enzyme-linked immunosorbent assay

The ELISA can be carried out on serum or on a measured volume of blood. The blood is collected by needle-prick on to suitable absorbent paper strips and allowed to dry. The sample is eluted and tested at a single dilution previously determined to give an acceptable sensitivity and specificity. This test can be used for seroepidemiological surveys under field conditions.

In the classical method, the antigen is prepared as follows: promastigotes harvested from cultures are washed four times with PBS, pH 7.2, at 1000 g for 15 minutes. The packed promastigotes are resuspended in twice their volume of distilled water, and then sonicated at medium amplitude in an ice bath. The suspension is left at 4°C overnight to allow the proteins to come into solution. After a final centrifugation at 4000 g for 10 minutes to eliminate the cellular debris, the overlay, representing the concentrated soluble antigen, is dispensed into vials and stored at −20°C until required. For use in the test, it is reconstituted with PBS to the predetermined optimal protein concentration (around 20 μg/ml) as measured by Lowry’s method. The ELISA is useful for the diagnosis of Old and New World leishmanioses. There is little or no cross-reaction with other diseases and, according to the Leishmania strain used, sensitivity can range from 86% to 99%.

A version of the ELISA called the Falcon assay screening test and enzyme-linked immunosorbent assay (FAST-ELISA) and which uses antigen-coated beads, is considered to be a sensitive, specific and field-adaptable test for visceral CanL with comparable sensitivity and specificity to the IFA test and ELISA. Whole blood or plasma can be evaluated quickly without the use of a microscope or spectrophotometer (Ashford et al., 1993).

A detergent-soluble promastigote antigen has been used in ELISA instead of the crude lysate, for the diagnosis of CanL. The detergent was Triton X-100 and the proteic extract was protected with protease inhibitors. Using this method, ELISA sensitivity increased to 99.5%, while its specificity was comparable with that of the IFA test (97%) (Mancianti et al., 1995).
The ELISA methods described above are all based on crude antigenic preparations. More recently, a recombinant antigen from a cloned protein of *L. infantum*, called rK39, has been reported to be highly reactive to sera from human and canine visceral leishmaniosis cases when run in an ELISA format. Using 25–50 ng of the antigen, 99% specificity and sensitivity was consistently found for immunocompetent human patients with clinical VL and for dogs with parasitologically proven disease (Badaro *et al.*, 1996; Scalone *et al.*, 2002). In HIV-positive patients, K39-ELISA showed higher sensitivity (82%) than the IFA test (54%) (Houghton *et al.*, 1998). The K39 antigen, which shows remarkable stability and reproducibility, is now produced commercially.

c) Direct agglutination test

The direct agglutination test (DAT) has been described for the diagnosis of VL and CanL. After test improvement, DAT has been validated as a specific and sensitive assay for field investigations (Boelaert *et al.*, 1999; Cardoso *et al.*, 2004; Ozbel *et al.*, 2000). The antigen consists of promastigotes harvested from cultures, washed in PBS, pH 7.2, treated with 0.4% trypsin (for 45 minutes at 37°C and then washed again), and stained with 0.02% Coomassie brilliant blue. Twofold serial dilutions of serum in PBS are made in V-bottomed microtitre-plate wells; 50 µl of antigen preparation is added to each well, and the plate is then carefully shaken by hand and left for 18 hours at room temperature. The test is read visually against a white background. Positive reactions are indicated by a clear sharp-edged blue spot.

A modified DAT for detection of specific anti-leishmanial antibodies in canine reservoir hosts is considered to be highly suitable for wide-scale epidemiological and ecological field work and diagnosis of CanL, having 100% sensitivity and 98.9% specificity (Harith *et al.*, 1988; 1989). The reliability of the test was improved by treating the test sera with 0.2 M 2-mercaptoethanol and incubating them at 37°C.

d) Rapid immunochromatographic assay (dipstick or strip-test)

A rapid immunochromatographic assay using rK39 as antigen (K39 dipstick or strip-test, commercially available) has been evaluated in different endemic settings of VL. The nitrocellulose membrane of the test kit holds an absorbent pad at one end, a band of immobilised anti-protein A antibody (used to detect IgG) at the other (control region), and a band of rK39 antigen in the middle (test region). A protein-A-colloidal gold conjugate is used as the immunochromatographic detection reagent. One small drop (20 µl) of the serum to be examined is placed on the absorbent pad before two large drops (100 µl) of test buffer are added to the pad, and the mixture is allowed to migrate up the strip by capillary action. After 2–10 minutes, the result is positive if two distinct red lines appear (one in the test region and another in the control region), it is negative when no red line appears in the test region, and it is invalid if the control line fails to appear.

In clinical cases of human VL, the K39 dipstick showed 100% sensitivity and 93% specificity in India (Sundar *et al.*, 2002), 90% sensitivity and 100% specificity in Brazil (Carvalho *et al.*, 2003), and 100% sensitivity and specificity in the Mediterranean basin (Brandonisio *et al.*, 2002). In parasitologically proven CanL, in both asymptomatic and symptomatic cases, the sensitivity of the K39 dipstick was 97% and the specificity 100% (Otranto *et al.*, 2005).

3. Delayed hypersensitivity test

Delayed hypersensitivity is an important feature of all forms of human leishmaniosis and can be measured by the leishmanin test, also known as the Montenegro reaction (Manson-Bahr, 1987). The leishmanin skin test has no value for the diagnosis of CanL. Leishmanin is a killed suspension of whole (0.5–1 × 10⁷/ml) or disrupted (250 µg protein/ml) promastigotes in pyrogen-free saline containing phenol. A delayed reaction develops and is read at 48–72 hours.

The false-positive reaction rate in otherwise healthy people is approximately 1%, but this can be higher in areas where there is a background of leishmaniosis, as many of the healthy population may show quite high rates of leishmanin sensitivity. Although there is complete cross-reactivity among all strains of *Leishmania*, although heterologous antigens often give smaller reactions, which may be caused by difficulty in standardisation. The leishmanin test is used in the clinical diagnosis of CL and MCL. In VL it will only measure past infections because during active disease, a complete anergy is found. Leishmanins are not available commercially.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Vaccine

There is no effective vaccine available for prophylactic immunisation against leishmaniosis. Until now, the only dependable vaccination against *Leishmania* has been limited to the protection of humans from both *L. tropica* and *L. major* by prior syringe-induced infection with *L. major* organisms. The promastigotes are injected into the arm
or other parts of the body. The living promastigotes used must either be freshly extracted from cultures or may be preserved in liquid nitrogen. The infection is allowed to run a natural course and after recovery, the individual is firmly immune to subsequent infection with both Leishmania species. This type of immunisation has been practised on a limited scale in hyperendemic areas of CL (due to \textit{L. major}) in Israel, Iran and the former Union of Soviet Socialist Republics (Shuikina et al., 2002). \textit{Leishmania major} causes cross-protection against \textit{L. tropica}, but the reverse is probably not true. However, this species cannot be considered to be totally safe and this type of immunisation should be used only for humans moving into high-risk areas. Moreover, it is not beneficial in highly endemic areas as individuals contract infection long before this type of preparation confers protection (i.e. approximately 3 months after vaccination). Standardisation and quality control of such vaccines, presently not available, are needed.

At present, a number of promising anti-leishmanial vaccines are under development (Coler & Reed, 2005; Gradoni, 2001). Among the first-generation vaccines, the glycoprotein-enriched fraction of \textit{L. donovani} known as ‘fucose-mannose ligand’ (FML), developed in Brazil, represents the first licensed veterinary vaccine against CanL. Field studies showed about 80% clinical protection conferred by the antigen administered with QuilA saponin as adjuvant (Borja-Cabrera et al., 2002), and also good immunotherapeutic efficacy when used in sick dogs (Borja-Cabrera et al., 2004). Killed \textit{Leishmania} organisms mixed with a low concentration of BCG as adjuvant have undergone phase I–II and phase III trials for immunisation against CL \textit{Leishmania} agents in humans and against VL in humans and in dogs, with limited success (Mohebali et al., 2004; Momeni et al., 1999).

Second-generation vaccines, most of which are at predevelopment stage, consist of genetically reconstructed \textit{Leishmania} parasites incapable of producing disease, recombinant molecules or their corresponding DNAs, or recombinant organisms carrying leishmanial genes and expressing parasite antigens. A chimeric antigen generated from three recombinant \textit{Leishmania} antigens screened for their ability to elicit cellular immune responses (known as Leish-111f), entered Phase I clinical testing in healthy volunteers in January 2003 (Reed & Campos-Neto, 2003). The same polyprotienic antigen, administered with monophosphoryl lipid A – stable emulsion (MPL-SE) or Adjuprime as adjuvants, failed to protect dogs from \textit{L. infantum} infection in a phase III trial (Gradoni et al., 2005).

2. Immunodiagnostic antigens

Neither the leishmanin used for skin tests nor the antigens commonly employed in serodiagnosis in leishmaniosis are internationally standardised (the recombinant K39 antigen, which is virtually standardised, is patent-protected and is not widely available). The leishmanin test is group-specific, not species-specific, and the leishmanin prepared from one clinical type of leishmaniosis will cause the development of delayed hypersensitivity to the same or other clinical types. Similarly, serological cross-reactions are common among leishmanial species.

a) Leishmanin

The leishmanin test is described in Section B.3. Sterility, safety and potency tests are required for leishmanin preparations.

b) Antigens for serological tests

Commercial antigens for the IFA tests and ELISAs have been produced are still under evaluation. The main reason for unsatisfactory results with these antigens is the poor stability of leishmanial antigens. They can be obtained in the laboratory by growing a \textit{Leishmania} strain in a suitable culture medium. For the IFA test and the DAT, crude particulate antigens, i.e. intact promastigotes, are required, whereas for ELISAs a soluble form of the antigen is needed.

3. Seed management

a) Characteristics of the seed

Strains of \textit{Leishmania} species used to prepare biological products should be identified at species and subspecies level by appropriate identification tests given in Section B.1. Once the organisms have been isolated and established in the laboratory, they must be assigned an International Code (WHO, 1989; 1990). This Code should consist of four elements separated by oblique strokes: (a) the type of host from which the strain was isolated (M for Mammalia and I for Insecta followed by three letters indicating the generic name of the host); (b) the country where isolation was made, indicated by a two-letter code; (c) the year of isolation indicated by the last two digits, and (d) the original laboratory code given to the isolate (for example, MHOM/IN/80/DD8). The parasites must be free from contaminating organisms and should be capable of yielding a product that conforms to the norms. Standard strains are available on request from the WHO Collaborating Centres in Madrid (Spain), Montpellier (France) and Jerusalem (Israel). A list of Identification Centres has been published by WHO (1990).
Chapter 2.1.8. – Leishmaniosis

b) Method of culture

The strain of the parasite used for preparing leishmanin should be capable of producing a product that conforms to national/international norms. It should be free from ingredients causing toxic or allergic reactions. There is no single specific antigen standardised for use in serodiagnostic tests, but when these antigens are prepared in the laboratory, they must be standardised for their sensitivity depending on the requirement. For the preparation of leishmanin as well as serodiagnostic antigens, the organisms should be grown in a suitable culture medium (such as those recommended in Section B.1 for *Leishmania* isolation and bulk cultivation). Normally, good growth of parasites is obtained 7 days after inoculation, and care must be taken that leishmanial stocks are not lost by overgrowth of the flagellates, which may occur after approximately 10 days.

c) Cryopreservation

Promastigote cultures and tissue infected with amastigotes may easily be conserved in the living state at low temperatures. Both forms can be cryopreserved for years at low temperatures in mechanical freezers (−70°C), in solid carbon dioxide containers (−76°C), or in liquid nitrogen containers (−196°C) (WHO, 1989). A sterile cryoprotectant is required – glycerol, to give a final concentration of 7.5–10%, or dimethyl sulphoxide (DMSO), to a final concentration of 5–7.5%. The cryoprotected samples are transferred to the sterile containers in which they are to be frozen. These may be 2 ml plastic freezing tubes with airtight screw-caps, hard glass, heat-sealed ampoules, or glass/plastic capillaries. A slow cooling rate (approximately 1°C/minute) is essential for the cryopreservation of *Leishmania*. This can be obtained by cooling samples to 4°C and keeping them at this temperature for a minimum of 1 hour; they are then transferred to a −20°C freezer and left for 24 hours, then removed to a −70°C freezer for at least 24 hours. They can be permanently stored at this temperature, or else transferred into liquid nitrogen or solid carbon dioxide. If possible, a programmable freezing unit should be used. When the cryopreserved material is required, the sample is taken out and thawed rapidly in a water bath at 37°C.

d) Validation

Cultures for leishmanin or serodiagnostic antigens should be checked for sterility before use. Leishmanin is stored at 4°C and serodiagnostic antigens at −20°C or −70°C until required. The latter should be reconstituted with PBS, pH 7.2, before use. Viable *Leishmania* cultures can be kept at −70°C for 3–4 years or at −196°C indefinitely. Because of nonavailability of suitable vaccine, it has not been possible to validate the currently developed immunising agents. Live or attenuated promastigotes of *L. major* used in some areas are far from being satisfactory. Leishmanin should be tested for allergenicity in guinea-pigs before use. Serodiagnostic antigens should be tested for their efficacy and sensitivity by proper standardisation for a particular test. If a batch of antigen has not been used for a long time, it should be rechecked before being used in the test.

4. Method of manufacture

As standardised immunodiagnostic antigens are not available commercially, they need to be prepared in the laboratory. Workers in the laboratory can be at risk of laboratory acquired infection, especially by injection. Appropriate biosafety precautions are therefore essential to minimise the risks (see Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

a) Leishmanin

*Leishmania* species are grown, preferably in blood-free liquid media such as Schneider’s Drosophila medium and RPMI (Rosewell Park Memorial Institute) medium, in order to avoid blood–antigen contamination. The promastigotes are harvested during the log phase, washed four times in pyrogen-free saline at 1000 g for 15 minutes, and resuspended in pyrogen-free saline containing 0.5% phenol (w/v) to obtain a final concentration of 0.5–1 × 10^7/ml. Leishmanin can also be made with disrupted promastigotes obtained as above and sonicated. The filtrate is adjusted to a final protein concentration of 250 µg/ml with pyrogen-free saline containing Tween 80 (0.0005% [v/v]) and phenol (0.28% [w/v]).

b) Antigens for serological tests

Methods of antigen preparation for various tests are given in Section B.2.

5. In-process control

One or more batches of leishmanin should be tested in guinea-pigs by allergic test. Sensitivity and specificity of the leishmanin should preferably be determined by performing the test in appropriate animal models (different inbred mice according to the *Leishmania* species), or in patients who have recovered from leishmanial infections, and in an unexposed control population.
6. Batch control

The WHO has suggested guidelines for the production of leishmanin (WHO, 1982; 1985b). It is recommended that the source material be controlled by using isoenzyme analysis to type the *Leishmania* strains used in preparing leishmanin.

a) Sterility

Each filling lot should be tested for bacterial and mycotic sterility according to WHO (1972). Absence of live leishmaniae is checked by inoculating one sample of each lot in an appropriate blood-agar medium, which is then incubated at 23°C for at least 15 days. One sample is injected intradermally (for dermotropic leishmaniae) or intraperitoneally (for viscerotropic leishmaniae) in mice or hamsters. These animals are observed during a period of 30–90 days.

b) Safety

Samples from each filling lot should be tested for abnormal toxicity by appropriate tests in guinea-pigs and mice. For each lot, five mice weighing 17–22 g and two guinea-pigs weighing 250–350 g are injected subcutaneously and intraperitoneally with one human dose of the product. The animals are then observed for at least 7 days for death or signs of disease.

c) Potency

The leishmanin is tested on animal models (according to the *Leishmania* species involved) that have been previously infected by the same strain used for leishmanin production. Lots of at least five infected animals and control animals are injected intradermally into one of the posterior footpads with 50 µl of leishmanin. After 2–3 days, all the infected animals should show a significant enlargement of the footpad compared with control animals.

7. Tests on the final product

a) Safety

See Section C.6.b.

b) Potency

See Section C.6.c.

REFERENCES


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CHAPTER 2.1.9.
LEPTOSPIROSIS

SUMMARY

Leptospirosis is a transmissible disease of animals and humans caused by infection with any of the pathogenic members of the genus Leptospira. Laboratory diagnosis of leptospirosis can be complex and involves tests which fall into two groups. One group of tests is designed to detect anti-leptospiral antibodies and the other group of tests is designed to detect leptospires, leptospiral antigens, or leptospiral nucleic acid in animal tissues or body fluids. The particular testing regimen selected depends on the purpose of testing (e.g. herd surveys or individual animal testing) and on the tests or expertise available in the area.

Identification of the agent: The isolation or demonstration of leptospires in:

a) the internal organs (such as liver, lung, brain, and kidney) and body fluids (blood, milk, cerebrospinal, thoracic and peritoneal fluids) of clinically infected animals gives a definitive diagnosis of acute clinical disease or, in the case of a fetus, chronic infection of its mother.

b) the kidney, urine, or genital tract of animals without clinical signs is diagnostic only of a chronic carrier state.

Isolation of leptospires from clinical material and identification of isolates is time-consuming and is a task for specialised reference laboratories. Isolation followed by typing from renal carriers is important and very useful in epidemiological studies to determine which serovars are present within a particular group of animals, an animal species, or a geographical region.

The demonstration of leptospires by immunochemical tests (immunofluorescence and immunohistochemistry) is more suited to most laboratory situations. However, the efficacy of these tests is dependent on the number of organisms present within the tissue, and these tests lack the sensitivity of culture. Unless specially prepared reagents are used, immunochemical tests do not identify the infecting serovar and results must be interpreted in conjunction with serological results. Reagents for immunofluorescence are best prepared with high IgG titre anti-leptospire sera, which are not available commercially. Rabbit leptospiral-typing serum or monoclonal antibodies can be used for immunohistochemistry and are available from leptospiral reference laboratories.

Genetic material of leptospires can be demonstrated in tissues or body fluids using a variety of assays based on the polymerase chain reaction (PCR), either in real-time or traditional formats. PCR assays are sensitive, but quality control procedures and sample processing for PCR are critical and must be adjusted to the tissue, fluid and species being tested. Like immunochemical tests, PCR assays do not identify the infecting serovar.

Serological tests: Serological testing is the most widely used means for diagnosing leptospirosis, and the microscopic agglutination test (MAT) is the standard serological test. Antigens selected for use in the MAT should include representative strains of the serogroups known to exist in the particular region plus those known to be maintained elsewhere by the host species under test.

The MAT is used to test individual animals and herds. As an individual animal test, the MAT is very useful for diagnosing acute infection: a four-fold rise in antibody titres in paired acute and convalescent serum samples is diagnostic. To obtain useful information from a herd of animals, at least ten animals, or 10% of the herd, whichever is greater, should be tested and the vaccination history of the animals documented.
Chapter 2.1.9. — Leptospirosis

The MAT has limitations in the diagnosis of chronic infection in individual animals and in the diagnosis of endemic infections in herds. Infected animals may abort or be renal/genital carriers with MAT titres below the widely accepted minimum significant titre of 1/100 (final dilution).

Enzyme-linked immunosorbent assays (ELISAs) can also be useful for detection of antibodies against leptospires. Numerous assays have been developed and are primarily used for the detection of recent infections and the screening of experimental animals for use in challenge studies. Animals that have been vaccinated against the serovar of interest may be positive in some ELISAs, thus complicating interpretation of the results.

Requirements for vaccines and diagnostic biologicals: Vaccines for veterinary use are most often suspensions of one or more serovars of Leptospira spp. inactivated in such a manner that immunogenic activity is retained. While a range of experimental vaccines based on cellular extracts have been tested, commercial vaccines are, with few exceptions, whole cell products. The leptospires are grown in suitable culture media, which often contain serum or serum proteins. If used, serum or serum proteins should be removed from the final products. Vaccines may contain suitable adjuvants.

A. INTRODUCTION

Leptospirosis is a transmissible disease of animals and humans caused by infection with the spirochete Leptospira. All the pathogenic leptospires were formerly classified as members of the species Leptospira interrogans, however the genus has recently been reorganised and pathogenic leptospires are now identified in 17 named species and four genomospecies of Leptospira (Brenner et al., 1999; Morey et al., 2006; Ramadass et al., 1992; Yasuda et al., 1987). There are more than 200 distinct leptospiral serovars recognised and these are arranged in 23 serogroups (Kmet & Dikken, 1993; Vijayachari et al., 2004).

The use, interpretation, and value of laboratory diagnostic procedures for leptospirosis vary with the clinical history of the animal or herd, the duration of infection, and the infecting serovar. Acute leptospirosis should be suspected in the following cases: sudden onset of agalactia (in adult milking cattle and sheep); icterus and haemoglobinuria, especially in young animals; meningitis; and acute renal failure or jaundice in dogs. Chronic leptospirosis should be considered in the following cases: abortion, stillbirth, birth of weak offspring (may be premature); infertility; chronic renal failure or chronic active hepatitis in dogs; and cases of periodic ophthalmia in horses. Two major chronic microbiological sequelae of leptospiral infection present particular diagnostic problems: the localisation and persistence of leptospires in the kidney and in the male and female genital tract. Chronically infected animals may remain carriers for years to life and serve as reservoirs of the infection for other animals and humans.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The demonstration of leptospires in blood and milk of animals showing clinical signs suggestive of acute leptospirosis is considered to be diagnostic. However, isolation from blood is not often successful because bacteraemia is transient and not always accompanied by clinical signs. Dogs are often treated with antibiotics before samples are collected for testing for Leptospira, which further decreases the likelihood of identifying the agent in blood. The demonstration of generalised leptospiral infection in a range of organs taken at necropsy is also considered to be diagnostic. However, if the animal lives long enough or has been treated with antibiotics, it may be difficult to detect intact organisms systemically; immunohistochemistry can be particularly helpful in identifying residual leptospiral antigen in these cases. Demonstration of leptospires in the genital tract, kidneys, or urine only must be interpreted with full consideration of the clinical signs and serological results as these findings may merely indicate that the animal was a carrier.

Failure to demonstrate leptospires in the urine of an animal does not eliminate the possibility that the animal is a chronic renal carrier, it merely indicates that the animal was not excreting detectable numbers of leptospires at the time of testing. Collection of urine following treatment of the animals with a diuretic enhances the chances of detecting the organism (Nervig & Garrett, 1979). In important cases involving individual animals (e.g. clearing an infected stallion to return to breeding), negative tests on three consecutive weekly urine samples has been considered to be good evidence that an animal is not shedding leptospires in the urine.
The demonstration of leptospires in body fluids or internal organs (usually kidney, liver, lung, brain, or adrenal gland) of aborted or stillborn fetuses is considered to be diagnostic of chronic leptospirosis of the mother, and is evidence of active infection of the fetus.

In experienced hands, the isolation of leptospires is the most sensitive method of demonstrating their presence, provided that antibiotic residues are absent, that tissue autolysis is not advanced, that tissues are processed for culture rapidly after collection, and – in the case of urine – at a suitable pH. If tissues or fluids cannot be transported promptly to the laboratory for leptospiral culture, the sample should be kept at 2–5°C to prevent overgrowth with other bacteria and autolysis of tissue samples. Liquid culture medium or 1% bovine serum albumin (BSA) solution containing 5-fluorouracil at 100–200 µg/ml should be used as transport medium for the submission of samples.

Culture should be carried out in a semisolid (0.1–0.2% agar) medium containing BSA and either Tween 80 (e.g. Tween 80/BSA medium or EMJH) (Hockey et al., 1992) or containing BSA and a combination of Tween 80 and Tween 40 (Ellis, 1986). Contamination may be controlled by the addition of a variety of selective agents, e.g. 5-fluorouracil (Johnson & Rogers, 1964), nalidixic acid (Johnson & Seiter, 1977), fosfomycin (Oie et al., 1986), and a mixture of rifamycin, polymyxin, neomycin, 5-fluorouracil, bacitracin, and actidione (Adler et al., 1986). However, use of selective agents may reduce the chances of isolation when there are only small numbers of viable leptospires, and some strains of leptospires will not grow in selective media containing multiple antibiotics. Addition of 0.4–1% rabbit serum to semisolid culture medium enhances the chances of isolating fastidious leptospiral serovars.

Cultures should be incubated at 29 ± 1°C for at least 16 weeks, and preferably for 26 weeks (Ellis, 1986). The time required for detection of a positive culture varies with the leptospiral serovar and the numbers of organisms present in the sample. Less fastidious serovars (e.g. Pomona and Grippotyphosa) may result in positive cultures as soon as 7–10 days after inoculation; other serovars (e.g. Hardjo and Bratislava) may take much longer. Cultures should be examined by dark-field microscopy every 1–2 weeks. It is important to use a 100 watt light source and a good quality dark-field microscope.

Leptospires may also be demonstrated by a variety of immunochemical staining techniques, e.g. immunofluorescence (Bolin et al., 1989a; Ellis et al., 1982a), and various immunohistochemical techniques (Barnett et al., 1999; Ellis et al., 1983; Scanziani, 1991; Skillbeck & Chappell, 1987; Wild et al., 2002; Zaki & Shieh, 1996). These are useful in diagnosing infection in pathological material that is unsuitable for culture or where a rapid diagnosis is required. As the success of these techniques is dependent on the number of organisms present, they are less suitable for diagnosing the chronic carrier state, where the numbers of organisms may be very low or localised. Leptospires do not stain satisfactorily with aniline dyes, and silver-staining techniques lack sensitivity and specificity, although they are a useful adjunct for histopathological diagnosis (Baskerville, 1986).

Polymerase chain reaction (PCR)-based assays are now used in some diagnostic and many reference laboratories for the detection of leptospires in tissues and body fluids of animals. A variety of primer sets for the conduct of PCR assays have been described (Alt et al., 2001; Barocchi et al., 2001; Branger et al., 2005; Gerritsen et al., 1991; Gravekamp et al., 1993; Hockey et al., 1992; Jouglard et al., 2006; Kawabata et al., 2001; Levet et al., 2005; Marien et al., 1992; Marien et al., 2005; Palaniappan et al., 2005; Smythe et al., 2002; Truccolo et al., 2002; van Eys et al., 1989; Woodward et al., 1991) with some primers only specific for the genus *Leptospira* and others designed to identify only pathogenic species. These assays do not identify the infecting serovar, although some primer sets may permit further identification to the species or serovar level if the PCR amplicons are sequenced. This further analysis is not a routine diagnostic method. Many of the PCR primer sets have been designed and evaluated for use in human rather than animal specimens and general agreement about the PCR primers to be used for testing of animal samples is lacking. Therefore, the individual laboratory is generally responsible for the validation of the particular assay they use for the tissue, fluid, and species being tested. PCR assays can be quite sensitive, but lack of specificity (i.e. false-positive results) can be a problem. Presence of amplification inhibitors in clinical samples can cause false-negative results, particularly in animal specimens that may be compromised by contamination with faeces or autolysis. Quality control of PCR assays used for diagnosis of leptospirosis requires careful attention to laboratory design and workflow to prevent contamination of reagents, and use of appropriate control samples (Dragun et al., 1993; McCready & Callawayth, 1993). In addition, sample processing for PCR is critical and must be suited to the tissue, fluid, and species being tested. A procedure for the preparation of urine samples for PCR using magnetic beads coated with anti-leptospiral antibody shows promise in enhancing the detection of pathogenic leptospires in urine (Taylor et al., 1997).

The identification of leptospiral isolates is a task for specialised reference laboratories. For complete identification, a combination of procedures is used to determine: 1) if the isolate is a pathogen or a saprophyte; 2) the species of *Leptospira* to which the isolate belongs; and 3) the serogroup and serovar of the isolate. A pure leptospiral culture may be identified as belonging to a pathogenic or saprophytic species by a variety of tests: the ability to infect animals; the relative resistance to 8-azaguanine; lipase activity; salt relative resistatre tolerance (Johnson & Faine, 1984; Johnson & Harris, 1967); PCR-assay-based amplification of 23S rDNA (Woo et al., 1997c); and G+C content of DNA (Johnson & Faine, 1984).
New leptospiral species have been identified based on DNA–DNA hybridisation analysis (Brenner et al., 1999; Ramadass et al., 1992; Yasuda et al., 1987). In most cases, the type strain for each serovar was used in these analyses; for a few serovars, clinical isolates have also been tested to determine the new species designations. Different isolates belonging to a single serovar usually belong to the same species, but this is not always the case. Species identification of field isolates is still cumbersome but can be done by sequence analysis of the 16S rDNA, by genetic analysis of the 16S or 23S ribosomal RNA genes (Brown & Levett, 1997; Letocart et al., 1992; Perolat et al., 1993; Ralph et al., 1995; Roy et al., 2006; Tolhurst et al., 1997a; 1997b), by multilocus sequence typing (Ahmed et al., 2006; Salaun et al., 2006), by sequencing the DNA–DNA hybridisation analysis (Slack et al., 2006a), or by PCR using species-specific ompL1 primer sets (Reitstetter, 2006).

Strains belonging to *Leptospira* can be differentiated to the serogroup level by cross-agglutination reactions (Dikken & Kmethy, 1978). Further differentiation to the serovar level was traditionally by cross-agglutination absorption, although for most isolates this is now being done using less time-consuming methods: factor analysis (Dikken & Kmethy, 1978), monoclonal antibodies (MAbs) (Terpstra et al., 1985; 1987), restriction endonuclease analysis (Herrmann et al., 1992; Marshall et al., 1981; Thiermann et al., 1985; 1986), and various other molecular strategies (Brown & Levett, 1997; Corney et al., 1993; De Caballero et al., 1994; Gerritsen et al., 1995; Morey et al., 2006; Perolat et al., 1994; Ralph et al., 1995; Ramadass et al., 1997; Roy et al., 2004; Salaun et al., 2006; Savio et al., 1994; Slack et al., 2006a; 2006b; Zuerner et al., 1995; Zuerner & Bolin, 1997). However, genetic-based tests may not always give the same results as the cross-agglutination absorption test.

2. Serological tests

Serological testing is the laboratory procedure most frequently used to confirm the clinical diagnosis, to determine herd prevalence, and to conduct epidemiological studies. Leptospiral antibodies appear within a few days of onset of illness and persist for weeks or months and, in some cases, years. Unfortunately, antibody titres may fall to undetectable levels while animals remain chronically infected. To overcome this problem, sensitive methods are needed to detect the organism in urine or the genital tract of chronic carriers.

A wide variety of serological tests, which show varying degrees of serogroup and serovar specificity, have been described. Two tests have a role in veterinary diagnosis: the microscopic agglutination test (MAT) and the enzyme-linked immunosorbent assay (ELISA).

a) Microscopic agglutination test

The MAT using live antigens is the most widely used serological test. It is the reference test against which all other serological tests are evaluated and is used for import/export testing. For optimum sensitivity, it should use antigens representative of all the serogroups known to exist in the region in which the animals are found and, preferably, strains representing all the known serogroups. The presence of a serogroup is usually indicated by frequent reaction in serological screening but can only be definitively identified by isolation of a serovar from clinically affected animals. The sensitivity of the test can be improved by using local isolates rather than reference strains, but reference strains assist in the interpretation of results between laboratories.

The specificity of the MAT is good; antibodies against other bacteria usually do not cross-react with *Leptospira* to a significant extent. However, there is significant serological cross-reactivity between serovars and serogroups of *Leptospira* and an animal infected with one serovar is likely to have antibodies against the infecting serovar that cross-react with other serovars (usually at a lower level) in the MAT. Therefore, serology cannot be used to definitively identify the infecting serovar in an individual infection or outbreak – this requires isolation of the agent. However, in areas where the serovars of *Leptospira* present have been well described by isolation studies, serological examination of the infected animal(s) may suggest, but not definitively identify, the infecting serovar. In addition, animals that have been vaccinated against leptospirosis may have antibodies against the serovars present in the vaccine used. Therefore, it is particularly important to consider the vaccination history of the animals under test. The two methods for carrying out the test have been described in detail (Faine et al., 2000; USDA, 1987).

The strains selected should be grown in liquid leptospiral culture medium (e.g., EMJH, Tween 80 BSA, or other suitable medium) at 29 ± 1°C and the culture should be at least 4 days old, but no more than 8 days. Live cultures with densities of approximately 2 × 10^8 leptospires per ml are to be used as the antigens. The culture density can be determined by counting the cells directly using a bacterial counting chamber and dark-field microscopy. Alternatively, cell counts can be estimated by measuring transmittance in a spectrophotometer with a 400 nm filter or by nephelometry. If indirect methods are used, direct bacterial cell counts should be correlated with the readings on the specific instrument being used. The number of antigens to be used is determined and a screening test may be performed with a 1/50 serum dilution (or a different starting dilution based on the purpose of the test). A volume of each antigen, equal to the diluted serum volume, is added to each well, making the final serum dilution 1/100 in the screening test. The microtiter plates are incubated at 29 ± 1°C for 2–4 hours. The plates are examined by dark-field microscopy.
Identity of antigens is a crucial factor in conducting the MAT. Antigens should be evaluated for identity, using hyperimmune rabbit sera, MAbs, or a molecular method that confirms passages over time, preferably each time the test is run, but at least twice a year. Hyperimmune rabbit serum for this purpose can be obtained from a reference laboratory or prepared using a protocol such as that given by the Subcommittee on the Taxonomy of Leptospira (International Committee on Systematic Bacteriology, 1984). Briefly, healthy rabbits weighing 3–4 kg that lack detectable anti-leptospiral antibodies are selected. Each rabbit is given an intravenous injection in a marginal vein of the ear with a well-growing live or formalin-treated cloned culture from a reference laboratory or prepared using a protocol such as that given by the Subcommittee on the Taxonomy of Leptospira. The culture should be grown in Tween 80 BSA medium or another appropriate medium. Five injections of 1 ml, 2 ml, 4 ml, 6 ml, and 6 ml each are given at 7-day intervals. One week following the final injection, the homologous antibody titre is determined by MAT. If the titre is ≥1/12,800, the rabbit is anaesthetised and bled by cardiac puncture 7 days later (i.e. 14 days after the final injection). If the titre is <1/12,800, a further injection of 6 ml of culture can be given; 7 days after this injection the homologous titre is again determined. Unless the titre is ≥1/12,800 the procedure should be repeated with another rabbit. Two rabbits are used to prepare each antiserum. If the titres are satisfactory in both rabbits, the sera may be pooled. To preserve potency, it is preferable to freeze-dry the serum; alternatively, the serum can be stored in 2 ml volumes at −15 to −20°C. All animal inoculations should be approved and conducted according to the relevant standards for animal care and use. Other immunisation protocols may be considered based on the intended use of the antiserum.

Purity of antigens used in the MAT should be checked regularly by culture on blood agar and in thioglycollate broth. Stock cultures of antigens may be stored at −70 to −80°C or in liquid nitrogen. There may be a low survival rate of leptospires after lyophilisation. Repeated passage of antigens in liquid medium results in a loss of antigenicity. In this case, a new liquid culture should be derived from the stock culture.

As an individual animal test, the MAT is very useful in diagnosing acute infection; the demonstration of a four-fold change in antibody titres in paired acute and convalescent serum samples is diagnostic. In addition, a diagnosis of leptospirosis is likely based on the finding of very high titres in an animal with a consistent clinical picture. This has limitations in diagnosis of chronic infection in individual animals, both in the diagnosis of abortion (Ellis et al., 1982b) and in the identification of renal or genital carriers (Ellis, 1986). This is particularly true with the host-adapted leptospiiral infections, e.g. serovar Hardjo infection in cattle: when a titre of 1/100 or greater is taken as significant, the sensitivity of the test is only 41%, and even when the minimum significant titre is reduced to 1/10, the sensitivity of the test is only 67% (Ellis, 1986). The demonstration of antibodies in fetal blood is diagnostic, but the titres are often very low, i.e. 1/10, requiring a modified testing procedure for most laboratories.

As leptospirosis is a herd problem, the MAT has much greater use as a herd test. To obtain useful information, Cole et al. (1980) suggested that samples be taken from at least ten animals, or 10% of the herd, whichever is the greater. In a study of Hardjo infection in cattle, Hathaway et al. (1986) found that a 10-cow sample usually indicated the presence or absence of infection in a herd. Increasing the sample size markedly improved epidemiological information, investigations of clinical disease, and public health tracebacks.

In making a serological diagnosis of leptospirosis, the infecting serovar and the clinical condition involved must be fully considered. In the case of serovar Pomona-induced abortion in cattle, a high titre is commonly found at the time of abortion because the clinical incident occurs relatively soon after infection. Abortion in cattle due to serovar Hardjo is a chronic event; in this case, the serological response at the time of abortion is more variable, with some animals seronegative and others showing high titres. Cattle may experience a drop in milk production during the acute phase of Hardjo infection and this clinical sign is associated with high titres. Vaccination history must also be considered in the interpretation of MAT results as widespread vaccination contributes significantly to the number of seropositive animals and may mask the presence of chronic infections in the herd – particularly with serovar Hardjo.

b) Enzyme-linked immunosorbant assays

ELISAs for detection of anti-leptospiral antibodies have been developed using a number of different antigen preparations, assay protocols and assay platforms, including plate tests and dipstick tests. Information regarding the surface antigens of Leptospira has been reviewed (Cullen et al., 2005). In general, ELISAs are quite sensitive, but lack the serovar specificity of the MAT. An ELISA that measures canine IgG and IgM against various leptospiral serovars has been developed and evaluated in Europe (Hartman et al., 1984;
1986). Anti-leptospiral IgM is detectable in this assay as early as 1 week after infection, before agglutinating antibodies are present. IgG antibodies are detectable in infected dogs beginning 2 weeks after infection and persist for long periods of time. Therefore, dogs with acute leptospirosis have high IgM titres and relatively low IgG titres; dogs that are vaccinated or have had previous leptospiral infections have high IgG titres but low IgM titres. Similar assays to detect anti-leptospiral bovine, porcine, and ovine antibodies have also been developed (Adler et al., 1981; Cho et al., 1989; Cousins et al., 1985; Mendoza & Prescott, 1992; Ribotta et al., 2000; Surujballi & Elmgren, 2000; Surujballi et al., 1997; Surujballi & Mallory, 2001; Trueba et al., 1990; Yan et al., 1999). The major identified role of ELISA in livestock species is the use of an IgM ELISA for identification of recent infections (Cousins et al., 1985) and for screening herds in regions where vaccination for leptospirosis is not practiced. A total-Ig ELISA is useful in identification of fully susceptible animals suitable for experimental challenge work (Ellis et al., 1986). ELISAs have also been developed for use in milk from individual cows or in bulk tank milk for the detection of serovar Hardjo antibodies. These tests have been helpful in identifying Hardjo-infected herds. However, herds that are vaccinated against serovar Hardjo will also be positive in these various ELISAs decreasing their usefulness in regions where vaccination is a routine practice. New ELISAs have been developed based on detection of antibodies against surface proteins or lipoproteins of *Leptospira* (Bomfim et al., 2005; Dey et al., 2004; Mariya et al., 2006; Okuda et al., 2005; Palaniappan et al., 2004) but these tests are not yet widely available.

### C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Leptospiral vaccines for veterinary use are suspensions of one or more strains of pathogenic *Leptospira* inactivated in such a manner that immunogenic activity is retained. While experimental vaccines based on cellular extracts have been tested (Bey & Johnson, 1986), commercial vaccines are, with few exceptions, whole-cell products. The leptospires are grown in suitable culture media that may contain serum or serum proteins. If used, serum or serum proteins should be removed from the final product. Vaccines may contain suitable adjuvants.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given here and in Chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

1. **Seed management**

   a) **Characteristics of the seed**

      Proper selection of vaccine production strains is of utmost importance. Immunity induced by vaccination is largely serovar specific (Chen, 1986). A vaccine should be formulated for use in a particular animal species in a particular geographical region. It should contain only those serovars – and preferably those genotypes – that cause problems in the animal species, or that are transmitted by the animal species to other species in the region. Strains selected for use as master seed culture should be cloned on solid medium to ensure the absence of saprophytic *Leptospira* contaminants and uniformity of the culture.

      Suitable strains should be further selected by their ability to grow to high yields under batch culture conditions.

   b) **Methods of culture**

      Each component strain to be included in the final vaccine should be grown separately in liquid medium; preferably in a protein-free (Bey & Johnson, 1978; Shenberg, 1967) or low-protein medium (Bey & Johnson, 1978).

      The volume of each master seed culture should be amplified by growth for 2–10 days at 29°C ±1°C in a series of subcultures until a volume sufficient for use as a production seed culture is achieved. Cultures should be aerated and agitated as required.

      Each subculture of the master seed culture should be checked for purity and for satisfactory growth. Purity can be checked by inoculating a loopful of culture into blood agar plates or into thioglycolate broth for incubation at 35–37°C for 2–5 days, and by examining a Gram-stained smear of culture sediment. Growth can be checked by dark-field microscopy. Each production seed culture should also be checked against its homologous rabbit antiserum (Dikken & Kmet, 1978) to ensure purity and homology. MAbs may also be used for this purpose.

   c) **Validation as a vaccine**

      There is a large volume of literature describing the efficacy of leptospiral vaccines. In most cases, vaccines provide significant protection against disease produced by homologous challenge under field conditions.
Vaccines are less efficacious at preventing infection in animals and a percentage of vaccinated animals will become infected with the relevant serovar and may shed the organism in their urine despite a lack of clinical signs of disease.

Efficacy trials and vaccine validation must be conducted in the target species for the vaccine. The vaccine should be administered as recommended on the label, and immunity should be tested by challenge with virulent field strains of each serovar by natural routes of infection, i.e. by conjunctival and/or vaginal challenge. Validation studies have often been conducted with challenge of immunity by intravenous or intramuscular injections of leptospires. Vaccines validated in this way have not always been shown to be protective against field challenge, which occurs by exposure of mucous membranes of the eye, mouth, and genital tract to leptospires. Most notably, commercial leptospiral vaccines containing serovar Hardjo have not always protected cattle from conjunctival or field challenge with serovar Hardjo (Bolin et al., 1989b). A draft monograph for the efficacy testing of serovar Hardjo vaccines has been prepared and specifies the use of more natural routes of challenge (European Pharmacopoeia monograph).

2. Method of manufacture

Manufacture is carried out by batch culture in appropriately sized fermentor vessels. These should be equipped with ports for the sterile addition of seed culture, air, and additional medium. They should also have sampling ports so that the purity and growth of the production culture can be monitored.

Ideally, low-protein or protein-free media are used for production. However, some strains require the presence of animal protein to achieve suitable yields; this is usually supplied as BSA. All media components that are not degraded by heat should be heat sterilised. This reduces the risk of contamination by water-borne saprophytic leptospires that are not removed by filter sterilisation.

After addition of the seed culture, the growth of the production culture is monitored at frequent intervals for the start of log-phase growth. Once this is observed, the vessel is then agitated and aerated. The final yield can often be improved by the addition of more Tween 80 to the culture when log-growth is first observed to be slowing down. Adequate growth may require up to 10 days of incubation at 29 ± 1°C.

Inactivation is usually by the addition of formalin, but phenol, merthiolate, and heat inactivation have also been used.

After the appropriate inactivation period, the culture may be concentrated and extraneous protein material may be removed by ultrafiltration. Suitable volumes of the various strains to be included in the final vaccine can then be blended, and adjuvant and preservative added, if appropriate.

3. In-process control

During production, daily or twice daily subsamples should be taken and monitored for growth of leptospires and absence of contaminants. Growth is monitored either by counting leptospires in a counting chamber under dark-field microscopy or by a nephelometer. The absence of contamination can be monitored by the microscopic examination of Gram-stained preparations of centrifuged culture.

Immediately prior to inactivation, a sample should be taken for checking against its homologous antibody in a MAT. The inactivated culture must be checked for freedom from viable leptospires. This is done by inoculating aliquots of inactivated culture into an appropriate growth medium, such as the medium of Johnson & Harris (1967), incubating at 29 ± 1°C for at least 4 weeks, and examining weekly by dark-field microscopy for the presence of viable leptospires.

After blending, the levels of free inactivating agents, minerals present in adjuvants (such as aluminum), and preservative (such as thiomersal) must be within prescribed limits.

4. Batch control

a) Sterility

Selected samples of the completed vaccine should be tested for the absence of viable bacteria and fungi (British Pharmacopoeia [Veterinary], 1985b; Council of Europe, 2002a; 2002b; United States Department of Agriculture Standard Requirements § 113.26). Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

b) Safety

Samples of completed product should be tested for safety. Methods for this have been described elsewhere (British Pharmacopoeia [Veterinary], 1985a; Council of Europe, 2002a; United States Department of
Agriculture Standard Requirements § 113.38). The test should be carried out for each route of inoculation indicated on the label and in two healthy animals of each category (e.g. pregnant animals, young stock) for which the vaccine is intended. The animals must be susceptible to the serovars used in the vaccine and their sera must be free from agglutinating antibodies to those serovars. Each animal is given an injection of the vaccine by the recommended route with twice the recommended dose, as stated on the label. The animals are observed for 14 days and should show no adverse local or systemic effects attributable to the vaccine.

c) Potency

Samples of completed vaccine should be tested for potency in hamsters or guinea-pigs. Potency is usually measured by the vaccine's ability to prevent the death of the animal when challenged with between 10 and 10,000 LD$_{50}$ (50% lethal dose). With some serovars that are not hamster or guinea-pig lethal, such as serovar Hardjo, potency is measured against prevention of renal infection when the animals are challenged with between 10 and 10,000 ID$_{50}$ (50% infectious dose) or by induction of a suitable antibody titre in rabbits.

An example protocol is to inject 1/40 dog dose of the vaccine into each of ten healthy hamsters no more than 3 months old. After 15–30 days, each vaccinated hamster, and each of ten unvaccinated hamsters of the same age, is injected intraperitoneally with a suitable quantity of a virulent culture of leptospires of the serovar used to make the vaccine (or a suspension of liver or kidney tissue collected from an experimentally infected animal). In the case of bivalent vaccines, each serovar is tested separately. For the vaccine to pass the test, at least 8/10 of the vaccinated animals should remain in good health for 14 days after the death of the controls. Other protocols may apply to cattle and pig vaccines, which contain as many as five or six components.

In-vitro potency tests for leptospiral vaccines are being developed based on quantifying the protective antigen in the vaccine using MAb in a capture ELISA (Ruby et al., 1992). These assays are being standardised using reference vaccines and correlation with existing hamster or antibody-based potency assays and target–host efficacy data.

d) Duration of immunity

Duration of immunity should be determined in the animal species for which the vaccine is intended using natural routes of challenge (Bolin et al., 1989b). Duration of immunity should not be estimated based on the duration of MAT titres in vaccinated animals as protection against clinical disease may be present with very low titres. Vaccinal immunity should persist for at least 6 months or longer depending on the label claim.

e) Stability

When stored under the prescribed conditions, the vaccines may be expected to retain their potency for 1–2 years. Stability should be assessed by determining potency after storage at 2–5°C, room temperature, and 35–37°C.

5. Tests on the final project

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

REFERENCES


Chapter 2.1.9. — Leptospirosis


European Pharmacopoeia Monograph: Bovine Leptospirosis vaccine (inactivated); PA/PH/Exp. 15V/T (01) 28.


Chapter 2.1.9. — Leptospirosis


Chapter 2.1.9. — Leptospirosis


UNITED STATES DEPARTMENT OF AGRICULTURE STANDARD REQUIREMENTS § 113.26.

UNITED STATES DEPARTMENT OF AGRICULTURE STANDARD REQUIREMENTS § 113.38.


Chapter 2.1.9. — Leptospirosis


*   *

**NB:** There are OIE Reference Laboratories for Leptospirosis
(see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for leptospirosis
CHAPTER 2.1.10.

NEW WORLD SCREWWORM (COCHLIOMYIA HOMINIVORAX) AND OLD WORLD SCREWWORM (CHRYSMOYA BEZZIANA)

SUMMARY

The New World screwworm\(^1\) (NWS), Cochliomyia hominivorax (Coquerel), and the Old World screwworm\(^1\) (OWS), Chrysomya bezziana Villeneuve, are both obligate parasites of mammals during their larval stages. Both species are in the subfamily Chrysomyinae of the family Calliphoridae of the order Diptera (true flies). Larvae feeding on the skin and underlying tissues of the host cause a condition known as wound or traumatic myiasis, which can be fatal. Infestations are generally acquired at sites of previous wounding, due to natural causes or to animal husbandry practices, but they may also occur in the mucous membranes of body orifices.

Female flies are attracted to wounds at the edges of which each female lays an average of 175 (OWS) to 340 (NWS) eggs. The larvae emerge within 12–24 hours and immediately begin to feed, burrowing head-downwards into the wound. After developing through three larval stages (instars) involving two moults, the larvae leave the wound and drop to the ground into which they burrow to pupate. The duration of the life-cycle off the host is temperature dependent, being shorter at higher temperatures, and the whole cycle may be completed in less than 3 weeks in the tropics.

Treatment is generally effected by application of organophosphorus insecticides into infested wounds, both to kill larvae and to provide a residual protection against reinfestation. Preventive measures include the spraying or dipping of susceptible livestock with organophosphorus compounds and, more recently, use of avermectins (especially doramectin) as subcutaneous injections to animals ‘at risk’. Strict control of the movement of animals out of affected areas also acts as a preventive measure.

Identification of the agent: The larvae of NWS and OWS can be easily confused with each other and with the larvae of other agents of myiasis. Accurate diagnosis involves the identification of larvae extracted from the deepest part of an infested wound. The mature, third instar larvae are most reliable for this purpose and those of NWS can be identified by their darkly pigmented dorsal tracheal trunks extending from the twelfth segment forward to the tenth or ninth. This pigmentation is unique to the larvae of NWS among the species encountered in wound myiasis. Confirmation of OWS relies on the recognition of a characteristic combination of spinulation, the number of lobes on the anterior spiracles (4–6), and pigmentation of secondary tracheal trunks.

In the adult stage, species in the genus Cochliomyia can be separated from other genera involved in wound myiasis by confirmation of a body colour that is usually a metallic blue/green with three dark longitudinal stripes always present on the thorax. The separation of NWS from the very similar C. macellaria and the identification of adult OWS are discussed in this chapter.

Serological tests: At present there are no applicable serological tests, nor are they indicated in the identification of this disease. However, serology may have a future role in studies of the prevalence of myiasis.

Requirements for vaccines and diagnostic biologicals: There are no vaccines or biological products available except for the use of sterilised male flies in the sterile insect technique (SIT). In this technique, vast numbers of sterilised male flies are sequentially released into the environment, where their matings with wild females produce infertile eggs, leading to an initial population reduction and, progressively, eradication.

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\(^1\) In this chapter, the term ‘New World’ refers to the Americas and the term ‘Old World’ refers to Europe, Africa and Asia.
A. INTRODUCTION

The New World screwworm fly (NWS), *Cochliomyia hominivorax* (Coquerel), and the Old World screwworm fly (OWS), *Chrysomya bezziana* Villeneuve, are species of two genera of the subfamily Chrysomyinae of the Dipteran family Calliphoridae (blowflies). Both species are obligate parasites of mammals including humans and, rarely, birds. Despite being in different genera and geographically separated, the two species have evolved in remarkable parallel. They have almost identical life histories because they fill identical parasitic niches in their respective geographical zones. The following discussion will relate to both species, except where indicated.

Unlike most other species of blowflies, adult female screwworms do not lay their eggs on carrion. Instead, they lay them at the edges of wounds on living, injured mammals or at their body orifices. Virtually any wound is attractive, whether natural (from fighting, predators, thorns, disease, and/or tick and insect bites) or man-made (from shearing, branding, castrating, de-horning, docking, and/or ear-tagging). Commonly infested natural wounds are the navels of newborn animals and the vulval and perineal regions of their mothers, especially if traumatised. If eggs are deposited on mucous membranes, the larvae can invade undamaged natural body openings such as the nostrils and associated sinuses, the eye orbits, mouth, ears, and genitalia.

Within 12–24 hours of the eggs being laid, larvae emerge and immediately begin to feed on the wound fluids and underlying tissues, burrowing gregariously head-downwards into the wound in a characteristic screwworm fashion. As they feed, tearing the tissue with their hook-like mouthparts, the wound is enlarged and deepened, resulting in extensive tissue destruction. Infested wounds often emit a characteristic odour, which can be the first indication that at least one animal in a group is infested. Although the odour is not always apparent to humans, it is obviously highly attractive to gravid females (Hall, 1995), which lay further batches of eggs so increasing the extent of the infestation. A severe infestation that is left untreated may result in the death of the host.

Screwworm larvae pass through three stages (or instars), separated by cuticular moults that facilitate rapid growth, and they reach maturity about 5–7 days after egg hatch. They then stop feeding and leave the wound, falling to the ground into which they burrow and pupariate. The pupa develops within the puparium, a barrel-shaped protective structure formed by hardening and darkening of the cuticle of the mature larva. On completion of development, adult flies usually emerge from the puparium in the morning and work their way up to the soil surface, where they extend their wings for hardening prior to flight. Males become sexually mature and able to mate within 24 hours, but the ovaries of females need to mature over 6–7 days, and females only become responsive towards males and mate when about 3 days old. About 4 days after mating, female flies are ready to oviposit. They seek a suitable host and lay their eggs, all oriented in the same direction, like a tiled roof, firmly attached to each other and to the oviposition substrate. The numbers of eggs laid per batch vary depending on many factors (e.g. fly strain, disturbance during oviposition), but the average first batch has in the order of 175 eggs for OWS and 340 for NWS (Spradbery, 1994). Following the first egg batch, further batches are laid at intervals of 3–4 days (Thomas & Mangan, 1989). Adult flies live on average for 2–3 weeks in the field during which time they feed at flowers, and the females also take in protein, e.g. from serous fluids at animal wounds.

The rate of development of the immature stages is influenced by environmental and wound temperatures, being slower at low temperatures, although true diapause does not occur. This effect is most pronounced in the off-host pupal stage, which can vary from 1 week to 2 months’ duration depending on the season (Laake *et al*., 1936). Thus, the complete life cycle of NWS may take 2–3 months in cold weather (Parman, 1945), whereas in temperate conditions with an average air temperature of 22°C, it is completed in about 24 days (James, 1947). In tropical conditions averaging 29°C it is completed in about 18 days (Thomas & Mangan, 1989).

The degree to which NWS and OWS can tolerate cold has had a major influence on their distributions, best documented for NWS. Historically, the range of NWS extended from the southern states of the United States of America (USA), through Mexico, Central America, the Caribbean islands and northern countries of South America to Uruguay, northern Chile and northern Argentina (James, 1947). This distribution contracted during the winter months but expanded during the summer months, producing a seasonality at its edges and year round populations in the central areas – the New World tropics. Use of the sterile insect technique (SIT) in major programmes has resulted in eradication of NWS from the USA (Baumhover, 2001), Mexico (Graham, 1985), Curacao, Puerto Rico, and the Virgin Islands and, in Central America, from Guatemala, Belize, El Salvador, Honduras, Nicaragua and, in 2000, Costa Rica (Wyss, 2001). The Central American eradication programme is continuing in Panama, where sterile flies were first released in July 1998. The ultimate objective is to establish a barrier zone in Panama that will become the future northern limit of NWS in the Americas. A NWS eradication programme was also officially launched in Jamaica in July 1998, as part of a plan to eradicate the species from the entire Caribbean. This programme has encountered severe setbacks due to a complex combination of management and technical difficulties, but is ongoing although with an uncertain future (Dyck *et al*., 2005; Vreyssen *et al*., 2007). Although NWS is a New World species, in 1988, it was detected in Libya in North Africa where it threatened to become firmly established. However, it was eradicated in 1991 by an intensive SIT campaign (Food and Agriculture Organization of the United Nations [FAO], 1992; Lindquist *et al*., 1992). The threat of spread of screwworms aided by modern rapid transport systems is ever present, necessitating constant vigilance from quarantine and other front-line animal health and medical officers in unaffected areas. Imported cases of NWS have been reported recently in Mexico, USA, and even in the United Kingdom (Mallon *et al*., 1999).
Organophosphorus insecticides such as dichlofenthion, fenchlorphos, and in particular, coumaphos are recommended for the treatment of wounds infested with OWS and NWS (Graham, 1979; Perkins, 1987; Spradbrey et al., 1991). They have the effect of expelling the larvae, which die on the ground. To provide residual protection against reinfection, they must be applied at 2–3-day intervals until the wound has healed. The contents of individual wound treatment sachets, e.g. 5 g of 5% coumaphos wettable powder, should be either sprinkled directly on to a wound or, more effectively, brushed into the wound as a paste after mixing with ordinary cooking oil (33 ml). Organophosphorus compounds may also be applied as aerosol sprays, in which marker dyes and bacteriostats are included, or as dusts that are puffed into the wound from plastic squeeze bottles. Dichlofenthion is used in South America as a 1% aerosol to treat NWS cases and is also effective against OWS (Perkins, 1987). Any larvae that die in the wound should be removed to prevent sepsis. Close attention should always be paid to the manufacturers’ safety instructions.

Direct prevention of screwworm infestation can be achieved by spraying or dipping of livestock with coumaphos (0.25% aqueous suspension of 50% wettable powder) or other organophosphorus insecticides at the maximum concentration prescribed for external parasite control. The effects of such treatment are twofold: firstly, the treatment kills larvae directly and provides residual protection; secondly, the treatment kills ticks and other external parasites, which means that there are fewer wounds available as sites for oviposition. Synthetic pyrethroids have potential for control of screwworm larvae in wounds, but there have been few reported trials of their effect on screwworms (e.g. Permethrin versus NWS; Silva et al., 1991). Dipping or spraying of a group of animals would be indicated if any member of the group was found to be infested, or if animals were traversing or leaving an infested area, or following wound-inducing animal husbandry practices, e.g. shearing.

A single subcutaneous injection of ivermectin (200 µg/kg) was effective against OWS in preventing navel strike of newborn calves (Perkins, 1987) and scrotal strike of castrated calves (Spradbrey et al., 1985). Ivermectin also prevented re-strike of treated wounds of adult cattle. Cattle treated with a sustained-release bolus of ivermectin developed no OWS myiasis from 14 to 102 days after treatment (Warthaug et al., 2001). However, because of the negative effects on dung-breeding fauna, it was recommended that boluses be reserved for use in containing outbreaks of OWS. Early results suggested that ivermectin may be ineffective against NWS (Mackley & Brown, in: Graham, 1985), but more recent studies demonstrated that it can produce a significant reduction in the incidence of navel and scrotal myiasis due to NWS (Benitez Usher et al., 1997; Lombardero et al., 1999). Although results of ivermectin trials show variation, results of doramectin trials are overwhelmingly positive (Guimaraes & Papavero, 1999). There has been an increasing number of publications reporting that a subcutaneous injection of doramectin (200 µg/kg) was up to 100% effective as a NWS prophylactic, preventing infestation of artificial wounds, umbilical or castration wounds of calves, and infestation of post-parturient cows, for up to 12–14 days post-treatment (Anziani et al., 2000; Moya-Borja et al., 1993; Muniz et al., 1995). This doramectin treatment does not reduce egg-laying and, therefore, is efficient because gravid adults are not repelled and driven towards untreated animals. Effectiveness depended on factors such as cattle breed and degree of challenge. In one comparative trial, doramectin and ivermectin, both at 200 µg/kg subcutaneous injection, gave 100% and 50% protection, respectively, against NWS myiasis, experimentally induced 2 hours after treatment (Moya-Borja et al., 1997). Doramectin also provided complete protection for 21 days and partial protection (56%) at 28 days post-treatment (Moya-Borja et al., 1997). In another, larger, comparative trial, doramectin had a mean efficacy of 94.6% (range 53.3–100%) compared with 43.7% (range 0–100%) for ivermectin (Caproni et al., 1998). Abamectin (subcutaneous injection, 200 µg/kg) gave good, but not 100%, prevention of post-castration myiasis by NWS (Anziani et al., 1995). Pour-on formulations of moxidectin, eprinomectin and doramectin gave poor protection against OWS myiasis (Warthaug et al., 2001) when compared with injectable formulations of doramectin against NWS. There are early indications that fipronil (a phenylpyrazole) might be effective as a preventive of post-castration myiasis of cattle. Topical application of 10 mg/kg bodyweight of a 1% fipronil solution did not prevent oviposition by NWS, but it reduced the proportion of bulls developing active myiasis over the critical 10-day post-castration period, when most ovipositions occurred, from 65% in untreated controls to just 3% in treated animals (Lima et al., 2004). Similarly, topical application of an insect growth regulator (IGR), dicyclanil, to castration wounds in cattle gave good protection (>90%) against NWS myiasis (Anziani et al., 1998). IGRs are very specific to insects and, therefore, are less hazardous in the environment than many other groups of insecticides. Spinosad, a formulation of products derived from the fermentation of a bacterium with low mammalian and avian toxicity, was effective in treating and preventing myiasis due to NWS and OWS when applied as an aerosol spray (Snyder et al., 2005).
Indirect prevention of screwworm flies infestation includes the avoidance of wounding procedures at the times of year when screwworm are numerous, the careful handling of livestock to minimise wounding, the removal of sharp objects (e.g. wire strands) from livestock pens, and the use of measures to reduce other wound-causing parasites, in particular ticks, e.g. by dipping and by insecticide impregnated ear-tags.

To prevent the spread of the screwworms beyond present limits, strict observation of the requirements for international trade, as set out in the OIE Terrestrial Animal Health Code, is necessary.

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

Identification of the eggs and first instar larvae of the agents of myiasis based on morphology is difficult, and, because these stages are relatively short lived and seldom encountered during the collection of specimens from infested wounds, they will not be considered further here.

Larvae collected for diagnosis should be removed from the deepest part of the wound to reduce the possibility of collecting non-screwworm species, which may infest the shallower parts of the wound. Living specimens should first be examined for pigmentation of the dorsal tracheal trunks (Figures 1 and 4) and then be preserved in 80% ethanol and returned to the laboratory for examination under a dissecting microscope at up to ×50 magnification (for further techniques see: FAO, 1991; Hall & Smith, 1993; Spradbery, 1991; Zumpt, 1965). If larvae are placed directly into most preservative solutions they contract and darken. However, optimal preservation of larvae, in their natural extended state, can be made by killing them in boiling water (15–30 seconds immersion) before storage in 80% ethanol. This killing method had no negative effect on subsequent extraction of mitochondrial DNA, amplified by polymerase chain reaction (PCR) (Hall et al., 2001), but it might impact other molecular techniques and this should be borne in mind.

*Second instar larvae:* Second instars have only two spiracular slits in each of the posterior spiracular plates compared with the three slits of third instars (Figures 2 and 3). Second instars of NWS can be diagnosed by the presence of dark pigmentation of the dorsal tracheal trunks, for over half their length in the terminal segment. Other species have less extensive pigmentation of the dorsal tracheal trunks, for example, these trunks are pigmented for no more than one-third of their length in the twelfth segment of OWS. The anterior spiracles of second instar NWS have from seven to nine branches compared with about four branches in OWS (Kitching, 1974). More positive identification may be gained by rearing living, immature larvae to third instars. This can be done on the standard meat medium used for large-scale rearing of NWS before the introduction of gel diets, i.e. in the proportion of 1 litre water, 1.3 kg ground horse or beef meat, 50 g dried bovine blood, and 1.5 ml formalin (Taylor & Mangan, 1987), mixed and maintained at 35–38°C and 70% relative humidity. For simply rearing up larvae for identification, the exact meat and blood types are not essential, and more readily available fresh blood could be used instead of dried blood.

*Third instar larvae:* Third instars of both NWS and OWS have a robust, typical maggot shape, with a cylindrical body from 6 to 17 mm long and from 1.1 to 3.6 mm in diameter, pointed at the anterior end (Laake et al., 1936; Spradbery, 1991). Fully mature larvae of both NWS and OWS develop a reddish-pink tinge over the creamy white colour of younger larvae. Both screwworm species have prominent rings of spines around the body and these spines appear large and conspicuous under a microscope when compared with most non-screwworm species, the longest averaging 130 μm. In NWS the spines can be either single or double pointed, but in OWS they are always single pointed and thorn-like (Figure 2). The anterior spiracles of NWS each have from six to eleven well separated branches, but usually from seven to nine (Figure 2). In OWS, the anterior spiracles each have from three to seven branches, but usually from four to six (Figure 2). The latter character should not be used on its own to identify OWS, because third instars of the obligate myiasis-causing species Wohlfahrtia magnifica (Diptera: Sarcophagidae), whose distribution overlaps that of OWS in the Middle East, have similarly branched anterior spiracles. Hence, in using any identification key, such as that in Figure 1, it is essential that each specimen be taken through the whole key to avoid misidentifications. On the posterior face of the terminal segment of both NWS and OWS, the posterior spiracular plates all have a darkly pigmented, incomplete peritreme partially enclosing three straight, slightly oval-shaped slits, which point towards the break in the peritreme. These diagnostic features are illustrated in Figure 3. Of greatest diagnostic value are the dorsal tracheal trunks, which extend forwards from the posterior spiracular plates and are darkly pigmented up to the tenth or ninth segment in NWS (Figure 1; see also: FAO, 1991; 1993; Guimaraes & Papavero, 1999; Hall & Smith, 1993; James, 1947; Spradbery, 1991; Zumpt, 1965 for identification keys). This feature is seen most easily in living larvae. Those in preservative may need dissection to remove opaque tissues covering the trunks. The dorsal tracheal trunks of OWS are darkly pigmented only in the twelfth segment. However, in OWS the secondary trachea branching off the dorsal tracheal trunks are pigmented from the twelfth segment forwards to at least the tenth segment (confirmed in specimens throughout the range, from Malaysia, Bahrain and Zimbabwe; M.J.R. Hall, unpublished). Conversely, in NWS these secondary trachea are not pigmented, only the dorsal trachea are. Hence, the tracheal pigmentation appears almost reversed between the two screwworm species (Figure 4).
### Chapter 2.1.10. – Screwworm (Cochliomyia hominivorax and Chrysomya bezziana)

Remove larva from wound and examine gross surface structure

- **‘Hairy’ larva with obvious body processes**
- **‘Smooth’ larva, with spine bands but no obvious body processes except on last segment**

**Chrysomya albiceps, C. rufifacies, C. varipes**

- Posterior spiracles almost concealed in deep cavity on posterior ‘face’ of last segment
- Posterior spiracles not in cavity but clearly exposed on posterior ‘face’ of last segment

**Sarcophagidae**

- Peritreme of posterior spiracle closed
- Peritreme of posterior spiracle open

**Muscidae and Lucilia/Calliphora species**

- Dorsal tracheal trunks darkly pigmented forwards from the 12th to the 10th or even 9th segment
- Dorsal tracheal trunks not darkly pigment except possibly in posterior half of 12th segment

**Cochliomyia hominivorax**

- Anterior spiracle with 4–6, rarely 7, lobes
- Anterior spiracle with nine or more lobes

**Chrysomya bezziana**

- Other species of Chrysomya, Cochliomyia, Phormia or Protophormia

**Fig. 1.** Identification key for the diagnosis of third instar larvae of Cochliomyia hominivorax and Chrysomya bezziana from cases of wound myiasis. To avoid misidentifications, it is essential that the key is worked through from the first step for each specimen.
Chapter 2.1.10. — Screwworm (Cochliomyia hominivorax and Chrysomya bezziana)

Fig. 2. Head and first two thoracic segments of third instar larvae of Cochliomyia hominivorax (left, viewed by scanning electron microscopy, inset is the anterior spiracle of Chrysomya bezziana) and of Chrysomya bezziana (right, viewed by compound light microscopy, note the thorn-like spines and that this slide preparation has been cleared using 10% KOH so that the anterior spiracles on both sides of the first thoracic segment are visible); as = anterior spiracle.

Fig. 3. Characteristics of third instar larvae of Cochliomyia hominivorax: (A) whole larva, lateral aspect; (B) posterior face of terminal segment; (C) posterior spiracular plate; a = anterior spiracle; b = button adjacent to opening in peritreme; p = peritreme; sl = spiracular slit; sp = spines. (After Laake et al. [1936].)

Adult: Adult flies needed for identification purposes are often collected using wind-oriented traps (Broce et al., 1977) and sticky traps (Spradbery, 1991) baited with a synthetic odour, swarmlure-4 (Mackley & Brown, 1984). A modified bucket-trap and newly developed attractant (‘Bezzi lure’) is being developed for surveillance of OWS in Australia (Rudolf Urech, pers. comm.). Alternative sampling systems, using electrocuting grids or sticky surfaces at odour-baited visual targets, have been used for research purposes (Hall, 1995). Identification of adult flies is seldom required for the diagnosis of myiasis, because the larval stages are those most apparent to livestock owners and veterinary personnel. However, a brief description follows.

i) **NWS**: The body length is usually 8–10 mm long and has a deep blue to blue-green metallic colour, with three dark longitudinal stripes on the dorsal surface of the thorax. This combination of colour and pattern is not shared by any other species commonly involved in wound myiasis except the secondary screwworm of the New World, *Cochliomyia macellaria* (Fabricius). These two *Cochliomyia* species can be separated by the presence of black setulae on the fronto-orbital plates of the head of NWS compared with only light yellow hairs on the fronto-orbital plates of *C. macellaria*. The fifth (=fourth visible) abdominal tergite of NWS has only a very slight lateral pollenose dusting, whereas that of *C. macellaria* has a dense dusting, producing a pair of distinct, lateral, silvery-white spots. In addition, females of NWS have a dark brown-black basicosta, whereas those of *C. macellaria* have a yellow basicosta (Figure 5; see also: Dear, 1985; FAO, 1993; Laake et al., 1936; Spradbery, 1991).

ii) **OWS**: The body is up to 10 mm long and has a metallic blue, bluish-purple or blue-green colour, i.e. it is very similar to NWS, but without the thoracic stripes. The lower squama (s in Figure 5) also differs from NWS, being distinctly covered with fine hairs over its entire upper surface in OWS and other *Chrysomya* species,
whereas in NWS it is hairless above, except near the base. Adults of OWS can be distinguished from other Chrysomya found in cases of myiasis by the combination of black-brown to dark-orange-coloured anterior thoracic spiracles (rather than pale yellow, creamy, or white), with waxy-white, lower squamae (rather than blackish-brown to dirty-grey) (Spradbery, 1991; Zumpt, 1965).

Fig. 4. Dorsal tracheal trunks of third instar larvae of Cochliomyia hominivorax (left) and Chrysomya bezziana (right) dissected forwards from the posterior spiracles (top) to ninth abdominal segment (bottom). Note that the pigmentation of the main dorsal trunks (DT) and the smaller secondary trunks (ST) is almost reversed between the species.

Fig. 5. Characteristics of adult Cochliomyia hominivorax; note longitudinal thoracic stripes; b = basicosta; p = fronto-orbital plate, indicated from above on whole Cochliomyia hominivorax and laterally on head of typical calliphorid fly; s = lower squama, surface hairless except at base; v = stem vein with hairs on dorsal posterior surface.

In addition to the standard morphological techniques discussed previously, more recent techniques for identification of screwworms and their geographical origins include cuticular hydrocarbon analysis (Brown et al., 1998), analysis of mitochondrial DNA (Hall et al., 2001; Litjens et al., 2001; Taylor et al., 1996), the complete 16,022 base-pair sequence of which is known for NWS, and use of random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) (Skoda et al., 2002). Problems with identification of larvae or adults from
cases of myiasis can be referred to the FAO Collaborating Centre on Myiasis-Causing Insects and Their Identification\(^2\).

2. Serological tests

No standardised serological tests are presently available, nor are they indicated for diagnosis of this disease. However, experimental studies have shown that serological techniques have potential value in future investigations of the prevalence of screwworm infestations in animal populations to detect antibodies to screwworm post-infestation (Thomas & Pruett, 1992).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products such as vaccines, available currently. However, research towards development of potential vaccines is being conducted (Sukarsih Partoutomo et al., 2000). The only proven method of eradication of NWS relies on a biological technique, the sterile insect technique, SIT (Graham, 1985; Lindquist et al., 1992), which has also been applied experimentally to OWS (Spradbery et al., 1989). In this technique, male flies sterilised in their late pupal stage by gamma or x-ray irradiation are sequentially released into the wild in vast numbers. Any of their matings with wild females result in infertile eggs only, leading to a progressive population reduction and, eventually, eradication. In operational situations, SIT is supported by the insecticide treatment of screwworm-infested wounds in livestock, by strict control of livestock movement, by the quarantining of infested animals and by an active publicity campaign. SIT is very expensive because of the cost of continuous production and aerial dispersion of sterile flies. Historically, it has been considered cost effective only when used as an eradication strategy in situations where the geography would favour such a programme (e.g. FAO, 1992; Lindquist et al., 1992). For many years there was only one New World sterile screwworm production facility, located at Tuxtla Gutiérrez in the south of Mexico. However, a second facility opened in Panama\(^3\) in late 2006. An experimental facility to produce sterile OWS opened in Malaysia in 1998\(^4\).

REFERENCES


BAUMHOVER A.H. (2001). A personal account of programs to eradicate the screwworm, Cochliomyia hominivorax, in the United States and Mexico with special emphasis on the Florida program. Florida Entomologist, 84, 162 (Ababstract only, full text online at www.fcla.edu/FlaEnt/fe84p162a.pdf).


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Chapter 2.1.10. — Screwworm (Cochliomyia hominivorax and Chrysomya bezziana)


Chapter 2.1.10. — Screwworm (Cochliomyia hominivorax and Chrysomya bezziana)


Parmar D.C. (1945). Effect of weather on Cochliomyia americana and a review of methods and economic applications of the study. J. Econ. Entomol., 38, 66–76.


Chapter 2.1.10. — Screwworm (Cochliomyia hominivorax and Chrysomya bezziana)


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**NB:** There is an OIE Reference Laboratory for New World screwworm (*Cochliomyia hominivorax*) (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)). Please contact the OIE Reference Laboratories for any further information on New World screwworm (*Cochliomyia hominivorax*)
CHAPTER 2.1.11.

PARATUBERCULOSIS

(Johne’s disease)

SUMMARY

Paratuberculosis (Johne’s disease) is a chronic enteritis of ruminants caused by Mycobacterium avium subsp. paratuberculosis (M. paratuberculosis).

Identification of the agent: The diagnosis of paratuberculosis is divided into two parts: the diagnosis of clinical disease and the detection of subclinical infection. The latter is essential for control of the disease at the farm, national or international level.

Diagnosis of paratuberculosis is made on clinical grounds confirmed by the demonstration of M. paratuberculosis in the faeces by microscopy, culture, or by the use of DNA probes and the polymerase chain reaction. Diagnosis is made at necropsy by the finding of the pathognomonic lesions of the disease in the intestines, either grossly with the demonstration of typical acid-fast organisms in impression smears of the lesions or histologically, and by isolation of M. paratuberculosis in culture.

The detection of subclinical infection depends on the detection of specific antibodies by serology, or culture of M. paratuberculosis from faeces or tissues collected at necropsy, or the demonstration of cell-mediated responses. The choice of test depends on the circumstances and the degree of sensitivity required at individual animal or herd level.

Cultures of M. paratuberculosis may be obtained from faeces or tissues, after treatment to eliminate contaminants, by inoculation into artificial media with and without the specific growth factor – mycobactin – that is essential for the growth of M. paratuberculosis.

Serological tests: Control of paratuberculosis is difficult because of the prolonged course of infection, the predominantly subclinical nature of the disease and lack of tests for accurate detection of subclinically infected animals.

The serological tests commonly used for paratuberculosis in cattle are complement fixation (CF), absorbed enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID). Sensitivity and specificity are often determined by reference to results of faecal culture, which itself has unknown sensitivity in subclinically infected cattle. When used to confirm diagnosis of paratuberculosis in cows with typical clinical signs, some tests, for example CF and absorbed ELISA, perform very well.

Requirements for vaccines and diagnostic biologicals: Vaccines for paratuberculosis may be live attenuated or killed bacteria either incorporated with an adjuvant or lyophilised and adjuvanted on reconstitution. Bacterial counting is difficult and bacterial content of vaccines may be based on weight, while vaccine potency may be judged by batch tests for sensitising ability in guinea-pigs.

Vaccine safety or abnormal toxicity may also be tested in guinea-pigs.

For diagnostic skin tests, Johnin and avian tuberculin are purified protein derivatives (PPD) of a heat-treated culture of M. paratuberculosis or M. avium, respectively. Johnin is standardised for content of PPD by chemical assay and its biological activity is identified in guinea-pigs sensitised with M. paratuberculosis. Avian tuberculin activity is determined in guinea-pigs sensitised with M. avium by comparison with a reference preparation calibrated in international units.
A. INTRODUCTION

*Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*) is an organism first observed by Johne & Frothingham in 1895. *Mycobacterium avium* subsp. *paratuberculosis* causes paratuberculosis or Johne’s disease, an intestinal granulomatous infection (Thorel et al., 1990). First recognised in cattle, then in sheep and later in goats, paratuberculosis is found most often among domestic and wild ruminants and has a global distribution. The disease has also been reported in horses, pigs, deer and alpaca, and recently in rabbits, stoat, fox and weasel (Beard et al., 1999; Greig et al., 1999). Under natural conditions, the disease in cattle spreads by ingestion of *M. paratuberculosis* from the contaminated environment. The disease persists after the introduction of infected animals. Infection can be spread vertically to the fetus (Larson & Kopecky, 1970) and semen can be infected with the organism (Sweeney et al., 1995). The primary source of infection in calves is milk from infected cows or milk that is contaminated with the faeces of diseased cattle.

The identification of *M. paratuberculosis* is based on its mycobactin requirement and its pathogenicity in the host. Mycobactin dependence has long been used as a taxonomic characteristic for *M. paratuberculosis* because most mycobacteria are able to make mycobactin for themselves. *Mycobacterium avium* subsp. *paratuberculosis*, *M. silvaticum* and some primary isolates of *M. avium* lack this capacity, however, and require mycobactin to grow in the laboratory. Thus, the mycobactin requirement is not confined to *M. paratuberculosis*; this characteristic exists to various degrees within the *M. avium* group (Thorel, 1991).

Clinical signs of paratuberculosis are a slowly progressive wasting and diarrhoea, which is intermittent at first, becoming progressively more severe until it is constantly present in bovines (Gilmour, 1954). Diarrhoea is less common in small ruminants.

Early lesions occur in the walls of the small intestine and the draining mesenteric lymph nodes, and infection is confined to these sites at this stage. As the disease progresses, gross lesions occur in the ileum, jejunum, terminal small intestine, caecum and colon, and in the mesenteric lymph nodes. *Mycobacterium avium* subsp. *paratuberculosis* is present in the lesions and, terminally, throughout the body. The intestinal lesions are responsible for a protein leak and a protein malabsorption syndrome, which lead to muscular wasting. Clinical signs usually first appear in young adulthood, but the disease can occur in animals at any age over 1–2 years.

Within a few weeks of infection, a phase of multiplication of *M. paratuberculosis* begins in the walls of the small intestine. Depending on the resistance of the individual, this infection is eliminated or the animal remains infected as a healthy carrier. The proportion of animals in these categories is unknown. A later phase of multiplication of the organisms in a proportion of carriers leads to the extension of lesions, interference with gut metabolism and clinical signs of disease. Subclinical carriers excrete variable numbers of *M. paratuberculosis* in the faeces. In most cases larger numbers of organisms are excreted as clinical disease develops.

Delayed-type hypersensitivity (DTH) is detectable early in the infection and remains present in a proportion of the subclinically infected carriers, but as the disease progresses, DTH wanes and may be absent in clinical cases. Serum antibodies are detectable later than DTH. They may also be present in carriers that have recovered from infection. Serum antibodies are present more constantly and are of higher titre as lesions become more extensive, reflecting the amount of antigen present. In sheep, there may be a serological response that is more likely to be detected in multibacillary than in the paucibacillary form of the disease.

Other mycobacterial diseases and infections, including mammalian and avian tuberculosis, cause DTH and the presence of serum antibodies. It follows therefore that these diseases need to be differentiated from paratuberculosis, both clinically and by the use of specific diagnostic tests. Exposure to environmental saprophytic mycobacteria may also sensitize livestock, resulting in nonspecific DTH reactions.

Animals vaccinated against paratuberculosis develop both DTH and serum antibodies. Vaccination is an aid to the prevention of clinical disease, but does not necessarily prevent infection. It also interferes with programmes for the diagnosis and control of bovine tuberculosis. Thus, if it is necessary to attempt a diagnosis of infection in vaccinates, only tests to detect *M. paratuberculosis* in the faeces can be used (Jorgensen, 1984).

In individual animals, especially from a farm in which the disease has not previously been diagnosed, a tentative clinical diagnosis must be confirmed by laboratory tests. However, a definitive diagnosis may be warranted on clinical grounds alone if the clinical signs are typical and the disease is known to be present in the herd. Confirmation of paratuberculosis depends on the finding of either gross lesions with the demonstration of typical acid-fast organisms in impression smears or microscopic pathognomonic lesions and the isolation in culture of *M. paratuberculosis*.
B. DIAGNOSTIC TECHNIQUES

To diagnose the presence of paratuberculosis in an individual clinically suspect animal, a number of laboratory tests can be used including: faecal smears, faecal and tissue culture, DNA probes using faeces or tissues, serology, necropsy and histology.

Herd tests to detect subclinical infection are carried out to determine the prevalence of the infection, usually so that control measures can be instituted. As no test is 100% sensitive or specific, control of the disease by the disposal of positive reactors depends on repeated tests at 6-month or yearly intervals over a number of years and the elimination of reactors to serological tests or faecal shedders; the removal of offspring from female reactors is also considered to be prudent. Even these procedures are not always successful without changes in hygiene and livestock management to reduce the transmission of infection within a herd (Argente, 1988).

1. Identification of the agent

a) Necropsy

Paratuberculosis cannot be diagnosed on superficial examination of the intestines for signs of thickening. The intestines should be opened from the duodenum to the rectum to expose the mucosa. There is not always a close correlation between the severity of clinical signs and the extent of intestinal lesions. The mucosa, especially of the terminal ileum, is inspected for pathognomonic thickening and corrugation. Early lesions are seen by holding the intestine up to the light, when discrete plaques can be visualised. Mucosal hyperaemia, erosions and petechiation have been observed in deer with paratuberculosis. The earliest lesions are thickening and cording of lymphatic. The mesenteric lymph nodes are usually enlarged and oedematous. Smears from the affected mucosa and cut surfaces of lymph nodes should be stained by Ziehl–Neelsen’s method and examined microscopically for acid-fast organisms that have the morphological characteristics of M. paratuberculosis. However, acid-fast organisms are not present in all cases. Diagnosis is therefore best confirmed by the collection of multiple intestinal wall and mesenteric lymph node samples into fixative (10% formol saline) for subsequent histology. Both haematoxylx-in-and-eosin-stained sections and Ziehl–Neelsen-stained sections should be examined. The typical lesions of paratuberculosis consist of infiltration of the intestinal mucosa, submucosa, Peyer’s patches and the cortex of the mesenteric lymph nodes with large macrophages, also known as epithelioid cells, and multinucleate giant cells, in both of which clumps or singly disposed acid-fast bacilli are usually, but not invariably, found.

b) Bacteriology (microscopy)

Ziehl–Neelsen-stained smears of faeces or intestinal mucosa are examined microscopically. A presumptive diagnosis of paratuberculosis can be made if clumps (three or more organisms) of small (0.5–1.5 µm), strongly acid-fast bacilli are found. The presence of single acid-fast bacilli in the absence of clumps indicates an inconclusive result. The disadvantages of this test are that it does not differentiate among other mycobacterial species and only a small proportion of cases can be confirmed on microscopic examination of a single faecal sample.

c) Bacteriology (culture)

The isolation of M. paratuberculosis from an animal provides the definitive diagnosis of infection with the organism. Although culture is technically difficult and time-consuming to carry out, it is the only test that does not produce false-positive results (100% specificity).

The faecal culture is the best test available for the diagnosis of paratuberculosis in live animals. It is believed that the faecal culture method involving the double incubation method for decontamination of samples and cultivation on solid media detects about 30–40% of infected cattle (Whitlock et al., 2000). The faecal culture is able to detect most animals in advanced stages of the disease but identifies only a few animals in early stages of infection (Whitlock et al., 2000). It will detect infected animals 6 months or more before they develop clinical signs, and during the clinical stage its sensitivity approaches 100%. The culture of bovine and caprine tissues for M. paratuberculosis is more sensitive than histopathological examination.

There are several culture methods, which vary with respect to media and sample processing protocols. The cultivation of M. paratuberculosis is always performed using special media supplemented with mycobactin J.

Mycobacterium avium subsp. paratuberculosis organisms are vastly outnumbered by other bacteria or fungi in faecal and intestinal tissue specimens. The successful isolation of M. paratuberculosis from such samples depends on efficient inactivation of these undesirable organisms. The optimal method of decontamination must have the least inhibitory effect on growth of M. paratuberculosis. Routine decontamination protocols

1 Mycobactin can be obtained commercially (mycobactin J) from Allied Monitor, P.O. Box 71, 201 Golden Drive, Fayette, MO 65248, United States of America, or Symbiotic Society, 299 av. Jean Jaurés, 69007 Lyon, France.
were shown to decrease the number of *M. paratuberculosis* organisms isolated per sample by about 2.7 log\(_{10}\) and 3.1 log\(_{10}\) for faeces and tissues, respectively (Reddacliff *et al*., 2003).

There are two basic methods in use for the conventional culture of *M. paratuberculosis* on solid media: the method using oxalic acid and NaOH for decontamination and Löwenstein–Jensen (LJ) medium for growth, and the method using hexadecylpyridinium chloride (HPC) for decontamination in combination with Herrolds’s egg yolk medium (HEYM) for growth. Both media contain mycobactin. Although it has been published that HEYM supports growth of bovine isolates of *M. paratuberculosis* significantly better than LJ (Nielsen *et al*., 2004), recent studies have shown that certain strains grow better on LJ or Middlebrook media (de Juan *et al*., 2006).

In addition, there is a technique of radiometric culture where growth in liquid medium BACTEC™ 12B (Middlebrook 7H12) supplemented with egg yolk and mycobactin is measured by the release of radioactive \(^{14}\)CO\(_2\) from palmitate, as a consequence of bacterial metabolism. This method reduces the time required for results and is considered more sensitive than the conventional culture methods on solid media for the detection of both ovine and bovine strains of *M. paratuberculosis* (Eamens *et al*., 2000; Whittington *et al*., 1999). The decontamination protocol involving double incubation of faecal samples in HPC and mixture of antibiotics may further improve culture sensitivity (Eamens *et al*., 2000). However, as the BACTEC™ system is radiometrically based, it is not feasible for use in some laboratories and has been phased out in others. The evaluation of the usefulness of alternative culture systems based on liquid media such as MGIT (Becton Dickinson) ESPII (Difco) and MB/BacT Alert (Organon Teknika) that do not use radioactive material for the detection of *M. paratuberculosis* is currently in progress.

Primary colonies of *M. paratuberculosis* on solid media may be expected to appear any time from 5 weeks to 6 months after inoculation (Gwozdz, 2010). Sheep strains, including the uncommon, bright yellow pigmented types, grow less well than cattle strains on commonly used media such as HEYM or LJ, and primary cultures should not be discarded as negative without prolonged incubation. The solid medium Middlebrook 7H10 and liquid medium BACTEC™ 12B both supplemented with egg yolk and mycobactin are excellent for cultivation of ovine strains of *M. paratuberculosis* (Whittington *et al*., 1999).

On modified 7H10 medium, colonies of the cattle strain are less convex than those on HEYM, especially in aged cultures. They are pinpoint to approximately 1 mm in diameter and, being buff coloured, are only slightly lighter than the media. Compared with colonies of cattle strains on HEYM, those on 7H10 are more difficult to detect. Colonies of the sheep strain of *M. paratuberculosis* on modified 7H10 are convex, soft, moist, glistening, off-white to buff, and very similar to the colour of the media. Colonies are typically between pinpoint and 0.5 mm, but can reach 1 mm, and rarely 1.5 mm if few colonies occur on a slope (Gwozdz, 2010).

Saprophytic mycobacteria may have a similar appearance on either medium but are often evident after 5–7 days (Gwozdz, 2010).

For identification of *M. paratuberculosis*, small inoculum of suspect colonies should be subcultured on the same medium with and without mycobactin, to demonstrate mycobactin dependency. Mycobactin is present in the cell wall of the organism, and heavy inoculum may contain enough mycobactin to support the growth of a mycobactin-independent mycobacterium on medium that contains no mycobactin.

**Media**

Examples of suitable media are:

1. **Herrold’s egg yolk medium with mycobactin** (Merkal, 1970)

   For 1 litre of medium: 9 g peptone; 4.5 g sodium chloride; 2.7 g beef extract; 27 ml glycerol; 4.1 g sodium pyruvate; 15.3 g agar; 2 mg mycobactin; 870 ml distilled water; six egg yolks (120 ml); and 5.1 ml of a 2% aqueous solution of malachite green. Measure the first six ingredients and dissolve by heating in distilled water. Adjust the pH of the liquid medium to 6.9–7.0 using 4% NaOH, and test to ensure the pH of the solid phase is 7.2–7.3. Add the mycobactin dissolved in 4 ml ethyl alcohol.
Autoclave at 121°C for 25 minutes. Cool to 56°C and aseptically add six sterile egg yolks and sterile malachite green solution. Blend gently and dispense into sterile tubes.

It is permissible to add 50 mg chloramphenicol, 100,000 U penicillin and 50 mg amphotericin B.

ii) Modified Dubos’s medium (Smith, 1953)

For 1 litre of medium: 2.5 g Difco casamino acids; 0.3 g asparagine; 2.5 g anhydrous disodium hydrogen phosphate; 1 g potassium dihydrogen phosphate; 1.5 g sodium citrate; 0.6 g crystalline magnesium sulphate; 25 ml glycerol; 50 ml of a 1% solution of Tween 80; and 15 g agar. Dissolve each salt in distilled water with minimum heat and make up to 800 ml. Add mycobactin in alcoholic solution at 0.05% (2 mg dissolved in 4 ml ethyl alcohol), heat the medium to 100°C by free-steaming, and then sterilise by autoclaving at 115°C for 15 minutes. Cool to 56°C in a water bath, add antibiotics (100,000 U penicillin; 50 mg chloramphenicol; and 50 mg amphotericin B) and serum (200 ml of bovine serum sterilised by filtering through a Seitz ‘EX’ pad and inactivated by heat at 56°C for 1 hour). The medium is kept thoroughly mixed and then dispensed into sterile tubes. An advantage of this medium is that it is transparent, which facilitates the early detection of colonies.

iii) Modified Middlebrook 7H10 (Gwozdz, 2010)

To prepare this medium 19 g Middlebrook 7H10 agar (Difco), 1g Casitone and 5 ml Glycerol are resuspended in 900 ml water, autoclaved at 121°C for 15 minutes and cooled to 58°C. Using an aseptic technique, the following additional ingredients are combined adding the egg yolk last: 50 ml PANTA PLUS (Becton Dickinson), 25 ml Mycobactin J solution (50 µg/ml), 100 ml ADC enrichment (Difco), 250 ml egg yolk. The mixture is thoroughly mixed using a slow swirling action and 10-ml volumes are dispensed into sterile tubes to form slopes. After a sterility check, media are stored at 4°C.

iv) BACTEC 12B vials (Gwozdz, 2010)

The following supplements are added to each vial to give final concentrations of 0.8–1 µg/ml Mycobactin J and a minimum of 16–17% egg yolk in a final volume of 5–6 ml. For the 6-ml volume, 0.1 ml Mycobactin J (50 µg/ml), 0.1 ml PANTA PLUS, 1 ml egg yolk and 0.8 ml water are added. For the 5-ml volume, 0.1 ml Mycobactin J (50 µg/ml), 0.1 ml PANTA PLUS and 0.8 ml egg yolk are added.

v) Middlebrook 7H9, 7H10 and 7H11 media (Difco)

This media enhanced with mycobactin in the same proportion as for Herrold’s medium can also be used. The advantage of this medium is that it is transparent, which facilitates the early detection of colonies.

vi) Löwenstein–Jensen medium with or without mycobactin (Jorgensen, 1982).

• Sample preparation
  • Processing tissue specimens

Chemical preservatives should not be used. The tissues can be frozen at –20°C.

To avoid contamination, the faeces should be rinsed from portions of intestinal tract before shipment to the laboratory.

i) Digestion/sedimentation method for decontamination of tissues

Approximately 4 g of mucosa from the ileocaecal valve or 4 g of mesenteric node are placed in a sterile blender jar containing 50 ml of trypsin (2.5%). The mixture is adjusted to neutrality using 4% NaOH and pH paper, and stirred for 30 minutes at room temperature on a magnetic mixer. The digested mixture is filtered through gauze. The filtrate is centrifuged at approximately 2000–3000 g for 30 minutes. The supernatant fluid is poured off and discarded. The sediment is resuspended in 20 ml of 0.75% HPC and allowed to stand undisturbed for 18 hours at room temperature. The particles that settle to the bottom of the tube are to be used as the inoculum and are removed by pipette without disturbing the supernatant fluid. Alternatively, other methods of decontamination can be used, such as treatment with 5% oxalic acid.

ii) Double incubation method for decontamination of tissues (Gwozdz, 2010)

About 2 g of tissue sample (trimmed of fat) is finely chopped using a sterile scalpel blade or scissors and homogenised in a stomacher for 1 minute in 25 ml 0.75% HPC. Allow the sample to stand so that foam dissipates and larger pieces of tissue settle. Pour tissue homogenate into a centrifuge tube taking
care to avoid carry over of fat or large tissue pieces. Allow to settle for 30 minutes then take 10 ml of the suspension from just above the sediment to a 30 ml tube and incubate for 3 hours at 37°C. Centrifuge for 30 minutes at 900 g, discard supernatant fluid and resuspend pellet in 1 ml antibiotic cocktail containing 100 µg of each of vancomycin, amphotericin and nalidixic acid (VAN). Incubate overnight at 37°C. Use the suspension to inoculate media as described below.

iii) Inoculation of culture media and incubation
Approximately 0.1 ml of inoculum is transferred to each of three slants of Herrold’s medium containing mycobactin and to one slant of Herrold’s medium without mycobactin. The inoculum is distributed evenly over the surface of the slants. The tubes are allowed to remain in a slanted position at 37°C for approximately 1 week with screw caps loose. The tubes are returned to a vertical position when the free moisture has evaporated from the slants. The lids are tightened and the tubes are placed in baskets in an incubator at 37°C.

The egg in Herrold’s medium contributes sufficient phospholipids to neutralise the bactericidal activity of residual HPC in the inoculum. The other media (Modified Dubos and Middlebrook) do not have this property. Other treatments can be used for sample decontamination, for example oxalic acid at 5%.

HPC is relatively ineffective in controlling the growth of contaminating fungi. Amphotericin B (fungizone) was found to control effectively fungal overgrowth of inoculated media (Merkal & Richards, 1972). Fungizone may be incorporated in the Herrold’s medium at a final concentration of 50 µg per ml of medium. Due to loss of antifungal activity, storage of Herrold’s medium containing fungizone should be limited to 1 month at 4°C.

The slants are incubated for at least 4 months and observed weekly from the sixth week onwards.

Variations in the above methods have been described (Collins et al., 1990; Lagadic et al., 1983; Merkal et al., 1968; Ridge, 1993; Singh et al., 1991; Whipple et al., 1991; Whitlock et al., 1991). The sensitivity of culture may be enhanced using liquid media and with centrifugation rather than sedimentation techniques. The double incubation method described by Whitlock et al. (1991) assists with decontamination of the inoculum (Stabel, 1997) and offers higher sensitivity than the sedimentation or filtration protocols (Eamens et al., 2000). The double incubation method involves mixing 2 g faeces with 15 ml saline or water followed by sedimentation for 30 minutes and transferring (avoiding fibrous matter) the top 5ml of the suspension to 25 ml of 0.9% HPC in half-strength brain–heart infusion (Difco). After incubating at 37°C for 16–24 hours, the mixture is centrifuged at 900 g for 30 minutes (room temperature), the supernatant is discarded and pellet resuspended in 1 ml VAN. The mixture is incubated for 24–72 hours at 37°C and used to inoculate media as described above (Gwozdz, 2010).

d) DNA probes and polymerase chain reaction
DNA probes are being developed that offer a means of detecting *M. paratuberculosis* in diagnostic samples and of rapidly identifying bacterial isolates (Ellingson et al., 1996; Merkal, 1984). They have been used to distinguish between *M. paratuberculosis* and other mycobacteria.

McFadden et al. have identified a sequence (McFadden et al., 1987a; 1987b), termed IS900, which is an insertion sequence specific for *M. paratuberculosis*. It has been reported that a small number of isolates other than *M. paratuberculosis* have produced amplified products the same size as expected from
M. paratuberculosis. A restriction enzyme digest may be applied to positive IS900 products to confirm that their sequence is consistent with M. paratuberculosis (Collins et al., 1999).

The identifications of new DNA sequences considered to be unique to M. paratuberculosis; ISMav2, f57, and ISMap02 sequences, offer additional tools for rapid identification of this organism using the polymerase chain reaction (PCR) technology (Stabel & Bannantine, 2005; Strommenger et al., 2001; Vansnick et al., 2004). The restriction enzyme analysis of IS1311, an insertion sequence common to M. avium and M. paratuberculosis can be used to distinguish between these species and for typing of ovine, bovine and bison strains of M. paratuberculosis (Sevilla et al., 2005; Whittington et al., 1998).

The use of IS900 as a DNA probe for specific identification of M. paratuberculosis in faecal samples from cattle by PCR has been reported (Vary et al., 1990). Commercial diagnostic PCR tests for the detection of M. paratuberculosis in milk and faecal samples have been developed.

2. Serological tests

The serological tests commonly used for paratuberculosis in cattle are complement fixation (CF), enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID) (Sockett et al., 1992) corresponding to humoral immunity, and the gamma interferon assay corresponding to cellular immunity.

a) Complement fixation test

The CF test has been the standard test used for cattle for many years. The CF test works well on clinically suspect animals, but does not have sufficient specificity to enable its use in the general population for control purposes. Nevertheless, it is often demanded by countries that import cattle. A variety of CF test procedures are used internationally. An example of a microtitre method for performing the CF test is as follows:

i) The antigen is an aqueous extract of bacteria from which lipid has been removed (strain M. paratuberculosis 316F). Mycobacterium avium D9 may also be used.

ii) All sera are inactivated in the water bath at 60°C for 30 minutes and diluted at 1/4, 1/8 and 1/16. A positive control serum and a negative control serum should be included on each plate. The following controls are also prepared: antigen control, complement control and haemolytic system control.

iii) Reconstituted, freeze-dried complement is diluted to contain six times H50 (50% haemolysing dose) as calculated by titration against the antigen.

iv) Sheep erythrocytes, 2.5%, are sensitised with 2 units of H100 haemolysin.

v) All dilutions and reagents are prepared in calcium/magnesium veronal buffer; 25 µl volumes of each reagent are used in 96-well round-bottom microtitration plates.

vi) Primary incubation is at 4°C overnight and secondary incubation is at 37°C for 30 minutes.

vii) Reading and interpreting the results: Plates may be left to settle or centrifuged and read as follows: 4+ = 100% fixation, 3+ = 75% fixation, 2+ = 50% fixation, 1+ = 25% fixation and 0 = complete haemolysis. The titre of test sera is given as the reciprocal of the highest dilution of serum giving 50% fixation. A reaction of 2+ at 1/8 is regarded as positive. Results should be interpreted in relation to clinical signs and other laboratory findings.

b) Enzyme-linked immunosorbent assay

The ELISA is, at present, the most sensitive and specific test for serum antibodies to M. paratuberculosis in cattle. Its sensitivity is comparable with that of the CF test in clinical cases, but is greater than that of the CF test in subclinically infected carriers. The specificity of the ELISA is increased by M. phlei absorption of sera. The absorbed ELISA, designed by Yokomizo et al. (Yokomizo et al., 1983; 1985) and modified by Milner et al. (2004), was developed into a commercial kit by Cox et al. (1991).

The ELISA detects about 30–40% cattle identified as infected by culture of faeces on solid media (Whitlock et al., 2000). Similarly to the culture methods, the sensitivity of the ELISA depends on the level of M. paratuberculosis shedding in faeces and the age of animals. A large study recently performed in Australia showed that the actual sensitivity of the ELISA in 2-, 3- and 4-year-old cows was 1.2%, 8.9% and 11.6%, respectively, but remained between 20 and 30% in older age-groups (Jubb et al., 2004). The overall actual sensitivity for all age-groups was calculated to be about 15% (Jubb et al., 2004; Whitlock et al., 2000).

In small ruminants the commercially available ELISA had a specificity of 98.2–99.5% (95% confidence intervals [CI]) and detected 35–54% (95% CI) of animals with histological evidence of infection (Hope et al.,
Chapter 2.1.11. – Paratuberculosis (Johne’s disease)

...2000). In another study the estimated specificity of an in-house ELISA was 99% and its sensitivity measured against histological results was 21.9% (Sergeant et al., 2003).

The absorbed ELISA combines the sensitivity of ELISA with the added specificity of an absorption step. Sera to be tested are diluted with buffer containing soluble M. phlei antigen prior to testing in an indirect ELISA. This procedure eliminates nonspecific cross-reacting antibodies. In early versions, sera were absorbed with whole M. phlei, which were removed by centrifugation prior to testing.

A microtitre plate format has been developed in which M. paratuberculosis antigen is coated on to 96-well plates. Samples are diluted in sample diluent containing M. phlei to remove cross-reacting antibodies. On incubation of the diluted sample in the coated well, antibody specific to M. paratuberculosis forms a complex with the coated antigens. After washing away unbound materials from the wells, horseradish peroxidase (HPR)-labelled anti-bovine immunoglobulin is added. This reacts with immunoglobulins bound to the solid-phase antigen. The rate of conversion of substrate is proportional to the amount of bound immunoglobulin. Subsequent colour, measured (at 450 nm) spectrophotometrically is proportional to the amount of antibody present in the test sample.

The antigen used to coat the ELISA plates is available commercially.

An anti-bovine IgG labelled with HRP in used as conjugate. The substrate chromogen solution is hydrogen peroxide tetramethyl benzidine. A solution of 0.5 M H₂SO₄ is used to stop the reaction when the absorbance of the positive control serum reaches a predetermined point.

Several absorbed ELISA kits are commercially available. The method and test materials needed, the interpretation of the results and calculations are fully described in the instructions accompanying the commercial kit. It has recently been reported that several commercially available ELISAs have similar sensitivities and specificities (Collins et al., 2005). Some commercial kits offer and option of testing milk samples. The ELISA on bovine and caprine milk has been found to have specificity similar to that of the serum ELISA, but less sensitive than the blood test (Hendrick et al., 2005; Salgado et al., 2005).

c) Agar gel immunodiffusion test

The AGID test is useful for the confirmation of the disease in clinically suspect cattle, sheep and goats (Shermann et al., 1984). It has been reported that in small ruminants in New Zealand and Australia the AGID offers slightly higher sensitivity and specificity than that obtained by the ELISAs (Gwozdz et al., 2000; Hope et al., 2000; Sergeant et al., 2003). The reported specificity and sensitivity of the AGID measured against histological results were 99–100% (95%CI) and 38–56% (95% CI), respectively (Hope et al., 2000).

The antigen employed is a crude protoplasmic extract of laboratory strain M. avium 18 (formally M. paratuberculosis 18) prepared by disruption of cells in a hydraulic press cell fractionator. Disrupted cells are centrifuged at 40,000 g for 2 hours to remove cell wall debris, and the supernatant fraction is retained and lyophilised. This antigen is resuspended in water at a concentration of 10 mg/ml.

Agarose is dissolved in barbital buffer, pH 8.6, containing sodium azide, to give a final agarose concentration of 0.75%. Agarose may be poured into Petri dishes or on to glass slides. Wells are cut in a hexagonal pattern. Wells are 4 mm in diameter, 4 mm apart, and the agar should be 3–4 mm deep. Antigen is added to centre wells. Test, positive and negative control sera are added to alternate peripheral wells.

Plates are incubated in a humid chamber at room temperature. Gels are examined for precipitation lines after 24 and 48 hours’ incubation. The appearance of one or more clearly definable precipitation line(s), showing identity with that of a control positive serum, before or at 48 hours, constitutes a positive test result. Absence of any precipitation lines is recorded as a negative test result. Nonspecific lines may occur.

Several variations of the method are in use.

3. Tests for cell-mediated immunity

The detection of a systemic cell-mediated response precedes detectable antibody production. Animals that are minimally infected frequently fail to react on serological testing but may react positively to tests that measure cell-mediated immunity.

a) Gamma interferon assay

The assay is based on the release of gamma interferon from sensitised lymphocytes during an 18–36-hour incubation period with specific antigen (avian purified protein derivative [PPD] tuberculin, bovine PPD...
tuberculin or johnin) (Wood et al., 1990). The quantitative detection of bovine gamma interferon is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine gamma interferon. A commercial diagnostic test based on the detection of gamma interferon has been developed for the diagnosis of bovine tuberculosis. The method and test materials needed are fully described in the instructions accompanying the commercial kit. This test has not been validated by the manufacturer (Prionics, Switzerland) for the diagnosis of paratuberculosis. As such, results derived from this assay are frequently difficult to interpret because there is no agreement with respect to the interpretation criteria and types and amounts of antigens used to stimulate blood lymphocytes. In cattle the reported specificity of the test varied from 94% to 67% depending on the interpretation criteria (Kalis et al., 2003).

b) Delayed-type hypersensitivity

The skin test for delayed-type hypersensitivity (DTH) is a measure of cell-mediated immunity, but has limited value. The test is carried out by the intradermal inoculation of 0.1 ml of antigen into a clipped or shaven site, usually on the side of the middle third of the neck. In the past, avian PPD tuberculin or johnin was used for this purpose as it was believed that avian tuberculin and johnin are of comparable sensitivity and specificity. The skin thickness is measured with calipers before and 72 hours after inoculation. Increases in skin thickness of over 2 mm should be regarded as indicating the presence of DTH. It should be noted that positive reactions in deer may take the form of diffuse plaques rather than discrete circumscribed swellings, thus making reading of the test more difficult. The presence of any swelling should be regarded as positive in this species. However, sensitisation to the M. avium complex is widespread in animals, and neither avian tuberculin nor johnin are highly specific (Inderlied et al., 1993). Furthermore the interpretation of the skin test results is complicated by the lack of agreement with respect to interpretation criteria. In a recent study in which johnin (ID-Lelystad, Lelystad, The Netherlands) was used to test cattle, the skin test specificity was 88.8% at the cut-off value of ≥2 mm, 91.3% at the cut-off value of ≥3 mm and 93.5% at the cut-off value of ≥4 mm (Kalis et al., 2003). The effect of these cut-off values on the sensitivity has not been determined. The performance of this test may also be significantly affected by minor antigenic differences that occur in different batches of antigen (Kalis et al., 2003). Further research is required to increase the value of the skin test.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Vaccines: Vaccines used against paratuberculosis are: live, attenuated, incorporated with oil and pumice; lyophilised, live, attenuated, which may be adjuvanted with, for example, oil after reconstitution; and heat-killed bacterins. Vaccines may be prepared from one strain of M. paratuberculosis 316F or 2E (Weybridge) or M. paratuberculosis 3 and 5 or II (Canadian strains), or as many as three strains may be used. The information below applies to a live, attenuated vaccine adjuvanted with oil and pumice (Doyle, 1945; Vallee et al., 1941; Wilesmith, 1982). Vaccination may cause a reaction at the site of injection. Vaccination may also interfere with eradication programmes based on immunological testing and elimination of animals identified as infected and can interfere with the interpretation of DTH skin tests for bovine tuberculosis.

Diagnostic products: Johnin PPD is a preparation of the heat-treated products of growth and lysis of M. paratuberculosis. Avian tuberculin PPD is a preparation of heat-treated products of growth and lysis of M. avium D4ER or TB 56. Details of avian tuberculin PPD are in Chapter 2.3.6 Avian tuberculosis. These two preparations are used, by intradermal injection, to reveal DTH as a means of identifying animals infected or sensitised with M. paratuberculosis.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

Vaccine: Seed strains should be of a prevalent type, which may be checked by biotyping or genetic analysis. They should have been demonstrated to be innocuous when administered by the recommended route of vaccination to intended target species.

Johnin: Strains of M. paratuberculosis used to prepare seed cultures should be identified by biotyping or genetic tests. They should be shown to be free from contaminating organisms.

3 Avian tuberculin can be obtained from AHVLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom, or from Symbiotic Society, 299 av. Jean Jaurès, 69007 Lyon, France.
b) Method of culture

**Vaccine:** Seed cultures may be made on potato slants partly immersed in a suitable medium, such as Reid’s synthetic medium (Watson, 1935). Cultures may be stored lyophilised. Active cultures are normally incubated at 37°C.

**Johnin:** The culture substrate should be shown to be capable of producing a product free from substances known to cause toxic or allergic reactions. A suitable medium for seed culture is that of Reid, solidified with 1.75% agar, in screw cap tubes. Cultures may also be stored lyophilised.

c) Validation as a vaccine

**Vaccine:** Purity tests should be carried out on seed cultures and final harvest by stained smears.

The vaccine should be used as part of a control programme and will not on its own provide complete protection against disease caused by *M. paratuberculosis* (Wilesmith, 1982). There is usually good control of clinical disease, but subclinical infection persists in vaccinated herds, albeit at a reduced level. Vaccine should be administered to animals in early life only, e.g. calves in their first month of life. It should be inoculated subcutaneously and causes a small inflammatory swelling. This is gradually replaced by a cold, painless, fibro-caseous nodule, which varies in size and which may persist for years. Vaccination has been used to control the disease in sheep and goats, including older animals. In order to get the best results from vaccination, management practices to control the disease should also be in place.

The use of vaccines may interfere with the outcome of diagnostic skin tests for tuberculosis, and this should be remembered when planning a control programme (Halgaard, 1984).

**Johnin:** Cultures should be checked by staining smears for the presence of contaminating organisms.

To test for lack of sensitising effect, three guinea-pigs that have not previously been treated with any material that could interfere with the test, are each injected intradermally on each of three occasions at 5-day intervals, with 0.01 mg of the preparation under test in a volume of 0.1 ml. Each guinea-pig, together with each of three control guinea-pigs that have not been injected previously, is injected intradermally 15–21 days after the third injection with the same dose of the same johnin. The reactions of the two groups of guinea-pigs should not be significantly different when measured 24–48 hours later.

2. Method of manufacture

**Vaccine:** For vaccine batches, the organisms may be grown on a liquid synthetic medium, such as Reid’s synthetic medium. The organisms grow as a pellicle on the liquid surface. To ensure a good surface area, it is convenient to use vessels such as conical flasks containing one-third of their nominal volume of liquid medium. These flasks may be seeded directly from potato slant cultures, but with some strains, one or more passages on liquid medium may be necessary to ensure adequate pellicle growth for the final, vaccine batch passage. Such passaging should usually take place at 2-week intervals as longer periods may result in over-maturation and sinking of the pellicle. Incubation is at 37°C.

To prepare the vaccine, the pellicle growth from 2-week-old cultures of each strain to be included may be separated from the liquid medium by decantation, filtration and pressing between filter paper pads. The moist *M. paratuberculosis* culture is blended with an adjuvant, such as liquid paraffin, olive oil and pumice (Doyle, 1945).

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4 L-Asparagine, 5.0 g  
Potassium dihydrogen phosphate (KH$_2$PO$_4$, anhydrous), 2.0 g  
Magnesium sulphate (MgSO$_4$·7H$_2$O), 1.0 g  
Ammonium citrate ([NH$_4$]$_3$ C$_6$H$_5$O$_7$), 2.0 g  
Sodium chloride, 2.0 g  
Ferric ammonium citrate, 0.075 g  
Dextrose monohydrate B.P., 10 g  
Glycerol B.P. (46 ml), 60 g  
Distilled water to 1000 ml  
PH (not adjusted) 5.6–5.8  
When required, the above medium is solidified by the addition of 1.5% granulated agar (Difco). Sterilised at 121°C for 15 minutes.
Johnin: Johnin for skin test diagnosis is a PPD prepared from one or more strains of *M. paratuberculosis* (available from AHVLA Weybridge or CDI, Lelystad, the Netherlands). It may be prepared by the following method.

*Mycobacterium paratuberculosis* strains are grown as a pellicle on liquid Reid’s medium. Production cultures are usually inoculated from liquid seeding cultures rather than directly from seed on solid medium (Reid’s synthetic medium). Production cultures are incubated at 37°C for 10 weeks.

At the end of the incubation period, the culture medium has a pH of about 5 and little or no johnin will be obtained unless the pH is raised, using sodium hydroxide, to about 7.3 before steaming. After thorough mixing, the cultures are free steamed for 3 hours. The bulk of the killed organisms is removed by coarse filtration and the filtrate is clarified by further filtration. Protein in the filtrate is precipitated chemically with 40% trichloroacetic acid, washed and redissolved (alkaline solvent). The product is sterilised by filtration. An antimicrobial preservative that does not give rise to false-positive reactions, such as phenol (not more than 0.5% [w/v]), may be added. Glycerol (not more than 10% [w/v]) may be added as a stabiliser. The product is dispensed aseptically into sterile glass containers, which are then sealed.

3. In-process control

**Vaccine:** Adequate growth of culture and cultural purity need to be checked. Presence of contaminating organisms may be detected by conventional sterility tests on harvests. Tests for pathogenic mycobacteria are carried out by injection of moist culture, taken prior to blending with adjuvant and diluted tenfold in saline, into two guinea-pigs, each receiving 1 ml. These are observed for 8 weeks, killed humanely, and examined for any abnormal lesions.

**Johnin:** After final filtration the sterility of each filtrate of the PPD solution is checked. Sterile filtrates are tested for protein content by a Kjeldahl method (British Pharmacopoeia [Veterinary], 1985). The protein content is adjusted to give between 0.475 and 0.525 mg/ml of protein in the final product. The pH is adjusted to the range 6.5–7.5.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination may be found in Chapter 1.1.7 Tests for sterility and freedom from contamination of biological materials. The vaccine organism will not normally grow to a detectable level in conventional sterility tests.

b) Safety

**Vaccine:** These tests are normally performed in laboratory animals, although multidose tests in target animals would also be satisfactory. A typical laboratory animal test would be as follows. Each of two guinea-pigs is inoculated, subcutaneously, with an acceptable batch of vaccine at a fraction of the cattle dose previously determined to give a nodule but no overt necrosis at the injection site. Animals are observed for 8 weeks, killed humanely and examined for any abnormal lesions.

**Johnin:** Two guinea-pigs should each be injected subcutaneously with 0.5 ml of the johnin under test. No significant local or systemic lesions should be seen within 7 days (British Pharmacopoeia [Veterinary], 1985).

Tests on johnin for living mycobacteria may be performed either on the material immediately before it is dispensed into final containers or on samples taken from final containers themselves. A sample of at least 10 ml should be taken, and this should be injected intraperitoneally or subcutaneously into at least two guinea-pigs, dividing the volume to be tested equally between the guinea-pigs. It is desirable to take a larger sample, say 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea-pigs are observed for at least 42 days and post-mortem examinations are carried out. Any macroscopic lesions are examined microscopically and culturally.

c) Potency

**Vaccine:** As protection tests appear to be impractical, a test of sensitising ability may be used. This may then be related to bacterial content based on weight. A typical test would be as follows: guinea-pigs are sensitised by intramuscular injection of 0.5 ml of a 100-fold dilution in liquid paraffin of the vaccine under test. Skin tests are performed 6 weeks after sensitisation using intradermal inoculations of 0.2 ml of at least three serial dilutions of an *M. paratuberculosis* antigen, such as johnin PPD, the dilutions being chosen to
give expected skin reactions of from 8 mm to 25 mm diameter. Each guinea-pig receives several dilutions per flank, their distribution being chosen by a Latin square design. After 24–48 hours, skin reactions are measured. A reference preparation for tests of this type has not yet been fully established. Avian tuberculin PPD of known international unitage may be used as a skin test antigen in tests of this type to ensure that the vaccine is capable of producing adequate sensitisation (corresponding to the vaccination).

**Johnin:** The potency of johnin is currently determined by chemical assay for protein using a Kjeldahl method. A PPD content of 0.5 ± 0.025 mg/ml of final product is recommended (British Pharmacopoeia [Veterinary], 1985).

The identity of the material should be confirmed by injecting intradermally into guinea-pigs sensitised by injections of killed *M. paratuberculosis* (100 mg powder mycobacteria + 25 ml vaseline + 100 mg pumice stone) 6 weeks previously.

It is possible to perform a potency test using dilutions of johnin in guinea-pigs sensitised with *M. paratuberculosis*, similar to such tests for the potency of bovine and avian tuberculin, but a standard preparation for this type of test has not yet been fully established.

d) **Duration of immunity**

**Vaccine:** After vaccination at the age of 14–30 days, the vaccination effect is expressed as the reduction in the rate of excretors among vaccinated animals as compared with nonvaccinated bovines (Jorgensen, 1984).

There is usually good control of clinical disease, but a reduced level of subclinical infection persists. The favourable results probably reflect a diminishing exposure to infection resulting from a reduction in the number of heavy excretors in the herd.

e) **Stability**

**Vaccine:** The vaccine may be stored at 2–8°C for 9–12 months without loss of potency. It should not be frozen.

**Johnin:** Johnin should be protected from light and stored at 2–8°C. Under these conditions it should retain its potency for at least 5 years.

f) **Preservatives**

A preservative is normally included for vaccine in multidose containers. For johnin, the phenol used is no more than 0.5% (w/v). The concentration of the preservative in the final product and its persistence through shelf life should be checked.

g) **Precautions (hazards)**

**Vaccine:** The vaccine causes some side-effects, nodule formation and sensitisation of animals to the tuberculin test (Gwozdz *et al*., 2000). In humans, accidental injection of vaccine has resulted in chronic inflammatory reactions requiring surgical treatment (Jorgensen, 1984).

5. **Tests on the final product**

a) **Safety**

See Section C.4.b.

b) **Potency**

See Section C.4.c.

REFERENCES


Chapter 2.1.11. — Paratuberculosis (Johne’s disease)


Chapter 2.1.11. – Paratuberculosis (Johne’s disease)


Whittington R., Marsh I., Choy E., Cousins D. (1998). Polymorphisms in IS1311, an insertion sequence common to Mycobacterium avium and M. avium subsp. paratuberculosis, can be used to distinguish between and within these species. Mol. Cell Probes., 12 (6), 349–358.


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NB: There are OIE Reference Laboratories for Paratuberculosis (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for paratuberculosis.
CHAPTER 2.1.12.

Q FEVER

SUMMARY

Query (Q) fever (or Coxiellosis) is a zoonosis that occurs in most countries. Humans generally acquire infection through air-borne transmission from animal reservoirs, especially from domestic ruminants, but other domestic and wildlife animals (pets, rabbits, birds, etc.) can be involved. The causal agent is the obligate intracellular bacterium, Coxiella burnetii, which displays different morphological forms in its developmental cycle. Some forms can survive extracellularly and even accumulate in the environment. Because this microorganism is classified as a Group 3 pathogen, handling viable C. burnetii must be done in biosafety level 3 facilities.

In humans, the disease exhibits a large polymorphism. Q fever occurs in either an acute form or a severe chronic form following an early infection that may go unnoticed. The acute form resolves quite quickly after appropriate antibiotic therapy, but the chronic form requires prolonged antibiotic therapy (for 2 years or more), coupled with serological monitoring. In Australia, a vaccine (named Q-Vax) is available for professionally exposed population groups.

In domestic ruminants, Q fever is mostly associated with sporadic abortions or outbreaks of abortions and dead or weak offspring, followed by recovery without complications. Moreover, data also suggests that Q fever plays a role in infertility or problems such as metritis in cattle. Coxiella burnetii infection persists for several years, and is probably lifelong. Sheep, goats and cows are mainly subclinical carriers, but can shed bacteria in various secretions and excreta.

Identification of the agent: For laboratory diagnosis in the context of serial abortions and/or stillbirths, samples can be taken from the placenta, vaginal discharges and tissues of aborted fetuses (liver, lung or stomach content). For investigation of bacterial shedding, samples can be taken from vagina, milk, colostrum and faeces.

As an obligate intracellular bacterium, Coxiella burnetii can be isolated by inoculation of specimens into conventional cell cultures, embryonated chicken yolk sacs or laboratory animals. Inoculation of laboratory animals (guinea-pig, mouse, hamster) is helpful in cases requiring isolation from tissues, faeces, milk or environmental samples contaminated with various microorganisms.

The bacteria can be visualised in stained tissue or vaginal mucus smears using a microscope with an oil-immersion objective lens. Because it is acid resistant, the bacteria can be stained by several methods: Stamp, modified Ziehl–Neelsen, Gimenez, Giemsa and modified Koster. Because of lack of specificity, a positive finding is presumptive evidence of Q fever.

To date, demonstration of the agent by immunohistochemical staining, by in-situ hybridisation or by polymerase chain reaction (PCR) has proven to be more specific and sensitive than classical staining methods. No specific antibodies for immunochemistry are commercially available, but PCR kits are proposed for ruminants and can be used easily in suitably equipped laboratories. PCR is considered to be a useful and reliable test for screening large numbers and various types of samples. Currently, PCR has become the tool of choice for Q fever diagnosis.

Two PCR-based typing methods have been described recently, MLVA (multi-locus variable number of tandem repeats analysis) and multispacer sequence typing (MST), permitting the typing of C. burnetii without the need for isolation of the organism.

Serological tests: A number of tests can be used, particularly the indirect immunofluorescence (IFA) test, the enzyme-linked immunosorbent assay (ELISA), and the complement fixation test (CFT). The presence of specific IgG antibodies provides evidence of a recent C. burnetii infection or a past exposure. ELISAs are preferred for practical reasons and for their higher sensitivity.
Requirements for diagnostic biologicals and vaccines: Serological antigens are based on the two major antigenic forms of C. burnetii: phase I, obtained from spleens after inoculation of laboratory animals, and phase II, obtained by repeated passages in embryonated eggs or in cell cultures. Currently available commercial tests allow the detection of phase II or of both phases II and I anti-C. burnetii antibodies.

Several inactivated vaccines against Q fever have been developed, but only vaccines containing or prepared from phase I C. burnetii should be considered protective.

An inactivated phase I vaccine (named Coxevac) is commercially available. Repeated annual vaccination, particularly of young animals, are recommended in at-risk areas.

A. INTRODUCTION

Q fever is widely distributed throughout the world with the exception of New Zealand. Although Q fever is present in virtually all ‘animal kingdoms’, including arthropods, the disease affects mostly humans, cattle, sheep and goats (Arricau-Bouvery & Rodolakis, 2005; EFSA, 2010; Lang, 1990; Maurin & Raoult, 1999). The aetiological agent, Coxiella burnetii, is a Gram-negative obligate intracellular bacterium, adapted to thrive within the phagolysosome of the phagocyte. It has been historically classified in the Rickettsiaceae family; however, phylogenetic investigations, based mainly on 16s rRNA sequence analysis, have shown that the Coxiella genus is distant from the Rickettsia genus in the alpha subdivision of Proteobacteria (Drancourt & Raoult, 2005). Coxiella burnetii has been placed in the Coxiellaceae family in the order Legionellales of the gamma subdivision of Proteobacteria. The complete genome sequencing of C. burnetii has been achieved and confirms its systematic position (Seshadri et al., 2003). Unlike rickettsiae, C. burnetii produces a small, dense, highly resistant spore-like form (Coleman et al., 2004; Heinzlen et al., 1999). This ability has been attributed to the existence of C. burnetii developmental cycle variants described from in-vitro studies: large-cell variants (LCV), small-cell variants (SCV), and small dense cells (SDC) measuring 0.2 µm wide and between 0.5 and 2 µm long or 0.4 to 0.7 µm diameter (Coleman et al., 2004; Heinzlen et al., 1999). The SDC and SCV represent the small morphological variants of the bacteria likely to survive extracellularly as infectious particles, a trait that is important for persistence in the environment and transmission (ECDC 2010; EFSA, 2010; Kersh et al., 2010). Another essential characteristic is that C. burnetii has two antigenic forms: the pathogenic phase I, isolated from infected animals or humans, and the attenuated phase II, obtained by repeated in-ovo or in-vitro passages. An LPS (lipopolysaccharide) change occurs during serial passages: phase I cells, with full-length LPS O-chains, change to intermediate phases with decreasing LPS O-chain lengths and then to phase II, with truncated LPS. Thus, the long phase I LPS contains the phase II part. The latter has been described as a major immunogenic determinant. Currently available commercial tests allow the detection of at least the anti-C. burnetii phase II antibodies, which appear to be present whatever the infection stage or form. In contrast, vaccination is effective without immunity. The infection is endemic in many areas leading to sporadic cases or explosive outbreaks. In the absence of any appropriate antibiotic treatment, complications of the chronic form may be severe and sometimes fatal. Moreover, C. burnetii infection of pregnant women can provoke placentitis and leads to premature birth, growth restriction, spontaneous abortion or fetal death. Overall, the chronic disease is more likely to develop in immuno-compromised individuals. The infection is endemic in many areas leading to sporadic cases or explosive epidemics. Its incidence is probably greater than reported. Awareness for Q fever is increased during human outbreaks, which are generally temporary and rarely comprise more than 300 acute Q fever cases. However, the largest community outbreaks of Q fever ever reported emerged in 2007 in the Netherlands. In the subsequent years, peak incidence from February to September has increased and the geographical area has expanded progressively. The country reported 982 and 2305 confirmed cases in 2008 and 2009, respectively. However, the factors leading to outbreaks are not fully understood (ECDC, 2010; EFSA, 2010).

Domestic ruminants are considered the main reservoirs for C. burnetii, but cats, dogs, rabbits, birds, etc., have also been reported to be implicated in human disease/infection. There is clear epidemiological and experimental evidence that the infection is principally transmitted by inhalation of desiccated aerosol particles, and through contact with infected animals, their reproductive tissues or other animal products, like wool (Arricau-Bouvery & Rodolakis, 2005; ECDC, 2010; Maurin & Raoult, 1999). Ingestion has been often suggested, particularly through the consumption of dairy products derived from contaminated raw milk, but no good evidence has shown a significant transmission to humans by food. Indeed, there are epidemiological indications of sero-conversion but no association with clinical Q
fever in humans. Q fever seems also very rarely transmissible from person to person, although exposure during childbirth, through sexual transmission or blood transfusion is possible. In animals, vertical transmission and sexual transmission could occur but their importance is not known. Finally, arthropods, principally ticks, may be involved in Q fever transmission. The risk of transmission seems to be linked to wildlife animals. It could be associated with bites as well as with contaminated dust from dried excrement.

In cows, ewes and goats, Q fever has been associated mostly with late abortion and reproductive disorders such as premature birth, dead or weak offspring (Arricau-Bouvery & Rodolakis, 2005; Lang, 1990). Moreover, C. burnetii might be associated with metritis and infertility in cattle. Given the lack of specificity of these latter signs, it is not recommended to retain them for clinical diagnosis of Q fever (EFSA, 2010). Domestic ruminants are mainly subclinically carriers but can shed bacteria in various secretions and excreta. In the environment, C. burnetii can survive for variable periods and can spread. The levels of bacterial contamination in the environment have been tackled using quantitative PCR (polymerase chain reaction) for detection of C. burnetii DNA, but a rapid test assessing viability is required (EFSA, 2010; Kersh, 2010). For now, the lack of knowledge on shedding patterns among ruminants has made the determination of Q fever status difficult. Concomitant shedding into the milk, the faeces and the vaginal mucus may be rare (Guatteo et al., 2007; Rousset et al., 2009a). The vaginal shedding at the day of kidding may be the most frequent (Arricau-Bouvery et al., 2005). Within herds or flocks experiencing abortion problems caused by C. burnetii, most of animals may be shedding massive numbers of bacteria whether they have aborted or not. The global quantities are thus clearly higher than within subclinically infected herds/flocks. At the parturitions following an abortion storm, higher bacterial discharges were measured among the primiparous compared with the other females (Guatteo et al., 2008; Rousset et al., 2009b). Moreover, the shedding may persist several months, describing either an intermittent or a continuous kinetic pattern. Animals with continuous shedding patterns might be heavy shedders. These latter animals seem to mostly exhibit a highly-seropositive serological profile (Guatteo et al., 2007).

Diagnosis of Q fever in ruminants, including differentiating it from other abortive diseases, traditionally has been made on the basis of microscopy on clinical samples, coupled with positive serological results (Lang, 1990). At present, direct detection and quantification by PCR and serological ELISA (enzyme-linked immunosorbent assay) should be considered as methods of choice for clinical diagnosis (Sidi-Boumedine et al., 2010). Proposals have been recently elaborated for the development of harmonised monitoring and reporting schemes for Q fever, so as to enable comparisons over time and between countries (EFSA, 2010; Sidi-Boumedine et al., 2010). However, no gold standard technique is available and efforts are encouraged both for the validation of the methods and for development of reference reagents for quality control, proficiency and harmonisation purposes (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases). The Q fever diagnostic tests are also required for epidemiological surveys of ‘at risk’ and suspected flocks in limited areas (following recent outbreaks in humans or animals), or for exchanges between herds or flocks.

Concerns about the risks posed by Q fever have been raised in Europe, where the European Commission requested scientific advice and risk assessment for humans and animals (ECDC, 2010; EFSA, 2010). The main conclusions were that the necessary actions to stop an outbreak must be carried out by health authorities together with veterinary authorities at the national and the local levels. The overall impact of C. burnetii infection on public health is limited but there is a need for a better surveillance system. In human epidemic situations, active surveillance of acute Q fever is the best strategy for avoiding chronic cases. Measures for the control of animal Q fever should be implemented, particularly for domestic ruminants. Only a combination of measures is expected to be effective. Long-term options include preventive vaccination, manure management, changes to farm characteristics, wool shearing management, a segregated kidding area, removal of risk material, visitor ban, control of other animal reservoirs and tick control. The culling of pregnant animals, a temporary breeding ban, stamping out, identifying and culling shedders and controlling animal movements are considered as suitable options in the case of human outbreaks.

Regarding biosafety and biosecurity, C. burnetii is extremely hazardous to humans. Because of its ability to cause incapacitating disease in large groups of people, its likely low infectious dose, resistance in the environment, and aerosol route of transmission, C. burnetii is currently considered a potential agent of bioterrorism and is classified by the Center for Disease Control and prevention as a group B biological agent (Drancourt & Raoult, 2005; Kersh et al., 2010). Appropriate precautions must be taken with this risk group 3 agent. Live culture or contaminated material from infected animals must only be handled in facilities that meet the requirements for Containment Group 3 pathogens as outlined in Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Coxiella burnetii can be demonstrated in various ways, depending on the type of sample and the purpose of investigations (Samuel & Hendrix, 2009; Sidi-Boumedine et al., 2010). The ability to detect and quantify C. burnetii DNA by real-time PCR has dramatically enhanced diagnostic and study approaches. For laboratory
diagnosis in the context of serial abortions and stillbirths, samples should be collected from aborted fetuses, placenta and vaginal discharges soon after abortion or parturition. The diagnosis should always include a differential investigation of major abortive agents. Early detection of a Q fever storm of abortions in a herd or flock and correct measures is essential to deal with both farm-based and environmental route of infection. A positive case is a herd or flock with clinical signs (abortion and/or stillbirth) for which the presence of the agent has been confirmed. If possible, vaginal swabs at the day of parturition (or taken less than 8 days after) should be collected in order to limit the number of false-negative PCR results. When difficulties in interpretation of diagnostic results are encountered, an association with a positive serological result at the herd or flock level is useful. Milk from the tank, individual milk or colostrum, vaginal and faecal samples can be taken for investigating bacterial shedding. However, detection of shedders is still fastidious as the shedding dynamics are not well known (EFSA, 2010; Guatteo et al., 2007; Kim et al., 2005; Rousset et al., 2009a). Testing bulk tank milk or pooled individual samples (i.e. vaginal swabs and/or milk samples) should be assessed in terms of the relationships with the intra-herd or flock prevalence of shedding.

a) Isolation of the agent

For specific laboratory investigations, it may be necessary to isolate the agent. Where microscopic examination has revealed large numbers of C. burnetii combined with a low contamination rate with other bacteria, direct isolation by inoculation of embryonated chicken eggs or cell culture is possible (Maurin & Raoult, 1999; Samuel & Hendrix, 2009). For example, a portion of placenta is homogenised in phosphate-buffered saline (PBS) containing antibiotics (streptomycin 100–200 µg/ml and penicillin or gentamicin 50–100 µg/ml). After low-speed centrifugation, dilutions of the supernatant fluid are inoculated into 6- to 7-day-old embryonated chicken eggs via the yolk sac. Eggs are preferably from specific pathogen free (SPF) hens. Embryos that die during the first 5 days after inoculation are discarded. The yolk sacs are harvested after 10–15 days of incubation. Stained smears of the yolk sac wall are examined to ensure the absence of bacterial contamination and to determine the presence of C. burnetii. PCR analysis can also be used to detect the presence of C. burnetii. Further passages may be required to obtain an isolate in pure culture.

A cell microculture system from a commercially available method used for virus culture, the shell vial cell culture1, has been adapted for isolating strict or facultative intracellular bacteria, including C. burnetii. Such a method was described for C. burnetii in 1990 (Raoult et al., 1990). Suspensions of samples are inoculated into human embryonic lung (HEL) fibroblasts grown on a 1 cm² cover-slip within a shell vial. Various cell lines may be used to allow the observation of characteristic vacuoles of C. burnetii multiplication. Centrifugation for 1 hour at 700 g enhances the attachment and penetration of bacteria into the cells. Three shell vials are used for the same sample, and by day 3, 10 and 21, the cytopathic effect (CPE) – C. burnetii characteristic vacuoles in cells – are examined using an inverted microscope. After 10 days, detection of growing C. burnetii within the cells is achieved directly on the cover-slip inside a shell vial by a direct immunofluorescence assay with polyclonal anti-C. burnetii antibodies and an appropriate secondary antibody conjugated to fluorescein isothiocyanate (FITC). Cells of the remaining shell vial are harvested and transferred in a 25 cm² culture flask. Incubation can be conducted for 3 months, with a culture medium change once a week. The infection can be monitored by microscopy of Gimenez-stained cells cyto-centrifuged from the culture supernatant and by PCR analysis of the culture supernatant. When the CPE observations and Gimenez staining or PCR results are positive, a passage in a 75 cm² culture flask is performed. Culture supernatant is then inoculated on confluent layers of Vero cells or L929 mouse fibroblasts in a 150 cm² culture flask in order to establish a C. burnetii isolate. This method was developed for humans but could be adapted for animals.

With heavily multi-contaminated samples, such as placenta, vaginal discharges, faeces, or milk, the inoculation of laboratory animals may be necessary as a filtration system. Biocontainment level 3 requirements are recommended for holding experimentally infected rodents (see chapter 1.1.3). Mice and guinea-pigs are the most appropriate laboratory animals for this purpose (Scott et al., 1987). Following intraperitoneal inoculation with a dose of 0.5 ml per animal, body temperature and antibody status can be monitored. This method should be performed in conjunction with serological tests on other guinea-pigs or mice that have been inoculated with the same samples. Sera are collected 21 days after inoculation. A positive result confirms a diagnosis of C. burnetii infection. If pyrexia develops, the animal is killed and the spleen is removed for isolation of the agent by inoculation into embryonated chicken eggs or in cell cultures. Microscopic examination of C. burnetii can be done using impressions and staining of the collected spleens. Alternatively, the process can be simplified by performing PCR for detection of C. burnetii DNA (see below) on spleens. In the mouse model, the spleens can be systematically collected around 9 days post-inoculation.

b) Staining

In a case of an abortion suspected to have an infectious origin, smears of placental cotyledon are prepared on microscope slides. Lung, liver and abomasal contents of the aborted fetus or vaginal discharge may be

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1 Sterlin, Bibby Sterlin Ltd, Stone, Staffordshire ST15 0SA, United Kingdom.
used in the same manner. These could be stained according to several methods: Stamp, Gimenez, Macchiavello, Giemsa and modified Koster (Gimenez, 1964; Quinn et al., 1994; Samuel & Hendrix, 2009). The first three techniques give the best results. These methods are close to the modified Ziehl–Neelsen method involving basic fuchs in to stain bacteria. For example, the Stamp staining method is performed with 0.4% basic fuchsin solution, followed by rapid decolourisation with 0.5% acetic acid solution, and counterstaining with 1% methylene blue or malachite green solution. The smears are examined microscopically with an oil-immersion objective lens (×500 or more). The Stamp method is preferred in veterinary laboratories while the Gimenez method is widespread for monitoring infected cultural cells in research laboratories. Gimenez is fastest because an acidic solution is not included for differentiation. Coxiella burnetii are characterised by a very large number of thin, pink-stained coccobacillary bacteria against a blue or green background. They may sometimes be difficult to detect because of their small size, but this is compensated for by their large numbers; often inclusions within the host cells appear as red masses against the blue or green background. Attention must be taken in the interpretation of the results as, microscopically, C. burnetii can be confused with Chlamydia abortus or Brucella spp. However, using the same staining procedure, Chlamydia abortus have sharper outlines, are round, small and may resemble globules. Brucella spp. are larger (0.6–1.5 µm long × 0.5–0.7 µm wide), more clearly defined and stain more intensely. Control positive slides of C. burnetii, Chlamydia abortus and Brucella must be used for comparison. Diagnosis made on the basis of microscopy, coupled with positive serological results, is usually adequate for routine purposes. When biological staining is inconclusive, one of the other methods may be used as a confirmatory test.

c) Specific detection methods

Detection of C. burnetii in samples can also be achieved by specific immunodetection (capture ELISA, immunohistochemistry), in-situ hybridisation or DNA amplification (Jensen et al., 2007; Samuel & Hendrix, 2009; Thiele et al., 1992). Immunohistology may be used with paraffin-embedded tissues or on acetone-fixed smears (Raoult et al., 1994). The method is an indirect immunofluorescence or immunoperoxidase assay using polyclonal C. burnetii specific antibodies produced in laboratory animals (rabbit or guinea-pig). An anti-species (rabbit or guinea-pig) anti-IgG conjugate, labelled with FITC or peroxidase, is then used to visualise the bacteria. Control positive slides of C. burnetii antigen should be available for comparison. No specific antibodies for immunohistochemistry are commercially available.

Fluorescent in-situ hybridisation using specific oligonucleotide probes targeting 16s rRNA may be used on paraffin embedded tissues, especially placenta samples (Jensen et al., 2007).

PCR methods have been used successfully to detect C. burnetii DNA in cell cultures and biological samples. The PCR methods of C. burnetii detection are generally performed for the health investigations of ruminant herds or flocks prone to abortions (Sidi-Boumedine et al., 2010). Nevertheless, as the number of C. burnetii is likely to be lower in milk, colostrums and faeces than in abortion material, PCR can be used for analysis of this large diversity of samples. Before performing the PCR, biological samples can be inactivated, for ensuring the safety of laboratory personnel, by heating at 90°C for 30–60 minutes, depending of the samples’ nature, their size or their weight. This technique can be performed in suitably equipped laboratories using primers derived from various targets, such as multicopy insertion sequence IS1111 (accession number M80806), the most largely employed (Berri et al., 2000). The use of these primers for the amplification of this sequence allows the sensitivity of the test to be increased and this because of the presence of several copies in the Coxiella genomes. The other target genes reported to be used in the PCR for specific C. burnetii identification are: superoxide dismutase (sodB) gene (accession number M74242); com1 encoding a 27 kDa outer membrane protein (accession number AB004712); and macrophage infectivity potentiator protein (cbmip) (accession number U14170).

The real-time PCR provides an additional means of detection and quantification (Kim et al., 2005; Klee et al., 2006; Stemmler & Meyer, 2002). As with the conventional PCR, various target genes are used: IS1111; IS30; com1; and icd. To quantify the bacteria in biological samples using the real-time PCR, it is recommended to amplify a unique and specific sequence. Indeed, recent data show that the number of the insertion sequence (IS1111) varied widely (between 7 and 110) depending on the isolate (Klee et al., 2006). Whereas the use of this sequence could increase the sensitivity of the test, it may not be accurate for quantification when different strains are involved. It is nevertheless sufficiently informative for high quantities of bacteria (i.e. >10^4 per vaginal swab) for abortive diagnosis (Sidi-Boumedine et al., 2010). Regarding complex matrices, the DNA eluates should be evaluated for their ability to inhibit a PCR by adding an internal DNA control (such as a GAPDH sequence target).

Different primers and probes used in PCR can be obtained on the web site (http://ifr48.timone.univ-mrs.fr/Fiches/Fievre_Q.html#toc22), regularly updated by the French Reference National Center for human Q fever. Ready-to-use kits are commercially available and can detect the bacteria in various sample types.
However, there is an urgent need for the development of a molecular method for the assessment of bacterial viability, especially in milk samples and environmental samples. The development of a multiplex PCR or microarray constitutes other techniques for screening all infectious abortive agents in a single assay.

d) Genotyping methods

Q fever epidemiology is complex as represented by its wide host range, its capacity to resist in the environment and its multifactorial air-borne transmission. Although characterisation of isolates seems necessary for understanding the varying epidemiology of Q fever in different geographical areas, assessment of discriminatory typing methods for molecular epidemiology are in progress (Chmielewski et al., 2009; Klaassen et al., 2009; Sidi-Boumedine et al., 2009). These tools are very useful for epidemiological investigation, particularly to clarify links regarding source of infection, for better understanding the epidemiological emerging factors, and to a lesser extent for evaluating control measures.

Several typing methods have been used for the characterisation of C. burnetii strains, such as restriction endonuclease of genomic DNA (Hendrix et al., 1991), PFGE (pulsed-field gel electrophoresis) (Heinzen et al., 1990; Jager et al., 1998), and sequence and/or PCR-RFLP (restriction fragment length polymorphism) analysis of icd, com1 and mucZ genes. More recently, two PCR-based typing methods have been described, MLVA (multi-locus variable number of tandem repeats analysis) (Arricau-Bouvery et al., 2006; Svraka et al., 2006) and multispace sequence typing (MST) (Giazunova et al., 2005) that permit the typing of C. burnetii without the need for isolation of the organism.

To date, MLVA and MST are considered to be the most discriminating methods for C. burnetii, allowing the identification of up to 36 distinct genotypes. Moreover, databases have been established http://minisatellites.u-psud.fr/MLVAnet/ and http://ifr48.timone.univ-mrs.fr, respectively for MLVA and MST. The availability of such databases allows interlaboratory comparisons to be made easily and this will lead to a better understanding of the propagation of the C. burnetii isolates. Furthermore, their use in the characterisation of field samples or isolates is increasing (Chmielewski et al., 2009; Klaassen et al., 2009) and efforts to produce a standardised scheme for MLVA (based on common decisions for allele calling and marker panels to be used) are in progress and should be available in the near future (Sidi-Boumedine et al., 2009).

2. Serological tests

Among the various techniques that can be employed, the three most often used are: the indirect immunofluorescence assay (IFA), the ELISA and the complement fixation test (CFT). Three older serological tests are no longer used in routine diagnosis: the microagglutination technique, the capillary agglutination test and the indirect haemolysis test. Overall, ELISAs are preferred for practical reasons. Currently, no IFA is commercially available for ruminants. The production of the commercial antigen for CFT will probably be interrupted in the future. Numerous reports showed a weak sensitivity of CFT compared with other methods (EFSA, 2010; Kittelberger et al., 2009; Rousset et al., 2007; 2009a). Serological tools allowing specific antibodies detection in sera from different animal species (not only ruminants) should be developed (Jasper et al., 1994; Soliman et al., 1992).

The presence of specific IgG anti-C. burnetii antibodies provides evidence of a recent infection as well as a past exposure. Serological assays are suitable for screening herds or flocks, but the interpretation at the individual animal level is not possible. Indeed, a significant proportion of animals shedding C. burnetii bacteria and even some Q fever aborted animals are found to be seronegative (Arricau-Bouvery et al., 2005; Guatteo et al., 2007; Rousset et al., 2007, 2009a). Serological cut-off values used to diagnose Q fever are given by the suppliers; it was proposed that interpretation of the results requires at least six ewes or goats and ten cows (those aborted in priority). Both serological responses and bacterial evidence are often necessary for establishing the presence of the infection.

a) Indirect immunofluorescence assay (IFA)

In human medicine, the IFA adapted as a micro-immunofluorescence technique is the current method for the serodiagnosis of Q fever (Tissot-Dupont et al., 1994). The procedure can be adapted to perform an immunoperoxidase assay. Briefly, both phase I and phase II C. burnetii antigens are used; phase II antigen is obtained by growing C. burnetii Nine Mile reference strain in cell culture, while phase I antigen is obtained from the spleens of laboratory animals inoculated with phase II C. burnetii in cell cultures. A few phase I cells may still be present in the phase II population and can be selected and propagated within animals. Antigen is diluted, dropped on to the wells of a glass microscope slide, allowed to dry, and fixed with acetone. The two forms of the infection, acute and chronic, have different serological profiles: during acute Q fever, IgG antibodies are elevated against phase II only whereas during chronic Q fever, high levels of IgG antibodies to both phase I and II of the bacteria are observed (Tissot-Dupont et al., 1994). In addition, antigen-spot slide wells may be purchased from a supplier providing the phase II form, or the phase I and II forms of
C. burnetii. These can be adapted by replacing the human conjugate by a conjugate adapted to the animal species.

Twofold dilutions of the serum under test are placed on immunofluorescence slides with wells previously coated with one or two antigens. If specific antibodies are present, they are fixed by the antigen on the slide. The complex is then detected by examination with a fluorescence microscope following the addition of the fluorescent conjugate recognising the species-specific immunoglobulins.

• Materials and reagents

Microscope equipped for fluorescence, humidified incubator, washing basin.

Slides suitable for the antigen are necessary. The latter may be either prepared in the laboratory or purchased from a supplier (see above). The method described is adapted from the BioMérieux kit, and is given as an example. Ready-to-use slides contain 12 wells per slide, each of 7 mm diameter, coated with phase II antigen obtained from culture on Vero cells and can be stored at 4°C or –20°C.

Concentrated fluorescent conjugate, to be diluted when required with PBS + 1% Evans blue at the dilution recommended by the manufacturer.

PBS, buffered glycerine, Evans blue dye 1% solution.

• Test procedure

i) Inactivate the sera under test for 30 minutes at 56°C, then dilute serially from 1/40 to 1/640 in PBS.

ii) Allow the previously antigen-coated slides to warm to room temperature. Do not touch the wells.

iii) Add 20 µl of each serum dilution to the wells. Add negative and positive control sera. To one well, add 20 µl of PBS to serve as antigen control.

iv) Incubate in a humid chamber for 30 minutes at 37°C. Wash the slide twice with PBS for 10 minutes each. Rinse with distilled water and air-dry.

v) Add to the wells, including the controls, 20 µl of the conjugate directed against the appropriate species (e.g. FITC-labelled rabbit anti-goat or anti-sheep IgG[H+L]), freshly diluted in PBS + Evans blue. Incubate in a humid chamber for 30 minutes at 37°C. Rinse with distilled water and air-dry. Add a few drops of buffered glycerine and cover with a cover-slip. Examine under a fluorescence microscope at magnification ×400 or more.

• Interpretation of the results

A positive reaction will consist of small brilliant points against a dark background. Verify that the conjugate by itself and the negative control serum give a negative result (absence of small brilliant points). Nonspecific fluorescence usually takes the form of spots of irregular shape. The positive control must give the known titre with ± one dilution.

Values for interpretations have to be validated.

b) Complement fixation test (CFT)

This cold fixation micromethod of the type developed by Kolmer is performed with 96-well U-bottomed microtitre plates. The test detects complement-fixing antibodies present in the serum. The CFT is specific but less sensitive than the ELISA or IFA (Kittelberger et al., 2009; Rousset et al., 2007; 2009a). The CFT is still used by laboratories in many countries. This method often uses antigen in phase II prepared from a mixture of two strains (Nine Mile and Henzerling)2 or antigen in phase I and II mixture prepared from Nine Mile strain3.

The reaction is done in two stages. Antigen and complement-fixing antibodies are first mixed, and sheep erythrocytes, sensitised by the anti-sheep erythrocyte serum, are added. Fixation of the complement by the antigen/antibody complex during the first step does not permit lysis of erythrocytes; in contrast, if there are no complement-fixing antibodies, the complement induces the lysis of the sensitised erythrocytes. Then the haemolysis rate is inversely proportional to the level of specific antibodies present in the sample serum.

• Reagents

Veronal/calcium/magnesium buffer (VB), pH 7.2.

2 Dade Behring, Marburg, Germany.
3 Virion, Zürich, Switzerland.
Chapter 2.1.12. – Q fever

The haemolytic system: a mixture of equal parts of a 2% suspension of sheep erythrocytes in VB; and haemolytic serum diluted to a specified titre in VB.

Complement: commercial freeze-dried preparation or fresh guinea-pig serum.

Antigen: use commercial antigens at the titre recommended by the manufacturer if the antigen titration is performed with this method.

Positive and negative control sera.

- Pretitrations
  i) Dilute the sheep erythrocytes to a final concentration of 2% in VB.
  ii) Titrate the haemolytic serum on a microplate: 25 µl of complement at a known haemolytic concentration (e.g. 1/30); 25 µl of increasing dilutions of haemolytic serum + 2% sheep erythrocytes. Include controls without complement. Incubate for 30 minutes at 37°C. Establish the dilution equivalent to 2 haemolytic units.
  iii) Dilute the antigen as recommended by the manufacturer. The antigen may also be titrated: make increasing dilutions of antigen (25 µl horizontally) and a positive serum of known titre (25 µl, vertically). Add 25 µl of the suspension of sensitised erythrocytes and incubate for 30 minutes at 37°C. The antigen titre is the highest dilution producing a positive reaction with the highest serum dilution. Verify the absence of anticomplementary activity of the antigen at different dilutions.
  iv) Titrate the complement on a microplate: serially dilute the complement or guinea-pig serum in VB, for example from 1/15 to 1/200. To each well containing 25 µl of this dilution, add 25 µl of antigen and 25 µl of the haemolytic system. Incubate for 30 minutes at 37°C and establish the dilution equivalent to 2 haemolytic units of complement.

- Test procedure
  i) Make twofold dilutions of decomplemented sample sera from 1/10 to 1/320 in six wells and in four additional wells at dilutions from 1/10 to 1/80 to detect anticomplementary activity (25 µl per well).
  ii) Add 25 µl of diluted antigen or 25 µl of VB to control serum wells.
  iii) Add 25 µl diluted complement to all wells. Cover the plate with plastic adhesive film and incubate for 18 hours at 4°C.
  iv) Remove the plates from the refrigerator, allow them to reach room temperature, and add 25 µl of freshly prepared haemolytic system. Incubate at 37°C for 30 minutes. Centrifuge the plates at 500 g for 5 minutes at 4°C. Examine the controls and read the results.

- Interpretation of the results
  Titres between 1/10 and 1/40 are characteristic of a latent infection. Titres of 1/80 or above in one or more sera from a group of from five to ten animals reveal an active phase of the infection.

c) Enzyme-linked immunosorbent assay (ELISA)

This technique has a high sensitivity and a good specificity (Kittelberger et al., 2009; Rousset et al., 2007; 2009a). It is easy to perform in laboratories that have the necessary equipment (a spectrophotometer) and reagents. The ELISA is preferred to IFA and CFT, particularly for veterinary diagnosis, because it is convenient for large-scale screening and, as it is a reliable technique for demonstrating C. burnetii antibody in various animal species (Jaspers et al., 1994; Soliman et al., 1992). Ready-to-use kits are commercially available and can detect anti-phase II antibodies or both anti-phase I and II antibodies.

Wells of the microplate are coated with C. burnetii whole-cell inactivated antigen. Diluted serum samples are added to the wells and react to antigens bound to the solid support. Unbound material is removed by washing after a suitable incubation period. Conjugate (horseradish-peroxidase-labelled anti-ruminant Ig) reacts with specific antibodies bound to the antigen. Unreacted conjugate is removed by washing after a suitable incubation period. Enzyme substrate is added. The rate of conversion of substrate is proportional to the amount of bound antibodies. The reaction is terminated after a suitable time and the amount of colour development is measured spectrophotometrically.

- Materials and reagents

Microtitre plates with 96 flat-bottomed wells, freshly coated or previously coated with Q fever antigen; microplate reader (spectrophotometer; 405 and/or 450 and/or 492 nm filters); 37°C humidified incubator; 8- and 12-channel pipettes with disposable plastic tips; microplate shaker (optional).
Positive and negative control sera; conjugate (ruminant anti-immunoglobulin labelled with peroxidase);
tenfold concentration of diluent (PBS–Tween); distilled water; substrate or chromogen (TMB
[tetramethylbenzidine], ABTS [2,2’-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)] for peroxidase);
hydrogen peroxide.

• **Test procedure**
  
  i) Dilute the serum samples, including control sera, to the appropriated dilution (usually 1/100) and
distribute 0.1 ml per well in duplicate. Control sera are positive and negative sera provided by the
manufacturer and an internal positive reference serum from the laboratory in order to compare the
titres between different tests.

  ii) Cover the plate with a lid and incubate at room temperature for 30–90 minutes. Empty out the contents
and wash three times in washing solution at room temperature.

  iii) Add the appropriate dilution of freshly prepared conjugate to the wells (0.1 ml per well).

  iv) Cover each plate and incubate as in step ii. Wash again three times.

  v) Add 0.1 ml of freshly prepared chromogen substrate solution to each well (for example: TMB in 0.1 M
acetic acid and 30% H₂O₂ solution [0.2 µl/ml]; or 0.25 mM ABTS in citrate phosphate buffer, pH 5.0,
and 30% H₂O₂ solution [0.1 µl/ml]).

  vi) Shake the plate; incubate according to the manufacturer recommendations, stop the reaction by adding
stopping solution to each well, e.g. 0.05 ml 2 M sulphuric acid for TMB or 10% sodium dodecyl
sulphate for ABTS.

  vii) Read the absorbance of each well with the microplate reader at 405 nm (ABTS) or 450 nm (TMB). The
absorbance values will be used to calculate the results.

• **Interpretation of the results**

  For commercial kits, interpretations and values are provided with the kit.

  For example: calculate the mean absorbance (Ab) of the sample serum and of the positive (Ab<sub>pos</sub>) and
negative (Ab<sub>neg</sub>) control sera, and for each serum, calculate the percentage

\[
\frac{\text{Ab} - \text{Ab}_{\text{neg}}}{\text{Ab}_{\text{pos}} - \text{Ab}_{\text{neg}}} \times 100
\]

Interpret the results as follows:
Ab <30% negative serum
Ab 30–40% doubtful serum
Ab >40% positive serum

C. **REQUIREMENTS FOR DIAGNOSTIC BIOLOGICALS AND VACCINES**

1. **Production of Coxiella burnetii antigen**

Growth and purification of C. burnetii should only be performed in facilities that meet the requirements outlined in
chapter 1.1.3. Precautions assigned to Containment Group 3 pathogens must be taken either for phase I or phase
II C. burnetii. As seen in the Nine Mile reference strain, the LPS phase variation could be accompanied by a
permanent chromosomal deletion that makes impossible a reversion from phase II to phase I. However, a variant
of the Australia QD isolate producing truncated LPS had no detectable large deletion. The molecular changes that
occurred in LPS phase variation are not clearly defined. Even with extensive repeated passage in non-
immunologically competent hosts (cultural cells, embryonated eggs), the majority of isolates are non-clonal as
growth from a single colony is difficult to establish (Samuel & Hendrix, 2009). The risk of aerosols must be taken
into account at all stages when working with viable C. burnetii. Sustained serological monitoring of Q fever should
be carried out for laboratory personnel.

2. **Diagnostic biologicals**

An example of C. burnetii preparation for IFA serological diagnostic based on phase II and phase I antigens is
given below, but other modified protocols are used around the world (Samuel et al., 2009). Significant amounts of
C. burnetii (>10<sup>10</sup> bacteria) can be obtained in 2 weeks in embryonated eggs or cell cultures. Purification of
bacteria from host material includes differential centrifugations and takes 1 or 2 days. An infection on mice can require 7–14 days.

Phase II C. burnetii Nine Mile are grown in confluent layers of Vero or L929 cells in 150 cm² culture flasks at 35°C under 5% CO₂ with minimal essential medium (MEM) supplemented with 2 mM L-glutamine and 4% fetal bovine serum. The infection is monitored by microscopic examination of intracellular vacuoles or by Gimenez-stained cells collected from the supernatants of the flasks. Recent specific real-time quantitative PCR has been extremely valuable in routine monitoring. When a heavy C. burnetii infection is seen, the supernatants of 15 flasks are individually pelleted by centrifugation (5000 g, 15 minutes) resuspended in 1 ml of PBS with 0.1% formaldehyde and incubated for 24 hours at 4°C. After pooling, the remaining cells are broken by sonication. Cellular debris is removed by two successive centrifugation steps (100 g, 10 minutes each). The 15 ml suspension is then centrifuged through 20 ml of PBS with 25% sucrose (6000 g, 30 minutes, without a break). The resulting pellet is washed three times in PBS (6000 g, 10 minutes), resuspended in the smallest possible volume of sterile distilled water, and adjusted to 2 mg/ml by UV spectroscopy. An antibacterial preservative, such as sodium azide at a final dilution of 0.1% or thiomersal at 0.01%, is added. Antigen prepared in this manner is frozen at –20°C.

In order to obtain phase I antigen, mice are inoculated with C. burnetii grown in cells (mainly in phase II). Nine days after infection, the spleens are removed. Each one is ground in 7.5 ml MEM, and inoculated into three 75 cm² culture flasks containing L929 or Vero cell monolayers (2.5 ml per flask). Amplification of phase I water, and adjusted to 2 mg/ml by UV spectroscopy. An antibacterial preservative, such as sodium azide at a final dilution of 0.1% or thiomersal at 0.01%, is added. Antigen prepared in this manner is frozen at –20°C.

In order to obtain phase I antigen, mice are inoculated with C. burnetii grown in cells (mainly in phase II). Nine days after infection, the spleens are removed. Each one is ground in 7.5 ml MEM, and inoculated into three 75 cm² culture flasks containing L929 or Vero cell monolayers (2.5 ml per flask). Amplification of phase I C. burnetii is conducted for 4 weeks, with a culture medium change once a week. The infected cells are then harvested and the bacteria are purified as described above (mainly in phase I).

Antigen production can also be performed by culture of C. burnetii in SPF embryonated eggs. At 6–7 days of age, the microorganism is inoculated into the yolk sac of the embryonated eggs, which are harvested after 10–15 days of incubation. Infected yolk sacs have a characteristic straw-yellow colour. Uninfected yolk sacs are orange in colour and have a viscous consistency. Any embryos that die before 5 days of incubation are discarded. The strain used for egg inoculation is a 1/100 homogenate of yolk sac in PBS containing penicillin (500 International Units/ml) and streptomycin (0.5 mg/ml). The yolk sacs are pooled and homogenised with three parts PBS. The suspension is inactivated with 1.6% formaldehyde for 24 hours at 37°C. The lipid supernatant fluid is discarded. The suspension is then centrifuged at moderate speed (~500 g) for 30 minutes. After removal of the supernatant fluid, more PBS is added and centrifugation is repeated. The final suspension is diluted with PBS. Sodium azide or thiomersal is added as an antibacterial preservative. The abundance of C. burnetii and the absence of bacterial contaminants in homogenates of yolk sacs suspended in PBS are verified by microscopic examination of a smear on a microscope slide, stained by Stamp’s method. In order to obtain phase I antigen, C. burnetii recovered from spleen material of infected laboratory animals can be propagated, as ground spleen extracts are subsequently transferred in the yolk sacs, given that the amount of phase I cells is still high until the sixth egg passage.

Titration of antigen with at least three different known sera (with high, moderate and low titres, respectively) is sufficient to determine the appropriate dilution for further immunofluorescence tests.

3. Vaccine

The protective antigen is composed of purified particles in phase I with the nontruncated phase I LPS structure. In some countries, vaccination is practised for occupationally exposed people, such as abattoir workers, veterinarians and laboratory personnel. A vaccine inactivated by formaldehyde (Q-VAX, CSL Ltd, Australia), prepared from the Henzerling strain of phase I C. burnetii, received the approval of the Australian authorities in 1989. Phase I vaccines are effective, but vaccination is contraindicated for individuals who had seroconverted or had been exposed to C. burnetii prior to immunisation.

Several vaccines have been developed against animal Q fever. Results converge today towards the use of a phase I vaccine that is helpful against Q fever in combination with other control measures. An inactivated phase I vaccine is commercially available (Coxevac, CEVA, Hungary) for vaccination of ruminants. A review on Q fever in Slovakia suggests that the decrease in the occurrence of human and animal Q fever could be the result of the large-scale vaccination of cattle that was carried out there over a 10 year period, together with improved veterinary control of domestic animal transport within the country (Serbezov et al., 1999). In the Netherlands, a large vaccination programme has been implemented in goat and sheep farms, accompanied by the controlled processing of manure and checks on animal transports, but it is not clear yet whether bacterial shedding by animals is prevented or at least reduced by vaccination. Controlling the epidemic is difficult and can be compromised by the prolonged stability of the bacterium in the environment and the possible role of animal species other than small ruminants (EFSA, 2010).

This vaccine consists of highly purified whole cells prepared from Nine Mile strain in the phase I (egg passage 3 to egg passage 5) and inactivated by formaldehyde. No adjuvant is used. Recently, a French study demonstrated the efficacy of this vaccine through experimental vaccination and challenge of pregnant goats: the vaccine prevented abortion and shedding in milk, and decreased considerably the shedding in the vaginal secretions and
faeces (Arricau-Bouvery et al., 2005). Ideally, vaccine efficacy must be demonstrated by tests on all the target species.

In the case of vaccination on already infected animals, some authors believe that it is preferable to select seronegative herds or animals for immunisation, and to continue vaccination over several years in young animals (Krauss, 1989). First follow-up studies on shedding herds or flocks show a contribution of the vaccination against the infection incidence and the shedding levels (Guatteo et al., 2008; Rousset et al., 2009b). Repeated annual vaccination, particularly of young animals, is recommended. However, the duration of immunity is not defined. The development of serological tools distinguishing between infected and vaccinated ruminants (DIVA) would be helpful. To date, no data are available for comparing the cost–benefit of this strategy with a nonselective strategy in the control of Q fever.

REFERENCES


Chapter 2.1.12. — Q fever


* * *
CHAPTER 2.1.13.

RABIES

SUMMARY

Rabies is a major zoonosis for which diagnostic techniques have been standardised internationally. As there are neither gross pathognomonic lesions nor specific and constant clinical signs for rabies, accurate diagnosis can only be made in the laboratory. Laboratory techniques are preferably undertaken on central nervous system (CNS) tissue removed from the cranium (specifically, brain stem, Ammon’s horn, thalamus, cerebral cortex and medulla oblongata). A composite of CNS samples should be tested and the brain stem is the most important component of the sample.

Identification of the agent: Agent identification is preferably undertaken using the fluorescent antibody test (FAT). A drop of purified immunoglobulin previously conjugated with fluorescein isothiocyanate (FITC) is added onto an acetone-fixed brain tissue smear, preferably made from several parts of the central nervous system. FAT provides a reliable diagnosis in 98–100% of cases for all rabies virus strains if a potent conjugate is used. For a large number of samples, as in an epidemiological survey, the polymerase chain reaction (PCR) can provide rapid results in specially equipped laboratories.

Infected neuronal cells have been demonstrated by histological tests and these procedures will reveal aggregates of viral material (‘Negri bodies’) in the cytoplasm of neurones. However, histological techniques are much less sensitive than immunological methods, especially in the case of autolysed specimens. Consequently, histological techniques can no longer be recommended for primary diagnosis.

In cases of inconclusive results from FAT, or in all cases of human exposure, further tests (cell culture or mouse inoculation tests) on the same sample or repeat FAT on other samples are recommended. A monolayer culture of susceptible cells is inoculated with a pool of several CNS tissues, including the brain stem. FAT undertaken after appropriate incubation will demonstrate the presence or absence of viral antigen. Alternatively, newborn or 3- to 4-week-old mice may be inoculated intracerebrally with a similar pool of tissues and then kept under observation for 28-days. For any mouse that dies between 5 and 28-days post-inoculation, the cause of death should be confirmed by FAT. Wherever possible, virus isolation in cell culture should replace mouse inoculation tests.

The identification of the agent can be supplemented in specialised laboratories by identifying any variant virus strains through the use of monoclonal antibodies, specific nucleic acid probes, or the polymerase chain reaction followed by DNA sequencing of genomic areas. Such techniques can distinguish between field and vaccine strains, and identify the geographical origin of the field strains. These very sensitive tests should be used by well trained personnel in specialised laboratories.

Serological tests: Virus neutralisation (VN) assays in cell cultures are prescribed tests for checking vaccination responses prior to international animal movement or trade. Results are expressed in International Units relative to an international standard antiserum. Alternatively, use may be made of validated tests that are known to correlate with these, notably enzyme-linked immunosorbent assays using antibody to the G protein or the whole virus.

Requirements for vaccines: Rabies vaccines for use in animals contain either live virus attenuated for the target species (such as Flury low egg passage, Flury high egg passage, Street-
Alabama-Dufferin or Kelev), or virus inactivated by chemical or physical means, or recombinant vaccines. The virus is cultivated in embryonated egg, or in cell cultures.

Rabies vaccines are usually lyophilised, but inactivated virus vaccines, preferably with an adjuvant, may be stored in liquid form.

Before newly developed vaccines can be licensed, the duration of immunity resulting from their use should be determined in vaccinated animals of the target species. Vaccines should confer protective immunity for at least 1 year.

For live virus vaccines, the minimum virus content that will elicit a protective immune response must be established.

The potency of inactivated virus vaccines is established and controlled using tests formulated by the United States Department of Agriculture in the United States of America or the European Pharmacopoeia elsewhere. The final products of both types of vaccine are subjected to tests for innocuity and absence of toxicity.

For live vaccines that are prepared for oral vaccination of wild (or domestic) animals, safety and efficacy in target animals and safety in non-target species must be demonstrated.

**A. INTRODUCTION**

Rabies is caused by neurotropic viruses of the genus *Lyssavirus* in the family *Rhabdoviridae*, and is transmissible to all mammals. As the viruses are transmissible to humans, all suspected infected material must be handled under the appropriate safety conditions specified by the World Health Organization (WHO, 1996).

Eleven distinct species can be distinguished within the genus, namely classical rabies virus (RABV), Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssaviruses type-1 (EBLV1) and type-2 (EBLV2), Australian bat lyssavirus (ABLV) and four lyssaviruses (Aravan virus [ARAV], Khujand virus [KHUV], Irkut virus [IRKV], and West Caucasian bat virus [WCBV]), which have been isolated from Eurasian bats, and have recently been ratified as new lyssavirus species (ICTV). In addition, a newly identified lyssavirus (Shimoni bat virus) has been isolated from a bat in Kenya (Kuzmin et al., 2010) and is awaiting official classification. RABV is found worldwide, and is responsible for the overwhelming majority of reported animal and human rabies cases. Other lyssaviruses appear to have more restricted geographical and host range, with the majority having been isolated from bats. However, all lyssaviruses tested to date cause clinical disease indistinguishable from classical rabies. Conserved antigenic sites on the nucleocapsid proteins permit recognition of all lyssaviruses with modern commercial preparations of anti-rabies antibody conjugates used for diagnostic tests on brain tissue.

The Lyssaviruses have been divided into two phylogroups with distinct pathogenicity and immunogenicity (Badrane et al., 2001). For RABV, DUVV, EBLV and ABLV, conserved antigenic sites on the surface glycoproteins allow cross-neutralisation and cross-protective immunity to be elicited by rabies vaccination. A reduced protection with pre-exposure vaccination and with conventional rabies post-exposure prophylaxis was observed against IRKV, ARAV, and KHUV (Hanlon et al., 2005) and all of the above-mentioned lyssavirus species were assigned to phylogroup 1. Little or no cross-protection against infection with the members of phylogroup 2 (MOKV and LBV) is elicited by rabies vaccination and most anti-rabies virus antisera do not neutralise these lyssaviruses (Badrane et al., 2001). WCBV does not cross-react serologically with any of the two phylogroups. Laboratories working with lyssaviruses or suspect material must comply with national biocontainment and biosafety regulations and they should also comply with the guidelines for Risk Group 3 pathogens in Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities.

The WHO recommends the preventive immunisation of all staff handling infected or suspect material. The immunisation protocol includes three injections, e.g. at days 0, 7, and 28. The serological evaluation of immunisation is made 3 weeks after the last injection, and checked every 6 months in the case of laboratory workers or every 2 years for other diagnosticians. Booster vaccination must be given when the titre falls below 0.5 International Units (IU) per ml. In the absence of serological monitoring, the vaccination regimen should consist of a booster vaccination at 1 year and thereafter every 1–3 years.

As no clinical sign or gross post-mortem lesion can be considered pathognomonic in domestic or wild animals, the diagnosis of rabies has to rely on laboratory testing. Serological testing is rarely useful for ante-mortem diagnosis because of late seroconversion and the high mortality rate of host species, but is very useful for assessing seroconversion following vaccination and for epidemiological studies.
B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Clinical observation may only lead to a suspicion of rabies because signs of the disease are not characteristic and may vary greatly from one animal to another. The only way to undertake a reliable diagnosis of rabies is to identify the virus or some of its specific components using laboratory tests.

As rabies virus is rapidly inactivated, refrigerated diagnostic specimens should be sent to the laboratory by the fastest means available. Shipment conditions must be considered to be part of the ‘rabies diagnostic chain’ and should follow international guidelines.

Several laboratory techniques may be used, and have been detailed and standardised in the fourth edition of the WHO Laboratory Techniques in Rabies (1996). The methods vary in their efficiency, specificity and reliability. They are classically applied to brain tissue, but they can also be applied with variable sensitivity and specificity to other organs (e.g. salivary glands). In the brain, rabies virus antigen is particularly abundant in the thalamus, pons and medulla. It is recommended that a pool of brain tissues that includes the brain stem should be collected and tested (Bingham & Van der Merwe, 2002). The most widely used test for rabies diagnosis is the fluorescent antibody test (FAT), which is recommended by both WHO and OIE, and is sensitive, specific and inexpensive.

Precautions should be taken when handling central nervous system tissues from suspected rabies cases. Gloves should always be worn and precautions must be taken to prevent aerosols. Cutting tools, scissors and scalpels, should be used with care to prevent injury and contamination.

a) Collection of brain samples

Usually the brain is collected following the opening of the skull in a necropsy room, and the appropriate samples are collected preferably Ammon’s horn, thalamus, cerebral cortex and medulla oblongata. Under some conditions (e.g. in the field or when sampling for large epidemiological studies, this step may be impractical. In such cases, there are two possible methods of collecting some brain samples without opening the skull:

i) Occipital foramen route for brain sampling

A 5 mm drinking straw (Barrat & Blancou, 1988) or a 2 ml disposable plastic pipette is introduced into the occipital foramen in the direction of an eye. Samples can be collected from the rachidian bulb, the base of the cerebellum, hippocampus, cortex, and medulla oblongata. When using a straw it should be pinched between the fingers to prevent material escaping when withdrawing. Brain specimens from cattle can also be sampled using the ‘brain scoop or tool’ developed for bovine spongiform encephalopathy (BSE) tissue sampling, yielding a sample suitable for diagnosis of both BSE and rabies.

ii) Retro-orbital route for brain sampling

In this technique (Montano Hirose et al., 1991), a trocar is used to make a hole in the posterior wall of the eye socket, and a plastic pipette or straw is then introduced through this hole. The sampled parts of the brain are the same as in the former technique, but they are taken in the opposite direction.

b) Shipment of samples

Suspect material should be shipped according to the International Air Transport Association (IATA) Dangerous Goods Regulations. These regulations are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens.

When it is not possible to send refrigerated samples, other preservation techniques may be used. The choice of the preservative is dependent on the tests to be used for diagnosis:

• Formalin inactivates the virus, thus virus isolation tests cannot be used and diagnosis depends on using a modified direct FAT, polymerase chain reaction (PCR), (less sensitive than these tests on fresh tissue), immunohistochemistry or histology (Warner et al., 1997);

• Infectivity at room temperature may be extended for several days if brain material is kept in a mixture of 50% glycerol in phosphate buffered saline (PBS). Glycerol/PBS slows bacterial action and therefore protects against the chemical and biological effects of putrefaction. It does not protect against titre decline due to thermal conditions and therefore, because rabies is thermo-labile, the virus titre will decline during glycerol/PBS storage. Under normal transport conditions in the tropics, this protection may only be effective for a matter of several days. Therefore, whenever possible samples in glycerol/PBS should be kept refrigerated. As the virus is not inactivated by glycerol/PBS, all laboratory tests can be used on these samples.
An alternative for the transport of samples for molecular techniques is the use of FTA Gene Guard system (Picard-Meyer et al., 2007). The FTA paper preserves rabies virus RNA within the fibre matrix allowing the transport of samples at ambient temperature without specific biohazard precautions for further characterisation of rabies strains.

c) Laboratory tests

i) Immunochemical identification of rabies virus antigen

   a) Fluorescent antibody test

   The most widely used test for rabies diagnosis is the FAT, which is recommended by both WHO and OIE. This ‘gold-standard’ test may be used directly on a smear, and can also be used to confirm the presence of rabies antigen in cell culture or in brain tissue of mice that have been inoculated for diagnosis. The FAT gives reliable results on fresh specimens within a few hours in more than 95–99% of cases. The FAT is sensitive, specific and cheap. The sensitivity of the FAT depends on the specimen (the degree of autolysis and how comprehensively the brain is sampled, see Section B.1) (Barrat & Aubert, 1995), on the type of lyssavirus and on the proficiency of the diagnostic staff. For direct rabies diagnosis, smears prepared from a composite sample of brain tissue, that includes the brain stem, are fixed in 100% high-grade cold acetone for at least 20 minutes, air dried and then stained with a drop of specific conjugate for 30 minutes at 37°C. Anti-rabies fluorescent conjugates available commercially are either polyclonal or monoclonal antibodies (MAbs), specific to the entire virus or to the rabies nucleocapsid protein, conjugated to a fluorophore such as fluorescein isothiocyanate (FITC). FAT slides should then be examined for specific fluorescence using a fluorescent microscope and filter appropriate for the wavelength of the fluorescent conjugate used, for instance FITC, the most commonly used, is excited at 490 nm and re-emits at 510 nm. Aggregates of nucleocapsid protein are identified by specific fluorescence of bound conjugate. It is recommended that two independent trained operators read each FAT slide. Fluorescent antibody conjugates may be made locally, but should be fully validated for specificity and sensitivity before use, including its ability to detect lyssaviruses other than rabies.

   The FAT may be applied to glycerol-preserved specimens after a washing step. If the specimen has been preserved in a formalin solution, the FAT may be used only after the specimen has been treated with a proteolytic enzyme (Warner et al., 1997). However, the FAT on formalin-fixed and digested samples is always less reliable and more cumbersome than when performed on fresh tissue (Barrat, 1992).

   In cases of inconclusive results from FAT, or in all cases of human exposure, further tests on the same sample or repeat FAT on other samples are recommended. This is particularly important where sample autolysis is confirmed or suspected.

   b) Immunoperoxidase methods

   Immunoperoxidase methods can be used as an alternative to FAT with the same sensitivity (Lembo et al., 2006), but attention should be paid to the risk of nonspecific false-positive results. This risk is considerably reduced by the thorough training of the technicians. It must also be emphasised that this technique needs one incubation step more than the FAT.

   Peroxidase conjugate may also be used on fresh brain tissue or sections of formalin-fixed tissue for immunohistochemical tests.

ii) Detection of the replication of rabies virus after inoculation

   These tests detect the infectivity of a tissue suspension in cell cultures or in laboratory animals. They should be used if the FAT gives an uncertain result or when the FAT is negative in the case of known
human exposure. Wherever possible, virus isolation on cell culture should be considered in preference to the mouse inoculation test (MIT). Cell culture tests are as sensitive as MIT (Rudd & Trimarchi, 1989) but are less expensive, give more rapid results and avoid the use of animals.

- **Cell culture test**

  Neuroblastoma cells e.g. N2a, CCL-131 in the American Type Culture Collection (ATCC)\(^1\) are highly susceptible to infection with lyssaviruses. The cells are grown in Dulbecco’s modified Eagle’s medium (DMEM) with 5% fetal calf serum (FCS), incubated at 36°C with 5% CO\(_2\). Baby hamster kidney (BHK-21) cells are also sensitive to most street isolates without any adaptation step, but should be checked for susceptibility to locally predominant virus variants before use. Cell culture tests may be undertaken in multi-well plastic plates, multi-chambered glass slides or on glass cover-slips. The use of one 4-day passage in four wells of a 96-well microtitre plate has been shown to have comparable sensitivity to MIT for rabies strains (Rudd & Trimarchi, 1989). However additional passages could be considered to increase sensitivity. Cytotoxicity is a commonly reported factor limiting test robustness. Techniques proposed to reduce cytotoxicity include adding antibiotics, reducing the time before changing media (to as short as 35 minutes) and dilution of samples. Cell culture tests and their variations should be fully validated before use.

  **Suggested protocol** for a 96-well plate: 100 µl of clarified brain homogenate (20% in phosphate buffered saline, 0.1 M, pH 7.4) is added to 200 µl of a 2 × 10\(^5\) cells/ml suspension, freshly prepared from a sub-confluent flask in four wells of a 96-well plate. After 24 hours incubation at 5% CO\(_2\) and 37°C, the supernatant from each well is removed and 200 µl of fresh medium is added to each well. After a further 72 hours incubation the supernatant is removed by pipette and kept. The cells are fixed with 80% acetone and stained with fluorescent antibody according to manufacturers’ recommendations. Variations include reduced incubation time before changing media to reduce cell toxicity, the use of cell permeability agents (e.g. DEAE-dextran), and further passages. Up to three passages may be considered to increase sensitivity.

  **Suggested protocol for use in 8-chamber Lab-Tek \(^\oplus\) slides:** 50 µl of clarified brain homogenate (20% in a grinding substrate made of PBS, 0.1 M, pH 7.4 with heat-inactivated new-born calf serum) is added to 400 µl of a 10\(^5\) cells/ml suspension, freshly prepared from a subconfluent flask. After 24 hours incubation at 5% CO\(_2\) and 35.5°C, the supernatant from each chamber is removed and 400 µl of fresh medium is added to each chamber. After a further 24 hours incubation (or more) the supernatant is removed, chamber structure removed, cells layer dried and fixed with pure high grade cold acetone. The fixed cell layer is then stained with fluorescent antibody according to laboratory procedures. Variations include incubation time, use of cell permeability agents and further passages. Removed supernatants should be kept for possible further passage.

- **Mouse inoculation test**

  Three-to-ten mice, 3–4 weeks old (12–14 g), or a litter of 2-day-old newborn mice, are inoculated intracerebrally. The inoculum is the clarified supernatant of a 10–20% (w/v) homogenate of brain material including brainstem (e.g. cortex, Ammon’s horn, thalamus, medulla oblongata) in an isotonic buffered solution containing antibiotics. Mice should be anaesthetised when inoculated. The mice are observed daily for 28 days, and every dead mouse is examined for rabies using the FAT. For faster results in newborn mice, it is possible to check one baby mouse by FAT on days 5, 7, 9 and 11 post-inoculation. Any deaths occurring during the first 4 days are regarded as nonspecific (due to stress/bacterial infection etc.).

  Once a validated and reliable cell culture unit exists in the laboratory, consideration should be given to replacing the mouse inoculation test with cell culture whenever possible as it avoids the use of live animals, is less expensive and gives more rapid results. However, advantages of MIT are that when the test is positive, a large amount of virus can be isolated from a single mouse brain for strain identification purposes and that it can be easily and practicably applied in situations where skills and facilities for other tests (e.g. cell culture) are not available. MIT may also detect viruses other than rabies virus.

- **Molecular techniques**

  Various molecular diagnostic tests, e.g. detection of viral RNA by reverse transcription PCR (RT-PCR), PCR-ELISA, hybridisation in situ and real-time PCR are used as rapid and sensitive additional techniques for rabies diagnosis (Fooks et al., 2009). The principle of lyssavirus-specific PCRs is a reverse transcription of the target RNA (usually parts of the N gene) into complementary DNA followed by the amplification of the cDNA by PCR. Although those molecular tests have the highest level of sensitivity, their use is currently not recommended for routine post-mortem diagnosis of rabies (WHO, 2005) due to high levels of false positive or false negative results without standardisation and very stringent quality control. Nevertheless, they are useful for confirmatory diagnosis, as a first step in virus typing (see below).
iv) **Histological identification of characteristic cell lesions**

Negri bodies correspond to the aggregation of viral proteins, but the classical staining techniques detect only an affinity of these structures for acidophilic stains. Techniques that stain sections of paraffin embedded brain tissues (e.g. Mann’s technique) are time consuming, less sensitive and more expensive than FAT. Seller’s method on unfixed tissue smears has a very low sensitivity is only suitable for perfectly fresh specimens. These methods are no longer recommended for routine diagnosis. Immunohistochemical tests are the only histological methods specific to rabies.

d) **Other identification tests**

The tests above describe methods to accurately diagnose rabies and to isolate and identify the virus. Typing of the virus can provide useful epidemiological information and should be undertaken in specialised laboratories (such as OIE or WHO Reference Laboratories). These techniques would include the use of MAbs, nucleic acid probes, or the PCR, followed by DNA sequencing of genomic areas for typing the virus (Bourhy et al., 1993). These characterisations enable, for instance, a distinction to be made between vaccine virus and a field strain of virus, and possibly identify the geographical origin of the latter.

Participation in inter-laboratory proficiency testing is highly encouraged as part of quality assurance schemes; such tests should be organised for regional laboratories by the national reference laboratories, while the latter in turn should participate in international proficiency tests organised by OIE Reference Laboratories.

2. **Serological tests**

The main application of serology for classical rabies is to determine responses to vaccination, either in domestic animals prior to international travel, or in wildlife populations following oral immunisation. In accordance with the WHO recommendations (WHO, 1985), 0.5 IU per ml of rabies antibodies is the minimum measurable antibody titre considered to represent a level of immunity in humans that correlates with the ability to protect against rabies infection. The same measure is used in dogs and cats to confirm a satisfactory response to vaccination. As neutralising antibodies are considered a key component of the adaptive immune response against rabies virus (Hooper et al., 1998) the gold standard tests are virus neutralisation (VN) tests. However, indirect ELISAs have been developed that do not require high-containment facilities and produce rapid results. Care should be taken when correlating results between virus neutralisation tests and ELISAs owing to the inherent differences between them. Multiple publications demonstrate a variable sensitivity and specificity for ELISAs in both humans and animals. Although VN tests are recommended where specific assessment of protection is required, both tests are useful for detecting responses to vaccination if appropriate cut-offs are used. Poor quality sera can cause cytotoxicity in VN tests, which could lead to false-positive results. For such samples, the use of an indirect ELISA has been shown to be as sensitive and specific as the VN test (Servat et al., 2007).

Serological surveys have also been used to provide information on dynamics of lyssaviruses in bats although standardisation of serological tests for bats is still needed.

a) **Virus neutralisation test in cell culture: fluorescent antibody virus neutralisation test (a prescribed test for international trade)**

The principle of the fluorescent antibody virus neutralisation (FAVN) test (Cliquet et al., 1998) is the in vitro neutralisation of a constant amount of rabies virus (‘challenge virus standard’ [CVS-11] strain adapted to cell culture) before inoculating cells susceptible to rabies virus: BHK-21 C13 cells (ATCC number: CCL-10).

The serum titre is the dilution at which 100% of the virus is neutralised in 50% of the wells. This titre is expressed in IU/ml by comparing it with the neutralising dilution of the OIE serum of dog origin under the same experimental conditions. The WHO standard for rabies immunoglobulin [human] No. 2, or an internal control calibrated against the international control may be used. The WHO standard or internal control should only be used as a control in the test and should not be used to calculate the IU/ml titre of the sera.

This microplate method uses 96-well plates, and is an adaptation of the technique of Smith et al. (1973). The FAVN test and the rapid fluorescent focus inhibition test (RFFIT) give equivalent results (Cliquet et al., 1998).

- **Essential equipment**

  Humidified incubator at 35°C/37°C with 5% CO₂; dry incubator at 37°C; biocontainment cabinet; fluorescence microscope suitable for FITC fluorescence equipped with ×10 eye-piece and ×10 objective.

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2 National Institute for Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, United Kingdom (UK).
Chapter 2.1.13. — Rabies

The global magnification of the microscope ranges between ×100 and ×125 due to the extra magnification of some epi-fluorescence systems.

- **Reagents and biologicals**

  **PBS buffer, pH 7.2, without Ca²⁺ and Mg²⁺,** stored at 4°C;

  Trypsin ethylene diamine tetra-acetic acid (EDTA);

  High-grade acetone 80% (diluted with deionised water), stored at 4°C;

  Dulbecco's modified Eagle's medium (DMEM) + 10% heat-inactivated FCS;

  FITC anti-rabies conjugate;

  **Cells:** BHK-21 C13 (ATCC CCL-10) maintained in GMEM with 10% FCS and antibiotics;

  **Virus:** CVS-11 (previously ATCC reference VR 959) strain, which is available from the ATCC or the OIE Reference Laboratory for Rabies, Nancy, France (see Table given in Part 4 of this *Terrestrial Manual*). Vials are stored at –80°C;

  **OIE Standard Serum of dog origin** (OIE Reference Laboratory for Rabies, Nancy, France [see Table given in Part 4 of this *Terrestrial Manual*] stored at +4°C and diluted to 0.5 IU/ml with sterile deionised or distilled water according to the titre of the batch). This control serum may be used to calibrate an internal control that is used for regular FAVN testing;

  **Naive serum:** The pool of negative dog sera is stored at –20°C.

- **CVS production**

  i) **Cell growth:** the BHK-21 C13 cells (ATCC CCL-10) used to produce the CVS virus (ATCC VR 959 CVS-11) are trypsinised during the rapid growth phase, i.e. cells are in the exponential phase of their kinetic growth. If the confluence of the layer is complete, a new passage should be made. The cells in the cell suspension should not be aggregated; 2 × 10⁷ cells are needed for a 75 cm² cell culture flask. Cells are collected within a volume of 20–30 ml in cell culture medium with 10% heat-inactivated FCS.

  ii) **Infection of cells:** the multiplicity of infection (number of infectious particles per cell) is adjusted to between 0.1 and 0.5. The glass bottle containing the virus/cell suspension is incubated for 60 minutes at 35.5–37°C. The contents of the bottle are gently stirred every 10–15 minutes.

  iii) **Virus growth:** the virus/cell suspension is then centrifuged at 800–1000 g for 15 minutes and the cell pellet is resuspended in cell culture medium mixed with 10% heat-inactivated FCS. Virus is harvested 2 days later.

  iv) **Harvest and storage:** the supernatant is centrifuged at 800–1000 g for 15 minutes at 4°C. If several flasks have been used, the different centrifuged supernatants are mixed and then aliquoted and frozen at –80°C. The infective titre of the harvest is established at least 3 days after freezing.

- **Titration of virus in TCID₅₀ (50% tissue culture infective dose)**

  This titration method uses BHK-21 C13 cells (ATCC CCL-10) in microtitre plates. Different steps in this procedure may be adapted according to the safety requirements and to the working practices of the laboratory, but the following should not be changed:

  i) **Cell suspension:** the day before titration, a cell suspension containing 10⁵ cells/ml is prepared in cell culture medium containing 10% heat-inactivated FCS, and is distributed, 200 µl per well, into 96-well microtitre plates. The plates are then incubated for 24 hours at 35.5°C–37°C with 5% CO₂.

  ii) **Dilution of the virus:** the serial dilutions are performed in 5 ml tubes using a cell culture medium without FCS as diluent. Ten-fold dilutions from 10⁻¹ to 10⁻¹² are prepared (0.9 ml of diluent with 0.1 ml of the previous dilution).

  iii) **Infection of the cells:** the medium in the microtitre plates is discarded using an aspiration system. Fifty µl of each virus dilution is distributed per well. Six replicates are used per dilution. The microtitre
plate is then incubated for 1 hour at 35.5–37°C with 5% CO₂. Then 200 µl of cell culture medium, containing 5% FCS, is added.

iv) **Incubation:** incubate for 3 days at 35.5–37°C in 5% CO₂.

v) **Staining and calculation of titre:** The cells are stained using the FAT, as detailed below. Reading is qualitative, every well that shows specific fluorescence is considered to be positive. The titre calculation is made using either the neoprobit graphic method or the Spearman–Kärber formula (WHO, 1996).

vi) The CVS titration must be performed by FAVN test to establish the infective dose in TCID₅₀.

- **Test procedure**
  i) The microplates are used according to the pattern shown in Figure 1. Plate No. 1 is used for the titration of CVS (rows 1 to 4), and for the controls, standard sera and naive dog serum are used. All other plates are used for the sera to be tested.
  ii) Medium is added to the wells as follows: plate 1, rows 1 to 4 and cells A9 to A12: add 150 µl per well; in the other plates, rows 6 and 12: add 200 µl per well; all other wells: add 100 µl.
  iii) Sera to be tested are heat inactivated for 30 minutes at 56°C. As indicated in Figure 1, 50 µl of each undiluted serum to be tested is added to four adjacent wells.
  iv) Dilutions of sera are conducted in the microplates as follows:
    - OIE serum, the WHO serum, the internal control and the naive dog serum: with a 50–200 µl multichannel pipette, mix the first dilution wells by sucking in and out at least eight times, transfer 50 µl from one row to the next one, until the last one is reached. Discard 50 µl from the last row.
    - If there is a serum to be tested on the control plate, see below for the dilution step.
    - A minimum of four three-fold dilutions is required.

    Serum being tested (all plates): as above, transfer successively 50 µl from one row to the following one until rows 5 and 11 (dil. 10⁻².₃⁹). With a 5–50 µl multichannel pipette, transfer 10 µl from rows 5 and 11 to rows 6 and 12, respectively (from dil. 10⁻².₃⁹ to dil. 10⁻⁴.₂₃). Using a multichannel pipette adjusted to 90 µl, mix rows 6 and 12 and discard 180 µl. Then add 70 µl of medium to these rows. This final step does not lend itself to high throughput testing. To attain or exceed the recommended final dilution alternative procedures may be used. These may require modifications to the plate layout.

Fig. 1. Proposed use of microplates for the fluorescent antibody virus neutralisation test. Wells to which undiluted sera must be added are filled with the indicated ‘50 µl’. Wells to which 50 µl of diluted challenge virus standard must be added are shaded. Dilutions are given in log₁₀.

<table>
<thead>
<tr>
<th>Plate 1: Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Challenge virus</strong> standard titration</td>
</tr>
<tr>
<td>H</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>OIE standard serum (0.5 IU/ml)</td>
</tr>
<tr>
<td>50 µl</td>
</tr>
<tr>
<td>50 µl</td>
</tr>
<tr>
<td>50 µl</td>
</tr>
<tr>
<td>50 µl</td>
</tr>
<tr>
<td>Naive dog serum (negative)</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>Serum or internal positive control or WHO Standard serum</td>
</tr>
<tr>
<td>50 µl</td>
</tr>
<tr>
<td>50 µl</td>
</tr>
<tr>
<td>50 µl</td>
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<tr>
<td>50 µl</td>
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<tr>
<td>9</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>11</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>log (dilution) 0.48 0.95 1.43 1.91 2.39 2.87 CVS virus control Cells control</td>
</tr>
</tbody>
</table>
**Plate 2: Sera to be tested**

<table>
<thead>
<tr>
<th>log dilution</th>
<th>0.48</th>
<th>0.95</th>
<th>1.43</th>
<th>1.91</th>
<th>2.39</th>
<th>4.23</th>
<th>0.48</th>
<th>0.95</th>
<th>1.43</th>
<th>1.91</th>
<th>2.39</th>
<th>4.23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>50 µl</td>
<td>50 µl</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Serum 2</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>B</td>
<td>50 µl</td>
<td>50 µl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>50 µl</td>
<td>50 µl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D</td>
<td>50 µl</td>
<td>50 µl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum 1</td>
<td></td>
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<td></td>
<td></td>
<td>E</td>
<td>50 µl</td>
<td>50 µl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F</td>
<td>50 µl</td>
<td>50 µl</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Serum 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>G</td>
<td>50 µl</td>
<td>50 µl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum 4</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>H</td>
<td>50 µl</td>
<td>50 µl</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Addition of challenge virus standard**
  1. Stock CVS is stored in 1 ml microtubes at –80°C. One tube is thawed rapidly under cold running water, and placed in melting ice.
  2. One dilution from this tube is prepared in order to obtain 100 TCID₅₀ in 50 µl. Of this dilution, 50 µl is added to each serum-filled well (see Figure 1). For virus titration, 50 µl is added to wells H1 to H4 (plate 1). Next, transfer 50 µl from row to row (plate 1, lines 1–4). Discard 50 µl from the last row (plate 1, wells A1 to A4). No virus is added to wells A9 to A12 of plate 1 (controls). The range allowed for the virus dose titre must be between 30 and 300 TCID₅₀/50 µl.
  3. Incubate the microplates at 35–37°C in a humid incubator with 5% CO₂ for 1 hour.
  4. **Addition of cells**: trypsinise a subconfluent culture of BHK-21 cells. Resuspend the cells to obtain a 4 × 10⁵ cells/ml suspension in DMEM supplemented with 10% heat-inactivated FCS. Add 50 µl of the cell suspension to each well.
  5. Incubate the microplates for 48 hours at 35–37°C in a humid incubator with 5% CO₂.

- **Fixation and staining**
  1. After the 48-hour incubation period, the medium is discarded, and the microplates are rinsed once in PBS, pH 7.2, and once in 80% acetone. The microplates are then fixed in 80% acetone at room temperature for 30 minutes, and are dried at room temperature for at least 30 minutes.
  2. Add 50 µl of the FITC anti-rabies conjugate, at the working dilution, to each well, gently rock the microplates and incubate at 35–37°C for 30 minutes. Discard the fluorescent conjugate and rinse the microplates twice with PBS. Excess PBS is removed by briefly inverting the microplates on absorbent paper.

- **Reading and interpreting the results**
  1. The total surface of each well is observed. The reading evaluation is qualitative (plus or minus): no fluorescent cell – a minus score is recorded for the well; fluorescent cells (one cell or more) – a plus score is recorded for the well.
  2. Cell and virus controls are read first. For titration of CVS, naïve serum, and OIE standard serum, titres are calculated according to the Spearman–Kärber method or the neoprobit graphic method (WHO, 1996).
  3. Results of titration of CVS (TCID₅₀), naïve serum (D₅₀ [median dose]) and positive standard (D₅₀) are reported on a control card for each of these three controls. The control results of the current test are compared with the accumulated control test results from previous tests using the same batch of control. The test is validated if the values obtained for the three controls in the current test are not statistically different from the mean (± 2 SD) of all the values obtained in the tests conducted previously according to this technique.
  4. The result of the test corresponds to the non-neutralised virus after incubation with the reference serum or with the serum to be tested. These titres are calculated with the neo-probit graphic method or with the Spearman–Kärber formula (WHO, 1996). The comparison of the measured titre of the tested sera
with that of the OIE positive standard serum of a known neutralising titre allows determination of the neutralising titre of the tested sera in IU/ml. The conversion to IU/ml can be made by using either the log $D_{50}$ value of the day or the mean value of the OIE standard serum.

- Formula to convert the log $D_{50}$ value in IU/ml titre:

$$\text{Serum titre (IU/ml)} = \frac{\left(10^{(\text{serum log } D_{50} \text{ value})} \times \text{theoretical titre of OIE serum 0.5 IU/ml}\right)}{\left(10^{(\text{log } D_{50} \text{ of OIE serum 0.5 IU/ml})}\right)}$$

Example of conversion:

- log $D_{50}$ of the serum = 2.27
- theoretical titre of OIE serum 0.5 IU/ml = 0.5 IU/ml
- log $D_{50}$ of OIE serum = 1.43

(for the log $D_{50}$ of OIE, the value of the day or the mean value can be considered)

$$\text{Serum titre (IU/ml)} = \frac{10^{2.27} \times 0.5}{(10^{1.43})} = 3.46 \text{ IU/ml}$$

The following parameters have to be strictly respected:

- Rabies virus: only the CVS-11 strain should be used.
- Cells culture: only BHK-21 cells (ATCC number – CCL 10) should be used.
- The FAVN test must be performed only in 96 wells microplate.
- Control charts should be used for rabies virus, naïve serum and positive standard serum of dog origin.
- The back titration of the CVS virus, as well as naïve serum and positive standard serum of dog origin, must be present on control plate.
- A minimum of four three-fold dilutions of sera are required. The reading method is ‘all or nothing’ only.
- Four replicates of each serum should be diluted.
- For the conversion of log $D_{50}$ in IU/ml, the laboratories should use only the log $D_{50}$ value of the positive standard serum of dog origin.

b) The rapid fluorescent focus inhibition test (RFFIT) for determining rabies virus-neutralising antibody (a prescribed test for international trade)

- Standard procedure (from WHO, 1996)

  - Preparation of seed virus suspension

    i) Trypsinise one 3-day-old 150 ml flask culture of mouse neuroblastoma (MNA) cells. A similar cell line (CCL-131) may be obtained on request from the ATCC (see footnote 1).
    ii) Resuspend $3 \times 10^7$ cells in a 50 ml conical centrifuge tube in 2.7 ml of Eagle’s minimal essential medium supplemented with 10% fetal bovine serum (EMEM-10).
    iii) Using standard rabies safety procedures, add $1 \times 10^7$ infectious units of CVS-11 rabies virus (previously ATCC reference VR959) and vortex/mix once. Incubate the cells and virus for 15 minutes at 37°C; vortex/mix the cells once during this time.
    iv) Add 10 ml EMEM-10, vortex/mix, and centrifuge the cells at 500 g for 10 minutes.
    v) Discard the supernatant. Resuspend the cells in 30 ml of growth medium and transfer to a 150 ml flask.
    vi) Gently rock the flask to mix the cell suspension, and then prepare three eight-well tissue-culture chamber slides by pipetting 0.2 ml of the cell suspension into one well of each slide.
    vii) Incubate the flask and slides at 37°C in a humidified incubator with 0.5% carbon dioxide ($CO_2$). The flask should be incubated as a closed culture (tighten the cap).
viii) At 20, 40 and 64 hours after infection, acetone fix and stain one slide using an immunofluorescence technique (Cliquet et al., 1998) to determine the virus infectivity. The supernatant should be harvested 24 hours after the cells reach 100% infectivity (typically 40 hours after infection).

ix) Transfer the supernatant to a 50 ml centrifuge tube and centrifuge at 4000 g for 10 minutes.

x) Distribute the supernatant into 0.5 ml aliquots and store at –70°C.

- **Titration of seed virus suspension**
  i) Thaw one aliquot of the seed virus and prepare serial tenfold dilutions (from $10^{-1}$ to $10^{-6}$) in EMEM-10.
  ii) Distribute 0.1 ml of each virus dilution into one well of an eight-well tissue-culture chamber slide. Add 0.2 ml of MNA cells suspended in EMEM-10 (concentration $5 \times 10^4$ cells per 0.2 ml) to each well.
  iii) Mix the cells and virus by gently rocking the slide, then incubate at 37°C in a humidified incubator with 0.5% CO$_2$ for 40 hours.
  iv) Acetone fix and stain the slide using an immunofluorescence technique. Evidence of virus infection should be observed at the $10^{-6}$ dilution of virus, indicating a virus stock suspension containing at least $1 \times 10^6$ infectious units per 0.1 ml. Prepare sufficient seed virus so that frequent serial passage of the virus is unnecessary.

- **Preparation of stock virus suspension**
  i) Infect $3 \times 10^7$ MNA cells with $1 \times 10^7$ infectious units of the seed virus preparation (see above).
  ii) Harvest the supernatant 24 hours after the cells reach 100% infectivity (typically 40 hours after infection).
  iii) Distribute the supernatant into 0.5 ml aliquots and store at –70°C.

- **Titration of stock virus suspension**
  i) Thaw one aliquot of the stock virus and use this to prepare serial tenfold dilutions (from $10^{-1}$ to $10^{-6}$) in EMEM-10.
  ii) Distribute 0.1 ml of each virus dilution into one well of an eight-well tissue-culture chamber slide. Add 0.2 ml of MNA cells suspended in EMEM-10 (concentration $1 \times 10^5$ cells per 0.2 ml) to each well.
  iii) Mix the cells and virus suspension by gently rocking the slide, then incubate at 37°C in a humidified incubator with 0.5% CO$_2$ for 20 hours.
  iv) Acetone fix and stain the slide using an immunofluorescence technique.

Each well of an eight-well tissue-culture chamber slide contains 25–50 distinct microscopic fields when observed at ×160–200 magnification. One unit of virus for the RFFIT is determined as the dilution at which 50% of the observed microscopic fields contain one or more foci of infected cells (the focus-forming dose, FFD$_{50}$). The stock virus suspension should contain at least $1 \times 10^4$ FFD$_{50}$ per 0.1 ml (i.e. the well with cells infected with the $10^{-4}$ dilution of the virus should contain at least one focus of infected cells in 50% of the observed microscopic fields). A stock virus suspension of this titre can then be diluted to $10^{-2.3}$ to obtain a challenge virus containing 50 FFD$_{50}$.

- **Reference sera**
  A national or international reference serum standard diluted to a potency of 2.0 IU/ml should be included in each test. The reference serum used at the Centres for Disease Control and Prevention is the second international standard for rabies immunoglobulin (Lyng, 1994), which may be obtained from the NIBSC (see footnote 2). The reference serum should be maintained as frozen aliquots in amounts sufficient for 1 week of tests. A positive serum control standard diluted to a potency of 0.5 IU/ml and a negative serum control standard with a potency of <0.1 IU/ml should also be prepared by the laboratory and included in each test.

- **Test sera**
  Serum samples should be heated at 56°C for 30 minutes before testing in order to inactivate complement. If sera are frozen, they should be reheated after thawing. Serial dilutions of test sera may be prepared in an eight-well tissue-culture chamber slide. Screening dilutions of 1/5 and 1/50 are sufficient for routine evaluation of vaccination efficacy and may be made as follows:
i) Prepare a 1/2.5 dilution by adding 0.1 ml of inactivated serum and 0.15 ml of EMEM-10 to one of the slides. Mix by gently rocking the slide.

ii) Transfer 0.05 ml of the 1/2.5 dilution to a second well containing 0.45 ml of EMEM-10. Discard all but 0.1 ml from the well containing the 1/2.5 dilution.

iii) Mix the second well and discard all but 0.1 ml.

iv) Add 0.1 ml of the challenge virus preparation (containing 32–100 FFD50) to all serum dilutions.

v) Mix and incubate at 35°C in a humidified incubator with 0.5% CO2 for 90 minutes.

- **Addition of cells**
  i) During the incubation period, trypsinise a stock culture of 3–5-day-old MNA cells.
  ii) Resuspend the cells in EMEM-10 to give a final concentration of 1 × 10^5 cells per 0.2 ml.
  iii) Distribute 0.2 ml of the cell suspension into each well of the slide and incubate at 35°C in a humidified incubator with 0.5% CO2 for a further 20 hours.

- **Acetone fixation and staining by immunofluorescence**
  i) After 20 hours, remove the slides from the incubator and pour off the medium into a virucidal solution.
  ii) Rinse the slides once in PBS and then fix for 10 minutes at room temperature in cold acetone (−20°C).
  iii) Leave the slides to dry for 10 minutes before adding FITC-conjugated anti-rabies serum. The conjugate may be prepared in EMEM-10 or PBS; there is no need to adsorb the conjugate with tissue or cells. The working dilution of the conjugate should be determined by titration. The slides should be stained for 20–30 minutes at 37°C and then rinsed in PBS and distilled water, respectively.

iv) Observe the slides under a fluorescence microscope.

- **Calculation of virus-neutralising antibody titres**

Residual virus is detected using a standard fluorescence microscope. The serum neutralisation end-point titre is defined as the dilution factor of the highest serum dilution at which 50% of the observed microscopic fields contain one or more infected cells (i.e. a 97% reduction in the virus inoculum). This value may be obtained by mathematical interpolation. Alternatively, a 100% neutralisation titre may be determined by recording the highest serum dilution at which 100% of the challenge inoculum is neutralised and there are no infected cells in any of the observed fields. For both titration methods, the titre of antibody in the test serum (in IU/ml) can be obtained by comparison with the titre of the national reference standard included in each test. It should be noted that it is also valid to perform the RFFIT using BHK-21 cells instead of neuroblastoma cells. A modified protocol for this has been published (WHO, 1996).

The following parameters have to be strictly adhered to:

- Rabies virus; only the CVS-11 strain should be used.

- Cells cultures: only BHK-21 cells (ATCC number CCL10) or MNA cells (ATCC number CCL131) should be used.

- The test should be performed only on Lab-tek chamber slides.

- Control charts should be used for rabies virus, naïve serum and positive standard dog serum.

- The back titration of the CVS virus, as well as the naïve serum and positive standard dog, must be present on control plate.

- Reading method for the test: each chamber slide should contain 25–50 fields and be observed at ×160–200 magnification.

- A minimum of three-to-five-fold dilutions of sera is required.

- For the conversion of log D_{50} to IU/ml, only the log D_{50} value of the positive standard serum of dog origin should be used.

c) **Virus neutralisation in mice**

This method is no longer recommended by either OIE or WHO.
d) Enzyme-linked immunosorbent assay (a prescribed test for international trade)

The ELISA provides a rapid (~4 hours) test that avoids the requirement to handle live rabies virus. Commercial indirect ELISA kits are available that allow detection of rabies antibodies in individual dog and cat serum samples following vaccination. In 2007, the International Committee adopted such methods as Prescribed Tests for evaluating vaccine responses in dogs and cats prior to international movement, provided a kit is used that has been validated and adopted on the OIE Register as fit for such specific purposes. The validation for this purpose should include a comparison with, and calibration against prescribed methods for virus neutralisation.

Other ELISA methods or kits should not be regarded as prescribed but may be useful for monitoring of vaccination campaigns in wildlife populations, provided the kit used has been validated for the wildlife species under study.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

NB: SECTION C IS “UNDER STUDY”. THIS IS THE LAST ADOPTED VERSION PUBLISHED IN 2008

Rabies vaccines prepared from Pasteur’s original 1885 strain and its derivative strains (Pasteur Virus, Challenge Virus Standard, Pitman-Moore, etc.), and strains isolated more recently (Flury, Street-Alabama-Dufferin [SAD], Vnukovo and Kelev), protect against all strains of genotype 1 isolated so far. Conventional rabies virus vaccines may not provide adequate cross-protection against other lyssaviruses, especially in phylogroup II; there is no protection provided against Mokola virus (Von Teichman et al., 1998) and the recently identified West Caucasian Bat Virus (Hanlon et al., 2005). Cross neutralisation using conventional rabies virus vaccines has been demonstrated against two phylogroup I viruses: EBLV type-1 and EVLV type-2 (Brookes et al., 2005). The principles governing the preparation of inactivated rabies vaccines are identical whether they are to be used in humans or animals, although an adjuvant may be added to vaccines for animal use.

Recombinant vaccine (e.g. vaccinia rabies-glycoprotein recombinant) has also proved to be effective (Brochier et al., 1991; Kieny, 1984). The rabies-glycoprotein recombinant vaccines are not live rabies vaccines. They are prepared by inserting non-infectious rabies nucleic acid into a vector such as vaccinia or canary pox. Since these do not contain live rabies virus, animals vaccinated with rabies-glycoprotein recombinant vaccines should not be restricted from entry into countries that have restrictions on entry of animals vaccinated with live rabies vaccines.

For animals, live and recombinant vaccines are effective by the oral route and can be distributed in baits in order to immunise wild (or domestic) animals.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

Different standards apply to vaccines containing live virus modified by passage in eggs or cell cultures to reduce its virulence for the target animal, and to vaccines prepared from inactivated virus. Both types of vaccine have their advantages and disadvantages (Baer, 1991), but they can both be used to immunise animals for periods of between 1 and 3 years. Live attenuated rabies vaccines are not accepted in some countries. They are not to be relied on to protect previously unvaccinated animals that have been exposed to infection (Blancou et al., 1991). Only in humans has the efficiency of post-exposure prophylaxis with vaccine alone been proven and even in these cases there is an additional strong recommendation to administer anti-rabies immunoglobulin.

All handling of the virus during manufacture and testing of vaccines must conform to the strict safety precautions specified by WHO (1992; 1996), the OIE (chapter 1.1.3) and to national guidelines and regulations.

1. Seed management

a) Characteristics of the seed

Any strain belonging to serotype 1, which has been proved to protect against field rabies viruses (currently found in the country where the vaccine is to be used), is suitable. The strain of virus used should have well-known biological (e.g. pathogenicity) and antigenic properties (typing by MAbs). If it is to be used as a live vaccine, the master seed virus must be shown not to cause clinical rabies. At least two animals (preferably five to six per group) of each of the species for which the vaccine is intended and, so far as possible, any species that might be in contact with vaccine or vaccinated animals, should be tested. This can be done by inoculating in or adjacent to a major nerve, a dose equivalent to ten times the intended viral titre in one dose

3 see http://www.oie.int/en/our-scientific-expertise/certification-of-diagnostic-tests/background-information/
of the proposed final product. Animals should be observed for at least 90 days for any adverse effect attributable to the master seed.

b) **Method of culture**

A master cell stock of the seed virus should be prepared and kept at or below –70°C. Subculture from this stock will be used for vaccine production. Virus multiplication is verified by titration during growth of the seed virus.

c) **Validation as a vaccine**

Before a vaccine is licensed, evidence of efficacy should be established by the challenge of vaccinated and control animals of each target species. The challenge should be performed at the end of the period after vaccination for which the manufacturer claims maintenance of immunity. Antibody kinetics should also be determined in order to establish the correlation between antibody titre and resistance to challenge.

The efficacy of the produced vaccine is assessed by studies on every target species previously vaccinated as recommended. Protection at the end of the period of immunity is monitored by a measurement of specific neutralising antibodies and by challenge with rabies virus. The experimental conditions of this challenge should mimic the natural conditions of infection. The challenge virus should preferably be prepared from locally isolated strains. In animals vaccinated with inactivated vaccines, the percentage of seroconversion and the mean level of antibody allow a good prognosis for survival to challenge (Aubert, 1992).

The correlation between potency in the target species and antigenic value as estimated in mice should be established (see Section C.4.c below).

For the purposes of licensing a vaccine, safety tests should be conducted in the target species. In the case of live virus vaccines used in oral vaccination campaigns (including recombinant vaccines), safety tests should also be carried out on those other species that live in the area of vaccination and could become exposed to the vaccine (Baer, 1991).

Vaccine stability is ascertained by testing batches after prolonged storage, usually 1–2 years. A process of accelerated ageing, by storage at 37°C for 1 week, is sometimes used. The storage life claimed by the manufacturer is checked by the national licensing authority. In general, it is 12–18 months for fluid vaccines, and possibly 24 months for lyophilised vaccines.

2. **Method of manufacture**

Whatever method is adopted, close attention should be paid to the quality of the substrate. Eggs should be of SPF origin, and the cell cultures, such as BHK cell lines, should conform to international standards of sterility and innocuity.

During manufacture, the multiplication of the virus in one of the substrates mentioned above is monitored, followed by harvesting at the most appropriate time, usually 4–6 days after inoculation of eggs or cell cultures. The virus harvest is suspended in a buffer solution at a dilution that will provide an optimum antigenicity of the end-product. If required, the suspension is either inactivated or lyophilised. An adjuvant is recommended for vaccines prepared from inactivated virus, as well as for other vaccine antigens that may be incorporated in polyvalent vaccines.

a) **In cell cultures**

 Cultures are infected with cell-culture-adapted strains of rabies virus and incubated at 35–36°C. These may then be used as live virus vaccines (as in Flury and SAD vaccines), or as inactivated vaccines after the addition of phenol (Semple vaccine) or some other chemical, such as beta-propiolactone.

Cell culture can also be used to grow the vector viruses (e.g. vaccinia virus) harbouring the gene coding for the expression of rabies virus glycoprotein (Kieny, 1984).

b) **In eggs**

A modified egg-adapted strain of virus is inoculated into SPF-embryonated chicken eggs, which are then incubated at 38°C for 5–6 days. The virus is harvested in the form of infective embryo tissues, and is usually lyophilised and used as a live vaccine. Examples of such vaccines include those that contain the Flury low egg passage (LEP), or the more desirable high egg passage (HEP) variant strain, which is safer for some animal species such as the cat.
c) **In animals**

Nervous tissue vaccines prepared in animals are no longer considered safe or effective, and their use should be discontinued.

3. **In-process control**

This consists of monitoring virus growth to provide an optimum titre and ensure the absence of undesirable microbial contamination.

In live virus vaccines, kinetics of virus growth should be established in order to ensure a final titre of virus correlated to the desired protection in target species.

In inactivated virus vaccines, immunogenic properties of the final product may be evaluated by *in-vitro* techniques (e.g. ELISA, agar gel immunodiffusion, antibody-binding tests or infected cell staining). These evaluations will indicate the best time for harvesting the virus in cell cultures.

4. **Batch control**

a) **Sterility**

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

b) **Safety**

Safety tests for batches of inactivated virus vaccines are carried out by inoculation of cell culture or intracerebrally into mice to detect viable virus. A suitable safety test for live rabies vaccines should be carried out on each lot of vaccine, in the intended host species. At least three, preferably five to six animals of the intended host species should be given a dose equivalent to ten times the recommended field dose, by the recommended route of administration. The animals should be observed for 180 days for adverse reactions attributable to the vaccine (Council of Europe, 2005).

c) **Potency/biological activity**

The amount of virus present in live attenuated and recombinant vaccines is determined by titration. Once a correlation has been established between the activity of the vaccine in the target species and virus titres, virus titrations become reliable indicators of vaccine efficacy. This is carried out using cell cultures or by the intracerebral inoculation of unweaned mice (in mice it is only possible with a few attenuated viruses). Recombinant vaccines should be monitored for the expressed rabies protein until assured that expression stability is maintained in the manufacturing process. Titre of the vector can then be used as a reliable indicator of vaccine efficacy.

For inactivated virus vaccines, correlation between potency in the target species and antigenic value as estimated in mice provides a reliable indicator of vaccine activity. The potency of the vaccine is established in the USA by the National Institutes of Health (NIH) test. Elsewhere, the European Pharmacopoeia test is widely adopted.

Groups of at least ten mice, aged 3–4 weeks, are inoculated with single, decreasing doses of vaccine in accordance with the European Pharmacopoeia (Council of Europe, 2005), or with two doses, 1-week apart, according to the NIH test (WHO, 1996). A sufficient number of dilutions of vaccine are compared to estimate the dilution at which 50% of the mice are protected against intracerebral challenge 14 days later (Council of Europe, 2005; WHO, 1996).

A WHO international standard vaccine is available (see footnote 2) for calibration of national standards, so that the results of testing for antigenicity can be expressed in IUs. The test is not valid unless:

i) For both the vaccine to be examined and the standard preparation, the PD₅₀ (50% protective dose) lies between the largest and smallest doses given to the mice.

ii) The titration of the challenge virus suspension shows that 0.03 ml of the suspension contained 25 mouse intra-cranial LD₅₀ (MIC LD₅₀). The challenge dose should be in the range 12–50 LD₅₀ for a valid test.
iii) The confidence interval ($p = 0.95$) for the test should not be less than 25% and not more than 400% of the estimated potency: statistical analysis should show a significant slope and no significant deviations from linearity or parallelism of the dose–response lines.

The vaccine passes the test if the estimated potency is not less than 1 IU per dose, in the smallest prescribed dose.

A simplified test can also be used for the purpose of anticipating which vaccines are likely to be of an antigenic value $\geq$1 IU per dose (Aubert & Blancou, 1982). This test used as a screening test is a good way to reduce the number of mice used in vaccine potency control tests.

d) Duration of immunity

Duration of immunity must be established for the product licence in the target species with a defined vaccination protocol.

e) Stability

The proposed shelf life must be verified by appropriate tests. These experiments include biological and physico–chemical stability tests, and should be performed on a sufficient number of batches of vaccine stored under recommended conditions.

The thermostability of live virus vaccines in liquid form is generally poor. For freeze-dried inactivated virus vaccines, stability is generally granted for 2 years at 4°C.

f) Preservatives

Inactivated virus vaccines may contain preservatives (formalin, merthiolate). The nature and quantity of these preservatives should comply with national control regulations.

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

6. Oral vaccination

The concept of oral vaccination is unique: as stray or wild animals are out of physical reach, dropping vaccine baits into their environment is the only way to immunise them. In the 1980s and 1990s, the Veterinary Public Health Department of WHO organised several meetings of rabies experts to define the requirements for guaranteeing the safety and efficacy of vaccines both for the target species (red fox, raccoon dog, skunk, dog, etc.) and nontarget species, namely wild rodents and any other wild and domestic species that might be in contact with baits or a recently vaccinated animal (WHO, 1989; 2005).

Several guidelines have been established for the quality criteria that vaccines have to satisfy before marketing; the most precise documents are those produced by WHO, the European Pharmacopoeia and the European Commission (European Commission, 2002; European Pharmacopoeia, 2007; WHO, 2005). Available oral vaccines have been extensively tested by different routes (cerebral, muscular and oral) in a variety of species: puppies and adults of carnivores, avian species, nonhuman primates, rodents and immunocompromised mice. Nonhuman primates have been added to this list since the discovery in 1992 that the original SAD Bern strain is highly pathogenic for baboons by the oral route (Bingham et al., 1992).

All vaccines currently used for oral vaccination programmes are either modified live-virus vaccines or live recombinant vaccines. At the present time, two oral vaccines are recommended by WHO (2005): a recombinant vaccine – VRG vaccine, and a highly attenuated vaccine – SAG2.

The production controls are closely related to the ones used for parenteral vaccines. The major differences concern three points:

i) Safety of the vaccine for man, target and non-target species.
ii) Efficacy of the protection induced by the vaccine.

iii) Monitoring of the impact of oral vaccination campaigns in the field.

a) Safety considerations

For oral vaccination, either attenuated rabies strains or live-recombinant vaccines may be used. The vaccine should not induce any adverse signs in target and nontarget species. For vaccines used for dog immunisation, saliva should be checked for the absence of vaccinal virus because of possible contact with humans.

The attenuated rabies virus-based vaccines must achieve the lowest residual pathogenicity for target and nontarget species (European Commission, 2002); this is of utmost importance in the case of oral vaccination of dogs as dogs are often in close contact with humans (WHO, 2005).

The recombinant vaccines cannot induce any risk of rabies; the safety controls concern only the possible residual pathogenicity of the recombined parental virus.

b) Protection induced by the vaccine

The protection induced by the vaccine must be tested not only with the virus itself (to determine the minimal vaccinating dose) but also with manufactured baits ready to be used in the field. For foxes for instance, the vaccine should have a minimal titre corresponding to at least ten times the 100% protective dose (obtained with the same vaccine experimentally by direct oral instillation) (Blancou et al., 1986).

The protection status cannot be then checked by serology only; a virulent challenge with the homologous street rabies virus is necessary because of the important implication of cell-mediated immunity in response to oral vaccination (European Pharmacopoeia, 2007).

c) Monitoring the impact of oral vaccination

The stability of the vaccine in the field is important. The European Commission stresses the importance of checking the 100% protective dose after 7 days of exposure at 25°C (European Commission, 2002). Each vaccine bait should be tested for stability with a melting point above 40°C, and the blister or sachet containing the vaccine should still be covered by the bait casing after 7 days exposure at 40°C (European Commission, 2002).

Aerial distribution of baits is the only way to perform an homogenous, rapid and sufficient distribution for wildlife vaccination. Quality control measures should be used to monitor different key points of baiting: control of vaccine titre, control of area coverage by air and of baiting density should at least be constantly monitored. The cross border cooperation between neighbouring countries is also needed to avoid any unvaccinated area along the border.

For wildlife in Europe, two campaigns are performed yearly: the spring one aims at vaccinating the young population of the target species, its period should then be fixed according to the biology of the target species. The autumn campaign concerns both adult and young animals. It is generally admitted that four campaigns (i.e. 2 years) should be conducted after the last rabies diagnosis.

The impact of vaccination on the host/vector population is monitored in two different ways:

- Directly by measuring the bait uptake by the wild target species. This supposes that a biomarker (generally tetracycline) is included in the bait casing. The same examination allows the age of animals to be determined.

- Directly by measuring the serological response of target animals. This serological control is better done using validated ELISA techniques (Cliquet et al., 2000; Servat et al., 2007) as they are more robust than seroneutralisation tests when testing poor quality field specimens.

- Indirectly by measuring the incidence of rabies in the vaccinated area. Typing of field isolates should be performed (WHO, 2005) either by using MAbs or by sequencing positive samples from areas where the target species have been vaccinated with attenuated vaccines to possibly distinguish vaccine and field virus strains.

The first two controls should be performed on specially killed animals to collect good quality samples. Rabies monitoring is more sensitive when performed in found dead or ill animals.
REFERENCES


Chapter 2.1.13. — Rabies


ICTV (INTERNATIONAL COMMITTEE ON TAXONOMY OF VIRUSES): http://www.ictvonline.org/virusTaxonomy.asp?bhcp=1


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**NB:** There are OIE Reference Laboratories for Rabies (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for rabies
CHAPTER 2.1.14.

RIFT VALLEY FEVER

SUMMARY

Rift Valley fever (RVF) is a peracute or acute zoonotic disease of domestic ruminants in Africa. It is caused by a single serotype of a mosquito-borne bunyavirus of the genus Phlebovirus. The disease occurs in climatic conditions favouring the breeding of mosquito vectors and is characterised by liver damage. The disease is most severe in sheep, goats and cattle, in which it produces abortions in pregnant animals and a high mortality rate in the newborn. Older nonpregnant animals, although susceptible to infection, are more resistant to clinical disease. There is considerable variation in the susceptibility to RVF of animals of different breeds. Those breeds or strains that are exotic to Africa or are from areas where RVF is not endemic, tend to be more susceptible. Camels suffer an inapparent infection with RVF, but abortion rates can be as high as in cattle. Among ruminant game, buffalo also abort during an inapparent RVF infection.

Humans are susceptible to infection through contact with infected material or mosquito bites. Infection of humans by vectors is a striking feature in countries with a relatively small population of animal hosts. In such areas, RVF may be recognised first in humans. It has caused serious disease in laboratory workers and must be handled with high level biosecurity. It is recommended that laboratory workers be vaccinated.

Identification of the agent: RVF virus consists of a single serotype of a bunyavirus of the genus Phlebovirus and has morphological and physicochemical properties typical of bunyaviruses.

The virus can be isolated from blood, preferably collected in an anticoagulant, during the febrile stage of the disease, or from liver, spleen and brain tissues of animals that have died and from the organs of aborted fetuses. Primary isolations are usually made on cell cultures of various types, such as African green monkey kidney (Vero) cells, baby hamster kidney cells, chicken embryo reticulum, or primary cells of sheep or cattle origin. Alternatively, hamsters, adult or suckling mice, embryonated chicken eggs or 2-day-old lambs may be used for primary virus isolation.

A rapid diagnosis can be achieved by using the supernatant of homogenised samples as antigen in virus neutralisation (VN) tests; immunofluorescent staining of impression smears of liver, spleen, brain or infected cell cultures; or by the demonstration of virus in serum, taken during the febrile stage of the disease, by enzyme immunoassay or immunodiffusion.

The presence of characteristic histopathological lesions in the liver assists in the diagnosis.

Serological tests: Infected animals develop specific antibodies that may become demonstrable by VN as early as 3 days following infection and after 6–7 days by enzyme-linked immunosorbent assay, and by haemagglutination inhibition. Serological tests used less often include immunofluorescence, complement fixation and immunodiffusion.

Requirements for vaccines and diagnostic biologicals: Live virus vaccines and antigens for use either in countries where RVF is endemic or during outbreaks, should be prepared from nonpathogenic mouse- or mutagen-attenuated strains of RVF virus grown in cell cultures. The mutagen-attenuated strain of RVF is not yet at a stage where it can be recommended for use.

In RVF-free countries, vaccines and diagnostic tests should be limited to those using inactivated virus. Suitable virus strains can be obtained from the OIE Reference Laboratory for RVF (see Table given in Part 4 of this Terrestrial Manual).
A. INTRODUCTION

RVF virus consists of a single serotype of a bunyavirus of the genus *Phlebovirus* and has morphological and physicochemical properties typical of bunyaviruses. The virus is enveloped, spherical and 80–120 nm in diameter. Short glycoprotein spikes project through a bilayered lipid envelope and the virus is readily inactivated by lipid solvents and acid conditions below pH 6. The virus has a three-segmented, single-stranded, negative-sense RNA genome, and consists of the three segments: L (large), M (medium) and S (small), each of which is contained in a separate nucleocapsid within the virion. The S segment is ambisense RNA, i.e. has bi-directional coding (Gentsch & Bishop, 1979).

Rift Valley fever (RVF) is a peracute or acute, febrile, mosquito-borne, zoonotic disease caused by a virus of the family *Bunyaviridae*, genus *Phlebovirus*. It usually presents in epizootic form over large areas of a country following heavy rains and flooding, and is characterised by high rates of abortion and neonatal mortality, primarily in sheep, goats and cattle. The susceptibility of different breeds to RVF varies considerably. Some indigenous African animals may have only inapparent infections, while exotic or other breeds suffer severe clinical disease with mortality and abortion. Susceptible, older nonpregnant animals and some other species usually do not show signs of disease. Camels have been regularly involved in the RVF epidemics in East Africa and Egypt. Clinical disease is not seen in adult camels, but abortion occurs and some early post-natal deaths have been observed.

Signs of the disease tend to be nonspecific, rendering it difficult to recognise individual cases (Coackley et al., 1967; Coetzer, 1982; Coetzer & Barnard, 1977; Easterday, 1965; Gerdes, 2004; Meegan & Bailey, 1989; Weiss, 1957) and during epidemics; however, the occurrence of numerous abortions and mortalities among young animals, together with disease in humans, is characteristic. RVF has a short incubation period: 12–36 hours in lambs. A biphasic fever of up to 41°C may develop, and the fever remains high until shortly before death. Affected animals are listless, disinclined to move or feed, and may show enlarged superficial lymph nodes and evidence of abdominal pain. Lambs rarely survive longer than 36 hours after the onset of signs of illness. Animals older than 2 weeks may die peracutely, acutely or may develop an inapparent infection. Some animals may regurgitate ingesta and may show melaena or bloody, foul-smelling diarrhoea and bloodstained mucopurulent nasal discharge. Icterus may sometimes be observed, particularly in cattle. In addition to these signs, adult cattle may show lachrymation, salivation and dysgalactia. In pregnant sheep, the mortality and abortion rates vary from 5% to almost 100% in different outbreaks and between different flocks. The death rate in cattle is usually less than 10%.

The hepatic lesions of RVF are very similar in all species, varying mainly with the age of the infected individual (Coetzer, 1982). The most severe lesion occurring in aborted fetuses and newborn lambs is a moderately to greatly enlarged, soft, friable liver with a yellowish-brown to dark reddish-brown colour with irregular congested patches. Numerous greyish-white necrotic foci are invariably present in the parenchyma, but may not be clearly discernible. In adult sheep, the lesions are less severe and pinpoint reddish to greyish-white necrotic foci are distributed throughout the parenchyma. Haemorrhage and oedema of the wall of the gallbladder are common. Hepatic lesions in lambs are almost invariably accompanied by numerous small haemorrhages in the mucosa of the abomasum. The contents of the small intestine and abomasum are dark chocolate-brown as a result of the presence of partially digested blood. In all animals, the spleen and peripheral lymph nodes are enlarged, oedematous and may have petechiae.

Microscopically, hepatic necrosis is the most obvious lesion of RVF in both animals and humans. In fetuses and neonates of cattle and sheep, foci of necrosis consist of dense aggregates of cellular and nuclear debris, some fibrin and a few inflammatory cells. There is a severe lytic necrosis of most hepatocytes and the normal architecture of the liver is lost. In about 50% of affected livers, intranuclear inclusion bodies that are eosinophilic and oval or rod-shaped are found. Mineralisation of necrotic hepatocytes is also seen. In adult animals, hepatic necrosis is less diffuse and in sheep, icterus is more common than in lambs (Swanepoel & Coetzer, 1994).

In humans, RVF infections are usually inapparent or associated with a moderate to severe, nonfatal, influenza-like illness (McIntosh et al., 1980; Meegan, 1981). A minority of patients may develop ocular lesions, encephalitis, or severe hepatic disease with haemorrhagic manifestations, which is generally fatal. RVF virus has caused serious human infection in laboratory workers. Staff should either be vaccinated and work under containment level 3, work under containment level 4 conditions, or wear respiratory protection. Particular care needs to be exercised when working with infected animals or when performing post-mortem examinations (see Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

No significant antigenic differences have been demonstrated between RVF isolates and laboratory-passaged strains from many countries, but differences in pathogenicity have been shown (Bird et al., 2007; Swanepoel et al., 1986).

Infection of humans by mosquito vectors is a striking feature in countries, such as Egypt, with a relatively small population of animal hosts and a large population of mosquitoes.
RVF usually occurs in epizootics in Africa, which may involve several countries in a region or even in all the countries of a continent at one and the same time. These follow the periodic cycles of exceptionally heavy rain, which may occur very rarely in semi-arid zones (25–35-year cycles), or more frequently (5–15-year cycles) in higher rainfall savannah grasslands. Low level undetectable RVF activity may take place in inter-epizootic periods. RVF should be suspected when unusually heavy rains are followed by the occurrence of abortions together with fatal disease marked by necrosis and haemorrhages in the liver that particularly affect newborn lambs, kids and calves, concurrent with the occurrence of an influenza-like illness in farm workers and people handling raw meat.

Preventative measures to protect workers from infection should be employed when there are suspicions that RVF-virus-infected meat and tissue samples are to be handled.

### B. DIAGNOSTIC TECHNIQUES

#### 1. Identification of the agent

RVF virus may be isolated from serum and blood collected in an anticoagulant during the febrile stage of the disease, from liver, spleen and brain of animals that have died, or from aborted fetuses. Primary isolation is usually performed in hamsters, infant or adult mice, or on cell cultures of various types.

**a) Culture**

Approximately 5 ml of blood collected during the febrile stage of the disease or approximately 5 g of liver, spleen and brain collected after death should be presented for virus isolation. The samples should be kept at 0–4°C during transit. If transport to the laboratory is likely to take more than 24 hours, the samples should be frozen and sent on dry ice.

Approximately 1 g of homogenised tissue is suspended 1/10 in cell culture medium or buffered saline, pH 7.5, containing sodium penicillin (1000 International Units [IU]/ml), streptomycin sulphate (1 mg/ml), mycostatin (100 IU/ml), or fungizone (2.5 µg/ml). The suspension is centrifuged at 1000 g for 10 minutes and the supernatant fluid is injected intracerebrally into 1–5-day-old mice or intraperitoneally into hamsters or adult mice. Infant mice will either die or be obviously ill by day 2. Adult mice are affected 1–3 days later. Although mice or hamsters are the laboratory animal of choice, lambs and embryonated chicken eggs may also be used.

A variety of cell monolayers including African green monkey kidney (Vero), baby hamster kidney (BHK), chicken embryo reticulum (CER: cells developed by Tsunemasa Motohashi at the Nippon Institute for Biological Science, Tokyo, Japan; recharacterised as a hamster line) (Barnard & Voges, 1986) and primary kidney or testis cells of calves and lambs may be inoculated with 1 ml of clarified sample supernates and incubated at 37°C for 1 hour. It is advisable to also inoculate some cultures with a further 1/100 dilution of the inoculum. Some tubes containing flying cover-slips should also be prepared. The cultures are washed with phosphate buffered saline at room temperature and covered with medium containing 2% serum free from antibodies against RVF. The cultures are observed microscopically for 5–6 days. RVF virus induces a cytopathic effect (CPE) characterised by slight rounding of cells followed by destruction of the whole cell sheet within 12–24 hours. Specific identification of RVF virus antigen may be made 18–24 hours after infection by immunofluorescent staining of the cover-slip preparations.

The virus may also be detected by immunofluorescence carried out on impression smears of liver, spleen and brain. A rapid diagnosis can sometimes be made by demonstrating viral antigen in tissues or in serum of febrile animals by a complement fixation or agar gel immunodiffusion (AGID) test. A rapid diagnosis can also be made by detection of viral RNA using a reverse-transcription polymerase chain reaction (RT-PCR).

**b) Agar gel immunodiffusion**

The AGID test is useful in laboratories without tissue-culture facilities. Approximately 1 gram of tissue, preferably liver, is homogenised and made up to a 10–20% suspension in borate saline buffer, pH 9.0. The material is centrifuged at 1000 g and the supernatant is used in the test. Micro-AGIDs are performed on standard microscope slides covered with 3 ml of 1% agarose in borate saline. Patterns of six peripheral wells and a central well are prepared and filled with reagents as follows: a positive, preferably hyperimmune serum in the central well, positive control antigen in wells 1 and 4, test tissues in wells 2 and 5 and negative tissues in wells 3 and 6. A precipitin line of continuity should be formed between control antigen and positive serum that extends to include a line between test tissue and serum for a case to be considered positive.
c) Polymerase chain reaction

A rapid diagnosis can also be made by detection of viral RNA (Sall et al., 2001) using RT-PCR. The PCR was used, among other techniques, for antigen detection in two recent RVF virus outbreaks in Africa – one in Kenya in 1998 and a limited outbreak in South Africa in 1999. It may also be used to detect RVF virus in mosquito pools (Jupp et al., 2000). RT-PCR followed by sequencing of the NS(S) protein-coding region has been used in phylogenetic analysis to characterise two distinct lineages of RVF virus – one Egyptian and the other sub-Saharan – making this technique a powerful molecular epidemiological tool (Sall et al., 1997).

d) Histopathology

Histopathological examination of the liver of affected animals will reveal characteristic cytopathology, and immunostaining will allow the specific identification of the RVF viral antigen in infected cells. This is an important diagnostic tool because liver or other tissue may be placed in formol saline in the field for diagnostic purposes, which facilitates handling and transport in areas remote from the laboratory.

2. Serological tests

Virus neutralisation (VN) tests including microneutralisation, plaque reduction neutralisation (PRN) and neutralisation in mice have been used to detect antibodies against RVF virus in the serum of a variety of species. Neutralisation tests are highly specific and will record the earliest response, but these tests can only be performed with live virus and are not recommended for use outside endemic areas or in laboratories without appropriate biosecurity facilities and vaccinated personnel.

Other available tests include enzyme-linked immunosorbent assay (ELISA), haemagglutination inhibition (HI), AGID, immunofluorescence, radioimmunoassay and complement fixation. In these tests, however, cross-reactions may occur between RVF virus and other phleboviruses. An advantage of these tests is the fact that they can be performed with inactivated antigen and can therefore be used in RVF-free countries.

The ELISA is a reliable and sensitive test that may be employed with several species to detect antibodies against RVF virus. An IgM-capture ELISA allows diagnosis of a recent infection to be made on a single serum sample.

The HI test can be employed with great confidence in nonendemic areas. However, sera from individuals that have had previous infections with phleboviruses other than RVF may react with RVF antigen to titres as high as 40 and, rarely, to titres of 320 (Swanepoel et al., 1986). In suspected cases, the OIE Reference Laboratory for RVF (see Table given in Part 4 of this Terrestrial Manual) can be of assistance in carrying out neutralisation tests for specificity. The HI antibody titre after vaccination with RVF virus vaccine may be as high as 640 or, rarely, 1280, whereas titres following natural infections with RVF virus are usually significantly higher.

a) Virus neutralisation (the prescribed test for international trade)

The VN test may be employed to determine the presence of antibodies in naturally infected animals and in animals vaccinated with RVF vaccine. The test is highly specific and can be used to test serum of any species. It is generally used to measure vaccine efficacy. Factors other than neutralising antibodies may play a part in resistance to RVF. The Smithburn neurotropic mouse brain strain of highly attenuated RVF virus (Smithburn, 1949), also referred to as modified live virus and adapted to cell culture, is used as antigen. The antigen is stored at −80°C or 4°C in freeze-dried form. The stock is titrated to determine the dilution that will give 100 TCID_{50} (50% tissue culture infective dose) in 25 µl under the conditions of the test.

- Test procedure
  i) Inactivate the test sera for 30 minutes in a water bath at 56°C.
  ii) Add 25 µl of cell culture medium with 5% RVF-negative serum and antibiotics to each well of a 96-well cell culture plate.
  iii) Add 25 µl of test serum to the first well of each row and make twofold dilutions. Titrate each serum in duplicate from 1/10 to 1/80 for screening purposes or in quadruplet and to higher dilutions for determination of end-point titres. Include known positive and negative control sera.
  iv) Add 25 µl per well of RVF virus antigen (diluted in cell culture medium and calculated to provide 100 TCID_{50} per well) to each well that contains diluted test serum and to wells in rows containing negative and positive control serum. In addition, make twofold dilutions of antigen in at least two rows each containing cell culture medium only.
  v) Incubate for 30 minutes at 37°C.
vi) Add 50 µl per well of Vero, CER or any other suitable cell suspension at 3 × 10^5 cells/ml or at a dilution known to produce a confluent monolayer within 12 hours.

vii) Incubate the plates in an atmosphere of 3–5% CO₂ for 3–5 days.

viii) Using an inverted microscope, the monolayers are examined daily for evidence of CPE. There should be no CPE in rows containing positive control serum and clear evidence of CPE in rows containing negative control serum indicating the presence of virus. Determine the results by the Spearman–Kärber method.

b) Enzyme-linked immunosorbent assay

For the serodiagnosis of RVFV a number of ELISAs using different formats have been published and are commercially available (Afetine et al., 2007; Paweska et al., 2005). The use of inactivated whole virus or mouse liver antigens has recently been replaced by recombinant nucleocapsid (N) protein as antigen.

These ELISAs are at present in an indirect format and apart from the very important safety consideration also have the advantage of antigen stability and the ability to test 40 sera in duplicate per plate instead of only 20.

An indirect ELISA with pre-coated plates using a nucleocapsid protein (NC) recombinant antigen and Protein G peroxidase conjugate is described below (Jansen Van Vuren et al., 2007).

- Test procedure

Unless otherwise stated, all dilutions are made with 10% (w/v) dried milk buffer and all washes performed three times with volumes of 250–300 µl/well.

i) Using pre-coated plates add 50 µl of diluted (1/100) serum in duplicate wells

ii) Add control sera at predetermined dilutions in duplicate wells. Incubate for 60 minutes at 37°C. Wash the plate.

iii) Add Protein G/horseradish peroxidase conjugate at a working dilution to all wells of the plate. Incubate for 60 minutes at 37°C. Wash the plate

iv) Add 50 µl of ready-to-use TMB Substrate to all wells of the plate. Cover the plate and incubate at room temperature in the darkness for 20–30 minutes.

v) Add 50 µl of ready-to-use Stop solution to all wells of the plate. Tap plate gently to allow contents to mix. Wait 5 minutes and read plate using a spectrophotometer equipped with a 450 nm filter.

vi) Suggested plate layout.

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CC: Conjugate control; C++: High positive control serum; C+: low positive control serum; C–: negative control serum; 1–40: test samples.

Newer ELISA formats are being introduced, including formats that are more specific for IgG and IgM (Paweska et al., 2003).

c) Haemagglutination inhibition

The HI test adapted to a microtechnique is based on Clarke & Casals (1958). A sucrose/acetone-extracted hamster liver antigen is used in a 96-well U-bottomed plate test and antigen is diluted so that 4 haemagglutinating units are used in the test. Nonspecific inhibitors of haemagglutinin are removed by kaolin extraction of sera followed by adsorption with packed goose erythrocytes (RBC) prior to testing.
Doubling dilutions of sera made in borate saline buffer, pH 9, are tested against equal volumes of antigen. Plates are held overnight at 4°C before the addition of 50 µl of 0.5% RBC to each of the wells. Plates are read after 30 minutes at room temperature and end-points are recorded as the reciprocal of the highest serum dilution producing complete inhibition of agglutination.

Positive and negative control sera are incorporated into each test. A test is considered to be valid only if the control sera give the expected results. Sera with titres below 1/40 are considered to be negative.

HI is an appropriate screening test for surveys although it is not specific. Marked cross-reactions do occur between the phleboviruses, but homologous titres exceed heterologous titres. Experimentally, African phleboviruses other than RVF have been shown to be nonpathogenic for ruminants, and antibodies that they might induce are unlikely to cause confusion in RVF diagnosis (Swanepoel et al., 1986).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

A live vaccine prepared from Smithburn’s attenuated strain of RVF virus has been used for the control of RVF in nonpregnant cattle and sheep in endemic areas and during outbreaks (Botros et al., 2006), while inactivated vaccines for use in pregnant animals and in RVF-free countries are prepared from virulent field strains (Barnard; 1979; Barnard & Botha, 1977). Inactivated virus vaccines should be prepared from highly immunogenic strains of RVF virus produced in cell culture. The virus should be inactivated with formaldehyde and mixed with an adjuvant to enhance immunogenicity. The inactivated vaccine should be carefully safety tested to ensure that there is no residual live virus.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

In humans, an inactivated experimental RVF vaccine has been used for 25 years with considerable success to protect persons at risk. This vaccine is currently produced on diploid cells. However, the limited availability of the vaccine precludes its use in the general population.

Two new vaccine candidates produced from human RVF virus isolates are undergoing extensive testing with a view to replacing existing vaccines.

The first, MV P12, is a mutagen-derived strain of virus passaged in the presence of 5-florouracil with serial mutagenesis resulting in attenuation for mice. Immunogenicity and pathogenicity have been tested in sheep and the virus found to be non-abortogenic in pregnant ewes (Morrill et al., 1987). MV P12 was protective in young lambs (Hubbard et al., 1991; Meadors et al., 1986) and in cattle (Morrill et al., 1997a, 1997b). In further testing in sheep, the vaccine, when used after 28 days of pregnancy, i.e. in the first trimester, resulted in abortion and severe fetal teratology (Hunter et al., 2002).

The second candidate, Clone 13, a small plaque variant that did not react with two specific monoclonal antibodies, was found to be avirulent in mice and hamsters and highly immunogenic. Immunogenicity and pathogenicity have been tested in lambs, sheep, young and adult goats (Muller et al., 1995). In further trials it was non-abortogenic in pregnant sheep and gave more than 80% protection from virulent challenge (Hunter & Bouloy, 2001). Clone 13 possesses a large deletion in the portion of the sRNA segment coding for the nonstructural proteins, which should result in a stable vaccine candidate.

In the following description of vaccine production, information is given on live vaccine production adjacent to information on inactivated vaccine production. It must be stressed that live and inactivated vaccines must never be produced in the same facility at the same time, because of the risk of contaminating the attenuated live vaccine with a virulent strain of virus before it is inactivated. Staff handling live RVF virus should be vaccinated and work at containment level 3 to minimise the risk of self infection.

1. Seed management

a) Characteristics of the seed virus

Live vaccine: The stock antigen is derived from Smithburn’s original neurotropic strain. This strain is not lethal to adult mice inoculated intraperitoneally and is safe for use in all breeds of cattle, sheep and goats. However, it may cause fetal abnormalities or abortion in pregnant animals.
Inactivated vaccine: For seed virus, a highly immunogenic strain of RVF virus adapted to growth in cell culture may be used. It differs from the attenuated strain in that it is lethal to adult mice when injected intraperitoneally.

b) Method of culture

Both attenuated and inactivated virus strains are produced on BHK, Vero or CER cell cultures. The viruses are stored in a lyophilised form in vials containing 1 ml of a cell culture suspension. The virus titre (following intracerebral inoculation of infant mice) should be at least $10^{6.5}$ mouse LD$_{50}$ (50% lethal dose) per ml.

c) Validation as a vaccine

Seed virus must be shown to be free from adventitious agents, safe for use and able to stimulate effective immunity in species and breeds for which it is intended.

- Tests

The lyophilised seed virus is reconstituted in sterile cell culture medium without antibiotics and tested for freedom from bacteria and fungi. The contents of a reconstituted vial are inoculated into two tubes of thioglycollate and two tubes of soybean casein digest medium. The thioglycollate cultures are incubated at 37°C for 7 days and the soybean casein digest medium cultures at 20°C for 14 days. The cultures should remain negative.

In addition, 5 ml of reconstituted seed virus is mixed with an equal volume of specific RVF antiserum produced in rabbits. After incubation of the serum/virus mixture at 37°C for 30 minutes, the virus suspensions are tested before and after neutralisation on cell cultures, as well as in adult and infant mice, embryonated eggs, and guinea-pigs. The neutralised virus is:

i) Seeded on to six roller tube cultures of primary lamb kidney cells and six roller tube cultures of BHK cells. The cell cultures are incubated at 37°C and observed daily for 7 days for CPE, after which they are subjected to the haemadsorption test with guinea-pig RBCs at 4°C and 37°C. There should be no evidence of CPE or haemadsorption. If cultures degenerate or show suspicious CPE, the material from these cultures should again be mixed with antiserum and subinoculated into new cell cultures, which are observed for a further period of 14 days. The presence of specific CPE or haemadsorption disqualifies the seed virus pool.

ii) Inoculated intraperitoneally (0.2 ml) into groups of at least six adult and six 2–5-day-old mice. The mice should remain healthy for 14 days. If any mice should die, appropriate tissue should be emulsified, mixed with antiserum and subinoculated into further groups of mice, which should again be observed for a further period of 14 days. If there is any evidence of specific mortality, the seed virus pool is disqualified.

iii) Inoculated into at least ten 8-day-old embryonated chicken eggs by means of the 'stab' method (combination of chorioallantoic membrane and allantoic sac route). The eggs are incubated at 37°C for 8 days and are candled daily. Embryos that die within 24 hours are discarded. However, the test should be repeated if <70% of the embryos are alive after 24 hours. The cause of embryo mortality during the subsequent observation period should be determined by setting up appropriate sterility and HI tests, and by examination of yolk-sac smears. If these tests are negative, subinoculation of embryo suspensions mixed with antiserum should be set up as before. On day 4 of incubation, at least four eggs are opened and allantoic fluid is collected. The remaining eggs are opened on day 8 of incubation. The membranes of both groups are examined for lesions and abnormalities of the embryos. The allantoic fluids are subjected to the HI test with guinea-pig and chicken RBCs at 4°C and 37°C. Specific embryo mortality, haemagglutinating activity of the allantoic fluids or any lesions on the membranes or embryo abnormalities disqualifies the seed virus pool.

iv) Injected intraperitoneally with 1.0 ml of seed virus into each of two guinea-pigs. The guinea-pigs should remain healthy over an observation period of 14 days.

Failure to pass any test disqualifies the antigen for use as seed virus.

2. Method of manufacture

A vial of lyophilised seed virus is reconstituted and diluted 1/100 to 1/1000 with sterile Eagle’s medium for the attenuated vaccine and 1/1000 for the inactivated vaccine. To prepare a working suspension, the diluted virus is seeded on to confluent BHK cell cultures in roller bottles and incubated at 37°C. When 70% of cells is affected (CPE), the medium and cells are harvested and the material is diluted 1/100 to 1/1000, after which 10 ml is again
seeded on to roller bottles with confluent BHK cells and again incubated. As soon as 70% CPE is observed, the medium and cells are harvested and pooled.

Virus suspensions for both attenuated and inactivated vaccines are titrated intracerebrally in infant mice and should have a titre of at least $10^{6.5}$ mouse LD$_{50}$/ml. Alternatively, a plaque titration on CER cells may be performed.

Attenuated vaccine is lyophilised immediately after completion of titration and testing for bacteria and fungi.

A stabiliser should be used, such as 5% peptone in 0.3 M phosphate buffer. The volume of inactivated vaccine is adjusted so that the final vaccine will contain at least $10^{6.5}$ mouse LD$_{50}$/ml. The adjusted virus suspension is then inactivated at 37°C for 24 hours with formaldehyde at a final concentration of 0.2%. After inactivation, an equal volume of aluminium hydroxide gel is added to the cell suspension. The vaccine should have a final pH of 7–7.5.

3. In-process control

Prior to inoculation of cell cultures, seed virus is subjected to tests for bacteria and fungi in thioglycollate and soybean casein digest medium (see Section C.1.c and Chapter 1.1.7 Tests for sterility and freedom from contamination of biological materials).

A representative sample from each batch of vaccine is selected and the contents of each are reconstituted with 5 ml sterile distilled water and tested for freedom from bacteria and fungi.

For inactivated vaccines, inactivation must be checked using two passages in the same type of cell culture as used in the production of the vaccine.

4. Batch control

a) Sterility

Prior to freeze-drying or inactivation, each container of pooled vaccine, and thereafter representative samples of the batch, are tested for sterility in thioglycollate and soybean casein digest medium (see also chapter 1.1.7).

b) Safety

Live vaccine: Final containers of lyophilised attenuated vaccine are selected at random, and each is reconstituted in distilled water as for vaccination. Four susceptible sheep are injected subcutaneously with one dose of vaccine. The sheep are observed daily for 14 days and the rectal temperatures are recorded. The sheep must remain healthy.

Vaccine is also injected intraperitoneally into six adult mice (0.25 ml each), two hamsters and two guinea-pigs (1 ml each). The animals are observed for a period of 14 days during which they should remain healthy. Mortality attributed to the vaccine disqualifies the batch.

Inactivated vaccine: In the case of inactivated RVF vaccine, each of four susceptible sheep is injected subcutaneously with 2.0 ml of vaccine, observed daily for 3 weeks and rectal temperatures are recorded. The sheep should remain healthy.

In addition, safety is also determined by intracerebral injection of six adult mice and two litters of at least six infant mice per litter, and by intraperitoneal injection of two guinea-pigs and two hamsters. The mice, hamsters and guinea-pigs are observed for a period of 14 days. They should remain healthy. Mortality attributed to the vaccine disqualifies the batch.

c) Potency

Live vaccine: Lyophilised attenuated vaccine from two final containers is reconstituted and titrated intracerebrally in infant mice. The final vaccine should contain at least $10^{4.4}$ mouse LD$_{50}$/dose. Alternatively, titrations may be done on cell cultures.

Two final containers are kept at 37°C for 1 week, reconstituted and titrated as before. Each should contain at least $10^{3.4}$ mouse LD$_{50}$/dose. Alternatively, titrations may be done on cell cultures.

Inoculated sheep (see Section C.4.b) are bled 2 and 3 weeks after vaccination, and their antibody response is determined by PRN. A virus neutralising antibody titre of 100 or more is regarded as satisfactory.
Inactivated vaccine: The sheep, injected subcutaneously to determine safety (Section C.4.b), are bled after 3 weeks and their antibody response is determined by VN test. A virus neutralising antibody titre of 100 or more is regarded as satisfactory.

d) Duration of immunity
Both the live attenuated and the inactivated vaccines have had extensive field use. The live vaccine is considered to induce lifelong immunity against clinical disease, although controversy exists over the immunogenicity of the Smithburn vaccine. Nevertheless, cattle can be immunised with the live virus vaccine using this strain. Experience of the field efficacy of inactivated vaccines is limited because they are used in areas where RVF is not endemic, consequently natural field challenge of the vaccine does not occur. However, in South Africa, during the outbreak of RVF in 1976–1978, observations by State Veterinarians supported the efficacy of the vaccine. In more recent epizootics elsewhere, the inactivated vaccine failed to protect animals against abortion, following two vaccinations. When using the inactivated vaccine, a booster dose should be given 3–6 months after the initial vaccination and thereafter vaccination should be repeated yearly (Barnard; 1979; Barnard & Botha, 1977).

e) Stability
When stored at 4°C, lyophilised attenuated vaccines are stable for at least 4 years, while inactivated vaccine may be stored for many years. Storage at higher temperatures is not recommended.

f) Preservatives
No preservatives are used.

g) Precautions (hazards)
Although humans can be infected by handling infected material, no case of disease is known to have occurred in humans infected with attenuated vaccine virus, but seroconversion often occurs. However, the strains used to prepare inactivated vaccine may cause disease. Therefore, all staff likely to be exposed to vaccine virus should be vaccinated with the human formalin-inactivated vaccine.

5. Tests on the final product

a) Sterility
Representative samples of the final product are collected and tested as in Section C.4.a.

b) Moisture content
The moisture content of the lyophilised attenuated vaccine should not exceed 3%.

REFERENCES


Chapter 2.1.14. – Rift Valley fever


Chapter 2.1.14. — Rift Valley fever


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*  *

NB: There are OIE Reference Laboratories for Rift Valley fever  
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:  
Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Rift Valley fever
CHAPTER 2.1.15.

RINDERPEST

SUMMARY

In the past, classical rinderpest was an acute, viral disease of domestic cattle, yaks and wild African buffaloes (Syncerus caffer) and Asian water buffaloes (Bubalus bubalis). It was characterised by high morbidity and mortality rates. Sheep, goats, pigs and wild ungulates might also be affected. Between 2002 and 2011, there were no reported field cases of rinderpest. The eradication campaign concluded in 2011 with an international declaration of global freedom from rinderpest. Existing collections of virulent and attenuated rinderpest viruses will remain under sequestration in research and approved vaccine manufacturing laboratories. To guard against the accidental release of virus from laboratory sources, the FAO1 and the OIE are collaborating in establishing the principle of international oversight and regulation of facilities holding rinderpest virus.

Rinderpest remains a notifiable disease and adequate surveillance systems must be maintained for the early detection of clinical cases should there be any accidental escape of the virus. The OIE (with FAO) will ensure the permanent availability of educational materials demonstrating the range of signs associated with rinderpest cases in live animals.

Description of the disease: Clinical recognition of classical rinderpest is based on the finding of an individual dead animal or small groups of extremely sick animals showing one or more of the following signs: pyrexia, inappetance, depression, emaciation, shallow erosions of the upper and lower lip and gum, erosions or blunting of the cheek papillae, serous or mucopurulent ocular discharges and/or nasal discharges, diarrhoea, and terminal recumbency. It is more than likely that the group will contain a number of dead animals with such lesions. The introductory section of this chapter provides a more detailed description.

Identification of the agent: Laboratory confirmation is based on demonstrating the presence of the virus, virus-specific RNA or precipitating antigens in samples from the spleen, lymph nodes, or ocular or nasal secretions of acutely infected animals.

Serological tests: A competitive enzyme-linked immunosorbent assay (ELISA) can be used to determine the presence of rinderpest antibodies in animals that have been infected with field virus or received rinderpest vaccine. The test used should be sensitive with respect to the lineage of virus likely to be present and be highly specific. Neutralising antibody estimations may be used for the same purpose. As with the virus, serum samples from rinderpest-suspected cases, and that may contain the virus or viral sequences, may only be examined in OIE approved high security laboratories.

Requirements for vaccines: A live attenuated cell culture rinderpest vaccine is available. At this time no animal outside a biosecure facility will be inoculated with a rinderpest vaccine. In compliance with international oversight and regulation of facilities holding rinderpest virus, the retention and further manipulation of vaccine seed viruses will be internationally regulated.

In order to prepare for the possibility of a rinderpest virus release, under the terms of the international sequestration agreement, FAO and OIE, in collaboration with member countries, have developed a strategic plan for the post-eradication era that includes an international contingency plan, designation of a minimum number of Reference Centres/Reference Laboratories and creation of emergency vaccine repositories to maintain preparedness.

1 FAO: Food and Agriculture Organization of the United Nations
A. INTRODUCTION

In the past, classical rinderpest was an acute, viral disease of domestic cattle, yaks and wild African buffaloes (Syncerus caffer) and Asian water buffaloes (Bubalus bubalis). It was characterised by high morbidity and mortality rates. Sheep, goats, pigs and wild ungulates might also be affected. Between 2002 and 2011 there were no reported field cases of rinderpest. Further, in the period leading up to January 2011, the OIE Scientific Commission for Animal Diseases scrutinised a comprehensive world-wide list of applications (evidence-based and historical) for national recognition of rinderpest-freedom. This process concluded in 2011 with an international declaration of global freedom from rinderpest.

For the immediate future, existing collections of virulent and attenuated rinderpest viruses will remain under sequestration in research and approved vaccine manufacturing laboratories. To guard against the accidental release of virus from laboratory sources, FAO and the OIE are collaborating in establishing the principle of international oversight and regulation of facilities holding rinderpest virus based on minimising the number of repositories.

Rinderpest remains a notifiable disease and adequate surveillance systems must be maintained for the early detection of clinical cases should there be any accidental escape of the virus. The OIE (with FAO) will ensure the permanent availability of educational materials demonstrating the range of signs associated with rinderpest cases in live animals. A recent account of the history of rinderpest, its eradication and its socio-economic impact is available (Roeder & Rich, 2009).

Rinderpest is caused by a negative-strand RNA virus of the Morbillivirus genus within the family Paramyxoviridae. The virus exists as three geographically restricted clades, described as African Lineages 1 and 2 and Asian Lineage 3, which cross-protect fully and are only differentiated by molecular characterisation. The tissue culture rinderpest vaccine virus was derived from another genetically-distinct virus which was introduced into Africa from Asia in the 19th Century. Classic descriptions of rinderpest refer to it as a highly fatal disease of domestic cattle, yaks and wild African and Asian water buffaloes. The virus also affects swine and a very large variety of wildlife species within the order Artiodactyla, although not always in a clinically apparent form; a recent review identifies sheep and goats as susceptible but largely epidemiologically unimportant hosts of rinderpest (Taylor & Barrett, 2007).

Although some strains of rinderpest evolved into a mild, nonfatal, infectious disease of cattle, all strains retain two very dangerous attributes. The first is an almost certain ability to undergo virulence modulations. The second is an ability to infect wild animal species and, in African buffaloes, eland, giraffe, lesser kudu and warthog, to cause an acute infection associated with high mortality.

Rinderpest is not a zoonotic disease, but the virus or virus-containing materials must be handled in accordance with strict biocontainment procedures as described in Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities and in conformity with the Guidelines for Rinderpest Virus Sequestration.

An illustrated description of the disease is given in the OIE Atlas of Transboundary Animal Diseases (Fernandez & White, 2010). Classical rinderpest has an incubation period of between 1 and 2 weeks, the clinical disease is characterised by an acute febrile attack within which prodromal and erosive phases can be distinguished. The prodromal period lasts approximately 3 days, during which affected animals develop a pyrexia of between 40 and 41.5°C together with partial anorexia, constipation, congestion of visible mucosae, serous ocular and nasal discharges, depression and drying of the muzzle. However, it is not until the onset of the erosive phase, and the development of necrotic mouth lesions, that a tentative clinical diagnosis of rinderpest can be made. At the height of fever, flecks of necrotic epithelium appear on the lower lip and gum and in rapid succession may appear on the upper gum and dental pad, on the underside of the tongue, on the cheeks and cheek papillae and on the hard palate. Through the enlargement of existing lesions and the development of new foci, the extent of the oral necrosis can increase dramatically over the following 2–3 days. Much of the necrotic material works loose giving rise to shallow, non-haemorrhagic mucosal erosions.

Diarrhoea is another characteristic feature of rinderpest and develops 1–2 days after the onset of mouth lesions. The diarrhoea is usually copious and watery at first, but later on may contain mucus, blood and shreds of epithelium and it may be accompanied, in severe cases, by tenesmus. During the erosive phase, necrosis may be observed in the nares, in the vulva and vagina, and on the preputial sheath. Anorexia develops, the muzzle dries out completely, the animal is depressed and emaciated, the breath is fetid and mucopurulent ocular and nasal discharges develop.

Deaths will occur but depending on the strain involved, the breed of cattle infected and environmental conditions, the mortality rate may vary from 100% (peracute strains in European breeds), to 20–30% (acute strains in zebu cattle), to zero (mild strains in zebu cattle). With both acute and mild strains, the mortality rate may be expected to
rise as the virus gains progressive access to large numbers of susceptible animals. In the terminal stages of the illness, animals may become recumbent for 24–48 hours prior to death. Some animals die while showing severe necrotic lesions, high fever, emaciation and diarrhoea, others after a sharp fall in body temperature, often to subnormal values. In survivors, the pyrexia may remit slightly in the middle of the erosive period and then, 2–3 days later, return rapidly to normal accompanied by a quick resolution of the mouth lesions, a halt to the diarrhoea and an uncomplicated convalescence.

In cases where rinderpest is suspected, post-mortem examinations should pay particular attention to the abomasum, which may be highly engorged or show a grey discoloration; to the Peyer’s patches, which may show lymphoid necrosis; and to the development of linear engorgement and blackening of the crests of the folds of the caecum, colon and rectum. The principal differential diagnoses in cattle are bovine viral diarrhoea/mucosal disease and malignant catarrhal fever; differentiation of these diseases requires the use of appropriate laboratory methods. Diagnosis (and differential diagnosis) of rinderpest suspect material will only be undertaken in OIE approved high security laboratories.

Typically the carcass of the dead animal is dehydrated, emaciated and soiled. The nose and cheeks bear evidence of mucopurulent discharges, the eye is sunken and the conjunctiva congested. In the oral cavity, there is often extensive desquamation of necrotic epithelium, which always appears sharply demarcated from adjacent areas of healthy mucosa. The lesions frequently extend to the soft palate and may also involve the pharynx and the upper portion of the oesophagus; the rumen, reticulum and omasum are usually unaffected, although necrotic plaques are occasionally encountered on the pillars of the rumen. The abomasum, especially the pyloric region, is severely affected and shows congestion, petechiation and oedema of the submucosa. Epithelial necrosis gives the mucous membrane a grey colour. The small intestine is not commonly involved except for striking changes to the Peyer’s patches where lymphoid necrosis and sloughing leaves the supporting architecture engorged or blackened. In the large intestine changes involve the ileocaecal valve, the caecal tonsil and the crests of the longitudinal folds of the caecal, colonic and rectal mucosae. The folds appear highly engorged in acute deaths or darkly discoloured in long-standing cases; in either event the lesions are referred to as ‘zebra striping’.

Taking the mild expression of rinderpest, which was associated with African lineage 2 rinderpest virus in endemic areas of eastern Africa, as an example, the incubation period could be between 1 and 2 weeks and the ensuing clinical disease little more than a subacute febrile attack in cattle. The fever was not variable; it was short-lived (3–4 days) and low (38–40°C). The depression that characterised more acute forms of rinderpest was absent from mildly affected animals and, as a result cattle often did not lose their appetite, and continued to graze, water and trek as well as unaffected animals. Diarrhoea, if present, was not marked. On close examination there might be some slight congestion of the visible mucous membranes and small, focal areas of raised, whitish epithelial necrosis might be found on the lower gum – sometimes no larger than a pin head – along with a few eroded cheek papillae. Some animals totally escaped the development of such erosions, the appearance of which was fleeting. Other animals might show a slight, serous, ocular or nasal secretion but, in contrast to the more severe forms of the disease, these did not progress to become mucopurulent.

Even though infections with mild rinderpest could pass unnoticed in cattle, the virus remained highly infectious for wildlife species, and among those generally regarded as highly susceptible (tragelaphine species, such as lesser kudu and eland, African buffalo, and giraffe) it caused fever, a nasal discharge, typical erosive stomatitis, gastroenteritis, and death. Kock (2006) observed that in addition, African buffaloes infected with lineage 2 showed enlarged peripheral lymph nodes, plaque-like keratinised skin lesions and keratoconjunctivitis. Lesser kudu were similarly affected, but whereas blindness – caused by a severe keratoconjunctivitis – was common, diarrhoea was unusual. Eland also showed necrosis and erosions of the buccal mucosa together with dehydration and emaciation. Therefore, in these circumstances, a diagnosis of rinderpest in any of these species points to the likelihood of the simultaneous transmission of the virus, even at a subclinical level, in neighbouring cattle and possible dissemination of infection through live animal trade.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Any suspicion of rinderpest must be viewed as a potential threat to international biosecurity and must be rapidly confirmed or differentiated. If confirmed, back-tracing measures must be immediately instigated but based on an understanding that the virus has been isolated, its lineage identified and its virulence in experimental cattle assessed (Anderson et al., 1996). A variety of suitable tests is available.

Blood in anticoagulant is the preferred sample wherever possible. On average, the onset of viraemia slightly precedes the onset of pyrexia, and may continue for 1–2 days after pyrexia begins to wane. Consequently, animals showing a pyrexia are probably viraemic and therefore the best source of blood with which to attempt virus isolation. However, as occasional febrile animals may no longer be viraemic, samples from several febrile
animals should be collected for submission. It is important to ensure that there is adequate tissue available for at least two virus isolation attempts from the initial submission of a suspected outbreak. The other procedures described should only be attempted if there is extra tissue available.

a) Virus isolation

Rinderpest virus can be cultured from the leukocyte fraction of whole blood that has been collected into heparin or EDTA (ethylene diamine tetra-acetic acid) at final concentrations of 10 international units (IU)/ml and 0.5 mg/ml, respectively. Samples should be thoroughly mixed and transferred to the laboratory on ice, but never frozen. Virus can also be isolated from samples of the spleen, prescapular or mesenteric lymph nodes of dead animals; these samples may be frozen for transportation; transportation must be under biosecure conditions in compliance with international transport regulations described in Chapter 1.1.1 Collection and shipment of diagnostic specimens, and with the Guidelines for Rinderpest Virus Sequestration.

To isolate the virus from blood, uncoagulated blood is centrifuged at 2500 g for 15 minutes to produce abuffy coat layer at the boundary between the plasma and erythrocytes. This is removed as cleanly as possible, mixed in 20 ml physiological saline and recentrifuged in a washing procedure designed to remove any neutralising antibody present in the plasma. The resulting cell pellet is suspended in cell culture maintenance medium and 2 ml aliquots are distributed on to established roller tube monolayers of primary calf kidney, B95a marmoset lymphoblastoid transformed bovine T lymphoblast or African green monkey kidney (Vero) cells. The culture maintenance medium should be decanted and replaced every 2 or 3 days and the monolayer observed microscopically for the development of cytopathic effects (CPE). These are characterised by refractility, cell rounding, cell retraction with elongated cytoplasmic bridges (stellate cells) and/or syncytial formation. The speed with which the CPE develops varies by substrate and probably by strain of virus also. Up to 12 days should be allowed in primary cells, a week in Vero and 2–4 days in B95a cells. Blind passages may be attempted before declaring an important sample negative, but a preferable technique would be to inoculate the cell suspension, and any residue of the original sample, intravenously into a rinderpest-susceptible ox and attempt to re-isolate the virus from its blood. Isolates of virus can be partially identified by the demonstration of morbillivirus-specific precipitinogens in infected cell debris, or completely identified by the demonstration of specific immunofluorescence using a conjugated monoclonal antibody (MAb).

Alternatively, 20% suspensions (w/v) of lymph node or spleen may be used. These should be made by macerating the solid tissues in serum-free culture maintenance medium using standard grinding or shearing techniques and inoculating monolayers as before. The release of virus from solid tissue can be achieved in several ways. Perhaps the easiest is with a pestle and mortar, but this technique requires the use of sterile sand as an abrasive. Alternatively, tissue may be ground without an abrasive using all-glass grinders, for example, a Ten Broeck grinder. Shearing techniques are equally applicable using, for example, Silverson or Waring blenders. Virus-containing suspensions are clarified by low-speed centrifugation. The volume of the inoculum is not critical; a working volume is between 1 and 2 ml. Commonly used antibiotics are penicillin and streptomycin in combination, each at a concentration of 100 IU/ml. A similar broad-spectrum cover can be obtained using neomycin at 50 µg/ml. Fungizone should be included at 2.5 µg/ml.

b) Antigen detection by agar gel immunodiffusion

The agar gel immunodiffusion (AGID) tests may be conducted in Petri dishes or on glass microscope slides (Foreman et al., 1983). In either instance the surface should be covered with agar to a depth of about 4 mm using a 1% aqueous solution of any high quality agar or agarose. Wells are usually cut in a hexagonal pattern of six peripheral wells around a single central well. For slides, wells should be 3 mm in diameter and 2 mm apart. For Petri dishes, the wells can be increased to 4 mm in diameter and the distance between wells to 3 mm. The closer the wells are placed from each other, the shorter the reaction time.

Using a small volume pipette, rinderpest hyperimmune rabbit serum should be placed in the central well. Similarly, control positive antigen, prepared from the macerated lymph nodes of rabbits infected with the Nakamura III lapinised strain of rinderpest, should be placed in alternate peripheral wells (i.e. one, three and five). Negative control antigen is placed in well four. Test antigens are obtained as exudates from the cut surface of spleen or lymph nodes submitted for testing; if no exudate can be obtained a small portion of the sample should be ground with a minimum of saline. Ocular exudates may be squeezed directly from the swabs or, alternatively, by compression in a microtip (the cotton wool should be cut off the swab and placed into the wide end of a plastic 50–250 µl pipette tip; the stem of the swab may then be used to compress the cotton wool and force a small volume of exudate out of the narrow end of the tip). Test samples are added to wells two and six. Tests are best developed at 4°C or low ambient temperatures. The reaction area should be inspected from 2 hours onwards for the appearance of clean, sharp lines of precipitation between the wells forming a line of identity with the controls. Tests should be discarded after 24 hours if no result has been obtained. The result is not acceptable unless precipitation reactions are also obtained giving a line of identity with the control positive antigen preparation.
Although the test is neither highly sensitive nor highly specific, it is robust and adaptable to field conditions. A positive reaction from a large domestic ruminant should be treated as if it were rinderpest. From a small ruminant, a positive result should be treated as having been derived from a case of rinderpest or peste des petits ruminants (PPR) and requiring further differentiation.

c) Histopathology and immunohistochemistry

At post-mortem examination, tissues should be collected and placed in 10% neutral buffered formalin for histopathology and immunohistochemistry; the base of the tongue, retropharyngeal lymph node and third eyelid are suitable tissues. Sections stained with haematoxylin and eosin should be examined for the presence of syncytial cell formation, and cells with intranuclear viral inclusion bodies. The presence of rinderpest antigens can be demonstrated in the same formalin-fixed tissues by immunoperoxidase staining following the quenching of endogenous peroxidase activity. If a polyclonal antiserum is used, this test will fail to differentiate between rinderpest and PPR. However, this problem can be circumvented by using monoclonal antibodies specific for rinderpest and PPR in duplicate tests (Brown, 1997).

d) Lineage identification using the reverse-transcription polymerase chain reaction

The reverse-transcription polymerase chain reaction (RT-PCR) (Forsyth & Barrett, 1995) produces DNA suitable for gene sequence analysis. Viral RNA can be purified from spleen (not ideal because of its high blood content), lymph node and tonsil (ideal), peripheral blood lymphocytes (PBLs), or swabs from eyes or mouth lesions (contingent). Solid tissues (0.5–1.0 g) are minced and homogenised with 4.0 ml denaturing solution, eye and mouth swabs are treated with 1.0 ml, and purified PBLs (from 5 to 10 ml whole blood) are treated with 0.4 ml according to the published procedure. Solution D (disruption solution): the procedure is that recommended to minimise the hazard of handling poisonous guanidium thiocyanate. It should be carried out in a chemical safety hood. The following are the amounts of guanidium thiocyanate for a 250 g bottle, but the volumes can be adjusted for other quantities. Do not attempt to weigh out the guanidium thiocyanate, but dissolve it in the manufacturer’s bottle by adding 293 ml sterile distilled water, 17.6 ml 0.75 M sodium citrate, pH 7.0, and 26.4 ml 10% sarcosyl, then heat to 65°C in a water bath to dissolve. This solution can be kept for several months in the dark at room temperature in a chemical safety cabinet. The final solution D is made by the addition of 0.36 ml 2-mercaptoethanol to 50 ml of the stock solution. This solution should not be kept for more than 1 month.

In the past few years, RNA extraction spin columns have become widely used for fast purification of high quality RNA (RNeasy kit, Qiagen) The resulting RNA is precipitated with 2.5 volumes of ethanol, washed in 70% ethanol, dissolved in sterile water, or TE buffer (Tris/EDTA, 10 mM, pH 7.5, 1 mM EDTA) and stored at −70°C or −20°C until required. The cDNA synthesis is carried out using random hexanucleotide primers to enable several different specific primer sets to be used in the PCR amplification step. Aliquots of the resulting cDNA are amplified using at least three primer sets that can detect and differentiate between PPR and rinderpest. These primer sets include two ‘universal’ sets based on highly conserved regions in the phosphoprotein and nucleoprotein genes that should detect all morbilliviruses, and rinderpest virus-specific sets based on sequences in the fusion protein genes of the virus. The PCR products are analysed on a 1.5% (w/v) agarose gel along with a suitable DNA marker to identify the specific DNA product. A positive control such as measles or canine distemper virus RNA, and a negative control using sterile distilled water instead of RNA, must be included in each RT-PCR. Positive reactions should be confirmed either by using ‘nested’ primer sets based on the F gene sequences or by sequence analysis of the DNA product. It is important to use more than one set of primers for the PCR step when testing for the presence of RNA viruses, as their nucleotide sequences can vary significantly and one change at the 3’-end of the primer sequence may result in failure of the primers to amplify the DNA. The World Reference Laboratory in the United Kingdom (UK), which is also an OIE Reference Laboratory for rinderpest, and the OIE Reference Laboratory in France (see Table given in Part 4 of this Terrestrial Manual), can advise on use of the technique for field sample analysis.

Most recently, a simple Taqman real-time RT-PCR assay for RPV diagnostic has been described. This real-time RT-PCR assay for rinderpest virus has been validated to be highly sensitive in infected tissue culture supernatant and clinical samples from experimentally infected cattle. The assay has proved to be able to detect isolates representative of all known phylogenetic lineages of the virus and clearly differentiate from PPR virus and other look-alike diseases (foot and mouth disease virus, bovine viral diarrhoea virus, bovine herpesvirus, vesicular stomatitis virus). The analytical sensitivity of the L10 primer-probe system exceeded 1–100 TCID50 (50% tissue culture infective dose)/ml, depending on the rinderpest virus strain. Comparison of samples from experimentally infected animals showed that white blood cells and conjunctival swabs are the sample of choice for epidemiological surveillance of the disease, allowing the preclinical detection of the disease by 2–4 days. In the event of a rinderpest virus outbreak, this portable, single-tube format, real-time RT-PCR has the capability of preclinical diagnosis, thus aiding efforts to prevent further transmission of disease.
e) Differential immunocapture ELISA

Neither clinical observations nor AGID tests can differentiate between rinderpest and PPR; consequently, if either disease is suspected in sheep or goats in countries where both diseases occur, other tests like the real-time PCR must be used. Rapid differentiation can be achieved using a differential immunocapture ELISA test (Libeau et al., 1994). This test employs MAbs directed against the N protein of the two viruses. One MAb, with a reactivity against both viruses, is used as a capture antibody, while a second biotinylated MAb specific for a nonoverlapping antigenic N protein site, and specific against either rinderpest or PPR, is used to determine which N protein has been captured.

High protein-binding ELISA plates (or strips) are coated with 100 µl/well of capture antibody. After three washes, the wells are loaded with 50 µl of test sample diluted 1/10 in a lysis buffer, 25 µl of the manufacturer’s recommended dilution of the virus-specific MAb and 25 µl of streptavidin peroxidase at a final dilution of 1/3000. The wells are then placed on an orbital shaker for 1 hour at 37°C, after which time they are again washed; following the addition of 100 µl of ortho-phenylenediamine (OPD), the wells are re-incubated at room temperature for 10 minutes. Reactions are halted by the addition of 100 µl of 1 N sulphuric acid, and the results, measured at 492 nm with an automated ELISA reader, are expressed as absorbance values.

f) Chromatographic strip test

A rapid chromatographic strip test (Bruning-Richardson et al., 2011a) has been developed for assisting field personnel in investigating suspected outbreaks of rinderpest. Any positive result should be treated as indicating a highly suspicious rinderpest case that must immediately be subjected to a thorough investigation. The test strip itself should be sent to the appropriate OIE/FAO Reference Laboratory along with other samples as viral nucleic acid can be harvested from used strips for characterisation (Bruning-Richardson et al., 2011b).

2. Serological tests

a) The competitive enzyme-linked immunosorbent assay

A competitive ELISA is available for the detection of rinderpest antibodies in the serum of animals of any species previously exposed to the virus. The test is based on the ability of positive test sera to compete with a rinderpest anti-H protein MAb for binding to rinderpest antigen. The presence of such antibodies in the test sample will block binding of the MAb, producing a reduction in the expected colour reaction following the addition of enzyme-labelled anti-mouse IgG conjugate and a substrate/chromogen solution. As this is a solid-phase assay, wash steps are required to ensure the removal of unbound reagents.

The rinderpest antigen is prepared from Madin–Darby bovine kidney cell cultures infected with the attenuated Kabete ‘O’ strain of rinderpest virus and inactivated at 56°C for 2 hours. The viral antigen is extracted from the infected cells by repeated cycles of sonication and centrifugation. The MAb was obtained by fusing the splenocytes of hyperimmunised mice with the NSO myeloma cell line, and then shown to be rinderpest H protein specific (Anderson et al., 1991); this MAb has now been designated as C1. Both C1 and standardised rinderpest antigen are directly available from the OIE Reference Laboratory for Rinderpest in the UK (see Table given in Part 4 of this Terrestrial Manual). Kits will continue to be available commercially.

- **Test procedure**
  i) Reconstitute the freeze dried rinderpest antigen with 1 ml of sterile water and further dilute it to the manufacturer’s recommended working dilution using 0.01 M phosphate buffered saline (PBS), pH 7.4.
  ii) Immediately dispense 50 µl volumes of the diluted antigen into an appropriate number of wells of a flat-bottomed, high protein-binding ELISA microplate using two wells per test serum. Tap the sides of the microplate to ensure that the antigen is evenly distributed over the bottom of each well and, having sealed the plate, incubate it on an orbital shaker for 1 hour at 37°C. Wash the wells three times with 0.002 M PBS, pH 7.4.
  iii) Add 40 µl of blocking buffer (0.01 M PBS, 0.1% [v/v] Tween 20 and 0.3% [v/v] normal bovine serum) to each test well followed by 10 µl volumes of all test sera.
  iv) Follow the manufacturer’s recommendations to prepare a working dilution of the MAb in blocking buffer, and add 50 µl of this to each test well. Seal the plates and re-incubate on an orbital shaker for 1 hour at 37°C.
  v) Follow the manufacturer’s recommendations to prepare a working dilution of rabbit anti-mouse immunoglobulin horseradish peroxidase conjugate in blocking buffer and add 50 µl to each test well. Seal the plates and re-incubate on an orbital shaker for 1 hour at 37°C.
vi) At the end of this period the plates are washed as before and immediately refilled with 50 µl volumes of substrate/chromogen mixture (1 part 3% H2O2 to 250 parts OPD), and incubate at room temperature for 10 minutes without shaking. Then add 50 µl of a stopping solution consisting of 1 M sulphuric acid.

vii) The test system must include known rinderpest positive and negative serum samples, a MAAb control and a conjugate control.

viii) Measure the resulting absorbance values on an ELISA reader with a 492 nm interference filter and express the test results as percentage inhibition values compared with the value obtained using the MAAb control. Inhibition values of 50% or more are considered to be positive and values below 50% are considered to be negative.

Lowering the positive/negative threshold to 40% or less increases the sensitivity of the test, but inevitably affects specificity by increasing the proportion of false-positive test results encountered. In practise, the 50% value is recommended by GREP at which level sensitivity is at least 70% and specificity exceeds 99%. The sensitivity needs to be taken into account when designing sampling frames for serosurveillance.

b) Virus neutralisation

The ‘gold standard’ virus neutralisation (VN) test is performed in roller-tube cultures of primary calf kidney cells following the method of Plowright & Ferris (1961); the test has been validated in experimentally infected cattle. In the roller tube procedure, sera, that has not been inactivated, are diluted at intervals of 1 in 10 and then, starting with undiluted serum, mixed with an equal volume of 10^{3.0} TCID_{50} per ml of the attenuated Kabete ‘O’ vaccine strain virus. Mixtures are held overnight at 4°C, after which 0.2 ml volumes are inoculated into each of five roller tubes, immediately followed by 1 ml of dispersed indicator cells suspended in growth medium at a rate of 2 × 10^5 cells per ml. Tubes are incubated at 37°C, sloped for the first 3 days, after which they are replenished with maintenance medium and placed on a roller apparatus. They are examined regularly for virus-specific cytopathology and positive tubes recorded and discarded; the final examination takes place on day 10.

For calculating end-points, the virus dose is regarded as satisfactory if the final dilution falls within the range 10^{1.8} to 10^{2.8} TCID_{50}/tube. This test should be used to examine the sera of ELISA reactors during national serosurveillance programmes designed to demonstrate freedom from infection, or to qualify susceptible cattle for vaccine testing. Under these circumstances, the presence of any detectable antibody in the 1/2 final serum dilution is considered to indicate previous infection with rinderpest virus. The VN test is the test of choice for the examination of wildlife serum samples.

A microplate method may be used as a screening test. In this procedure, an initial serum dilution of 1/5 is further diluted at twofold intervals. Thereafter, 50 µl volumes of serum are incubated with 50 µl volumes of virus diluted to contain between 10^{1.8} and 10^{2.8} TCID_{50} (Taylor & Rowe, 1984). Following a 45-minute or an overnight incubation period, between 1 and 2 × 10^5 calf kidney, lamb kidney or Vero cells are added as indicators. Tests are terminated after 6 or 7 days. Such tests may give indications of nonspecific neutralisation at high serum concentrations. There appear to be factors in some normal (with respect to prior rinderpest exposure) sera that bring about the failure of the virus to penetrate and replicate in indicator cells. In the tube test, these factors were probably removed during changes in maintenance medium; in the microplate method, they remain present the whole time. If the most concentrated final serum dilution is limited to 1/10, the effect disappears.

C. REQUIREMENTS FOR VACCINES

1. Background

a) Rationale and intended use of the product

The live attenuated tissue culture rinderpest vaccine (TCRV) described in previous editions of the Terrestrial Manual (Plowright, 1962) was developed in Kenya through the serial passage in primary bovine calf kidney cells of RBOK (rinderpest bovine old Kabete, or “Kabete O”), a virulent bovine rinderpest field strain isolated in 1911. While the modern division of rinderpest viruses into four lineages (Africa 1 and 2 and an old African one which includes Kabete O, and Asian) was unknown until 1995 (Wamwayi et al., 1995) (the RBOK virus undoubtedly cross-protects against all strains of all lineages. Since its development, the RBOK vaccine seed was widely distributed and hundreds of millions of doses of it have been used on the Indian subcontinent, the Middle and Near East, and Africa in the control and eradication of rinderpest.
2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed

i) Biological characteristics

Seed lots used in the manufacture of TCRV must produce a cell-culture vaccine that is safe, that confers an immunity in cattle lasting at least 5 years. The immunogenicity of seed virus was demonstrated up to the 122nd BK passage level, which should not be exceeded.

Vaccine seed must be maintained in a seed-lot system between passage levels 90 and 120. Seed-lot virus must be preserved in a freeze-dried state at a temperature of –20°C or lower. The virus must be cultured in Vero cells or primary or serially cultivated kidney cells derived from a normal bovine foetus or a very young calf. Serially cultivated cells may not be more than ten passages removed from the primary cultivation.

The seed virus produces a vaccine that is safe to use in a variety of European, African and Indian cattle breeds. Its safety and efficacy have never been assessed in Chinese or Japanese cattle breeds.

ii) Quality criteria

Seed lots must be shown to be:

a) Pure: Free from contamination with viruses, bacteria, fungi or mycoplasmas.

b) Safe: Inducing no abnormal clinical reaction on inoculation into rinderpest-susceptible cattle.

c) Efficacious: Inducing an immunity to rinderpest in rinderpest-susceptible cattle.

b) Method of manufacture

i) Procedure

Individual vaccine batches are prepared by infecting cell cultures and, after an appropriate incubation period, harvesting the overlying media into which large numbers of live virus particles have been released. Virus may be grown in primary kidney cells from bovine embryos or calves, or cells derived in a homogeneous manner by up to ten serial subcultures from either of these sources. In addition, vaccine may be manufactured in approved continuous cell lines provided the cells are known to be non-infected with bovine viral diarrhoea (BVD) virus and are maintained in a seed lot system; Vero cells have been used for this purpose. To constitute a batch, infected cultures must have been inoculated with the same seed virus and incubated and harvested together.

Virus should be harvested from cultures maintained in roller bottles not more than 10 days after the date that these cultures were infected. The decision to harvest should be based on the development of extensive characteristic CPEs within the cell monolayer. To form a bulk suspension the harvest should be clarified by low-speed centrifugation before mixing with cryoprotectant.

Two harvests are permissible from the same set of cultures and may be pooled to form a single bulk suspension. Written records must accompany all stages of vaccine manufacture.

For long-term storage and cold-chain distribution, bulk suspensions are freeze dried.

ii) Requirements for substrates and media

Cells: Primary cells, serially cultivated primary cell s or continuous cell lines must have been derived from normal looking animals or embryos, and must retain a normal morphology during cultivation. They must be shown to be free of contamination with adventitious viruses, particularly BVD virus (see below).

Media: calf kidney are grown and maintained in Earle’s Balanced Salts Solution or Eagles Minimum Essential Medium supplemented with 0.5% lactalbumin hydrolysate and 0.1% yeast extract together with 5% new-born calf serum which must come from rinderpest-susceptible animals.

Vero cells are grown and maintained under Glasgow Modified Eagle’s medium (GMEM) supplemented with 14% TPB and 6% (rinderpest antibody-free) unheated bovine serum and antibiotics.

Cryoprotectant: the freeze drying stabiliser should consist of an equal volume of a solution containing 5% lactalbumin hydrolysate and 10% sucrose.

iii) In-process controls

A virus titration must be undertaken on the seed lot using tenfold virus dilutions in a microplate or roller tube system and employing ten replicates per dilution.
Chapter 2.1.15. – Rinderpest

Prior to lyophilisation the final bulk may be held for not more than 5 days at 4°C but considerably longer storage is permitted if frozen at −20°C to −60°C.

Checks for adventitious viral contamination should be undertaken on two uninfected control cell cultures prepared from the cell suspension used in batch production, after having been maintained using the same media and incubation conditions as the rinderpest-infected cells. They must be subjected to frequent in-process microscopic observations with negative results. After virus harvesting, the control cultures should be washed to remove ox serum and re-incubated for 10 days in media containing ox serum substitutes during which period they are again subject to frequent microscopic observations for evidence of cytopathic change. At the end of this period one culture should be examined for the presence of noncytopathic BVD virus using an immunofluorescence or immunoperoxidase test or RT-PCR.

The potency of the final bulk must be assessed by the same titration method used for the seed virus.

iv) **Final product batch tests**

*Sterility and purity*

Tests for sterility and freedom of contamination of biological materials may be found in chapter 1.1.7.

The final batch product consists of the freeze dried vials produced from a single bulk harvest; a batch may contain several filling lots. The contents of one container from each filling lot must be exposed to neutralisation by rabbit rinderpest antiserum, using a varying virus/constant serum method, and inoculated into bovine kidney cells. The identity of the product is established if no rinderpest-specific CPE develop.

*Safety and efficacy*

Using rinderpest susceptible cattle, the contents of five randomly selected vials are pooled and used to inoculate one ox with a volume equivalent to 100 cattle field doses (where a field dose is taken to be ≥ 300 TCID₅₀) and one ox with a volume equivalent to 1/10th of a cattle field dose. These animals are maintained in close contact with a contact-control ox for the following 3 weeks. During this period the animals are subjected to daily temperature recording and frequent clinical inspections. At the end of the 3 weeks, the cattle are examined for rinderpest neutralising antibodies and challenged with a strain of rinderpest capable of inducing a pyrexia. The vaccine is considered safe and efficacious if it does not induce any abnormal clinical reaction, if both animals receiving vaccine are protected and if there is no evidence that the vaccine virus has been transmitted. Each vaccine lot must also be tested for innocuity in small animals.

In general terms the safety of the RBOK vaccine has been widely demonstrated in both European and Indian breeds of cattle and Dwarf West African breeds. It has not been tested in Japanese or Chinese breeds and its safety in such animals cannot be guaranteed.

*Batch potency*

The close relationship between immunising potency and infectivity allows the latter to be used as the basis for potency estimations. Three infectivity titrations are undertaken using cells of an approved continuous line or cells grown from each of three different bovine calf or embryonic kidneys. For the first titration, the pool of vials used for the safety test may be employed. The second and third estimates are made on further pools, each of three final containers. The sensitivity of the cells used in each working session must be measured using a standard laboratory rinderpest virus preparation. The final titre is the geometric mean of the three estimates, each undertaken using tenfold dilutions and ten observations per dilution. Potent vaccine should contain 100 field doses per vial.

c) **Requirements for authorisation**

i) **Safety requirements**

*Target and non-target animal safety*

RBOK vaccine causes no clinical signs in rinderpest susceptible cattle or Asian water buffaloes. It does not spread by contact transmission to rinderpest susceptible cattle housed in close proximity to vaccinates.

*Reversion to virulence*

RBOK vaccine virus retains its attenuated characteristics during at least five back passages in cattle and lacks the ability to spread by contact. Any sub-strain of RBOK used in the manufacture of TCRV must be identifiable by written historical records which trace its origins to either of these sub-strains.
Environmental considerations

There are no environmental considerations with respect to either the manufacture or application of rinderpest vaccine.

ii) Efficacy requirements

For animal production

It protects vaccinated animals from infection with virulent rinderpest.

For control and eradication

For eradication purposes the object should be to use vaccine to immunise all susceptible animals in and around the vicinity of an outbreak in as short a period of time as possible (Taylor et al., 2002).

iii) Stability

TCRV is highly stable when correctly freeze-dried and will keep for long periods at either +4 or −20°C provided the product retains a vacuum. The rate of degradation of lyophilised TCRV can be altered by the choice of stabiliser and by variations in the drying cycle. The most advantageous results were associated with the use of a 5% lactalbumin hydrolysate/10% sucrose stabiliser, a 72–74 hour drying cycle under reduced vacuum (100 milliTorr), initial drying for 16 hours at −30°C, and a final shelf temperature of 35°C. With high release titres, such vaccine can be used in the field for 30 days without refrigeration. Following reconstitution in either normal saline or 1M magnesium sulphate, the virus becomes much more thermodilable. The period for field distribution of reconstituted vaccine should not exceed its half-life, but as this parameter is temperature dependent and varies between 8 and 24 hours over a range from 4°C to 37°C, a common sense limit must be applied; a universal period of 4 hours can be recommended.

3. Vaccines based on biotechnology

No biotechnology-based vaccines have so far been approved.

REFERENCES


Chapter 2.1.15. – Rinderpest


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NB: There are OIE Reference Laboratories for Rinderpest (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Rinderpest
CHAPTER 2.1.16.

TRICHINELLOSIS

SUMMARY

Trichinella spiralis is the only species known to cause trichinellosis in humans. It is a parasitic nematode that infects a variety of host species, including humans, pigs, rats, bears, walruses, horses (occasionally) and many other flesh-eating mammals, and birds and reptiles. Ingested infective larvae mature and mate in the small intestine of host species including humans, pigs, rats, bears, walruses, horses (occasionally) and many other flesh-eating mammals, and birds and reptiles. The adult worms survive less than 2 months. The larvae produced migrate and persist in the muscles of their hosts. Susceptible new hosts become infected by ingestion of muscle tissue that contains these larvae.

**Identification of the agent:** Tests for detecting Trichinella spp. fall into two categories: 1) direct detection of first-stage larvae encysted or free in striated muscle tissue, and 2) indirect detection of infection by tests for specific antibodies.

Tissue digestion and tissue compression methods have been used for the direct detection of Trichinella larvae in tissues. Trichinella larvae usually localise in preferred muscle sites, particularly in low level infections, and these sites may vary by host species. It is important that preferred sites be sampled to maximise test sensitivity. For example, in pigs, the diaphragm (crus) and tongue muscles are the two most preferred sites, whereas in horses, the tongue harbours the most worms, followed by the masseter, diaphragm and neck muscles.

The artificial digestion methods involve enzymatic digestion of individual or pooled muscle tissue samples incorporating mechanical homogenisation or grinding, stirring, and incubation. This is followed by filtration and sedimentation procedures to recover and concentrate any larvae that are released from muscle during digestion. Samples processed by these methods are examined under a stereomicroscope for the presence of larvae. Digestion tests can detect <1 larva per gram (lpg) of tissue, but at these low levels of infection, uneven distribution of larvae within tissues is a limiting factor. This is compensated for by testing larger samples per carcass, such as a minimum of 3–5 g for pigs and 5–10 g for horses, game and indicator wildlife species such as foxes. Digestion methods are recommended for the inspection of individual carcass of food animals such as pigs, horses and game.

The compression method (trichinoscopy) is less sensitive than artificial digestion and is not recommended as a reliable test for inspection of carcasses for either food safety or disease surveillance.

**Serological tests:** Serological assays are the most common tests used for indirect detection. The sensitivity and specificity of serological methods are mainly dependent upon the type and quality of antigen used. Most serological test performance (validation) data are from pigs. False negative serological results may occur 3 weeks or longer after muscle larvae become infective in pigs with light or moderate infections. A low rate of false-positive results has also been reported for serological tests. For surveillance or verification of Trichinella-free herds or regions, serological methods are acceptable. For the purposes of individual carcass inspection, only direct methods can be recommended. Pigs harbouring as few as one larva/100 g of tissue have been detected by enzyme-linked immunosorbent assays (ELISA). The specificity of ELISA for Trichinella infection is directed linked to the type and quality of the antigen employed in the test. Secretory antigens collected by short-term (18–20 hours) maintenance of T. spiralis muscle larvae in vitro and synthetic carbohydrate antigens currently provide the most specific and economical source. It is critical that appropriate positive and negative control sera be used to ensure that ELISAs are performing at a minimum acceptable level of sensitivity and specificity. The digestion of 100 g or
more of tissue is recommended as a confirmatory test for serologically positive animals. There is a critical need for an international bank of validated reference sera to provide a common standard for Trichinella serological assays.

Requirements for vaccines: There are no suitable vaccines for Trichinella infection in food animals.

A. Introduction

Clinical signs of trichinellosis are not generally recognised in animals, and its main importance is as a zoonosis. Trichinellosis in humans is caused by eating raw or undercooked meat from Trichinella-infected food animals or game (Gajadhar et al., 2006). The short-lived adult worms live in the small intestine of host species including humans, pigs, rats, bears, walruses, horses, many other flesh-eating mammals, and some birds and reptiles. The parasite has a direct life cycle. Within hours following consumption of infected muscle by a suitable host, first stage muscle larvae (L1) are released by digestion and burrow into the villi of the small intestine. They develop rapidly into adults (males up to 1.8 mm long, females up to 3.7 mm long) and survive for less than 2 months. During this time, copulation takes place and the ovo-viviparous females release new-born larvae (NBL), which migrate via venules and lymphatics into the general circulation. NBL are distributed throughout the body where they invade striated muscles, showing predilection for specific muscle groups. For example, in pigs, the diaphragm pillar and tongue usually contain the highest concentration of larvae, followed by the masseter, and in horses, the tongue followed by masseter, diaphragm and neck muscles. Predilection sites vary by host species, but in general, tongue, masseter and diaphragm are optimal sites for sampling. Current knowledge on predilection sites is available for several host species (Nockler et al., 2000). In cases of severe infection most voluntary muscles contain high numbers of larvae. The larvae of most Trichinella species become encapsulated in collagen in host musculature where they remain infective for years.

Within the genus Trichinella twelve genotypes have been identified, eight of which have been designated as species (Gajadhar et al., 2006; Krivokapich et al., 2008; Murrell et al., 2000; Pozio & Zarlenga, 2005). Trichinella spiralis (T-1) is distributed in temperate regions world-wide and is commonly associated with domestic pigs. It is highly infective for domestic and sylvatic swine, mice and rats, but it can also be detected in other mammalian carnivores and horses. Trichinella nativa (T-2) occurs in mammalian carnivores of arctic and sub-arctic regions of North America, Europe and Asia. Trichinella britovi (T-3) is found predominantly in wild animals and pigs, and occasionally in horses. It occurs in temperate regions of Europe, Asia, and in Northern and Western Africa. Trichinella pseudospiralis (T-4) is cosmopolitan in distribution and has been recovered from raptorial birds, wild carnivores and omnivores, including rats and marsupials in Asia, North America, Europe and Australia. Unlike most other Trichinella genotypes, T-4 is not enclosed within a collagen capsule in muscle. Trichinella murrelli (T-5) is found in mammalian carnivores of North America. It has low infectivity for domestic pigs, but poses a risk to humans who eat game meats and has been reported in a horse. Trichinella T-6 is cold-climate-adapted and appears to be closely associated with T. nativa in northern North America (Pozio & Zarlenga, 2005). Both T. nativa and T-6 are highly resistant to freezing. They have not been found in pigs or horses and experimentally shown to have limited infectivity for these host species. Trichinella nelsoni (T-7) has been isolated from mammalian carnivores and sporadically from wild pigs in Eastern Africa. Trichinella T-8 has been detected in mammalian carnivores in Namibia and South Africa and Trichinella T-9 in mammalian carnivores in Japan (Pozio & Zarlenga, 2005). T-8 and T-9 have some intermediate characteristics with T. britovi and T. murrelli, respectively. Like T. pseudospiralis, T. papuae (T-10) and T. zimbabwensis (T-11) are non-encapsulated muscle parasites. Trichinella papuae has been reported from wild and domestic pigs and farmed crocodiles in Papua, New Guinea. Trichinella zimbabwensis has been described in farmed and wild crocodiles in Zimbabwe, Ethiopia and Mozambique and in monitor lizards in Zimbabwe. Experimentally, it shows a high infectivity for a wide spectrum of mammalian hosts including pigs and rats (Pozio & Zarlenga, 2005). The putative twelfth genotype is based on larvae from a single mountain lion from Argentina (Krivokapich et al., 2008). All species and genotypes of Trichinella cause disease in humans, but not all naturally infect pigs. Risk of establishing Trichinella infection in pig herds is presented by T. spiralis, T. britovi, T. pseudospiralis, and T. papuae, whereas there is no evidence that other species and genotypes can play such a role.

Human trichinellosis can be a debilitating disease and may result in death, but in animals the infection is clinically inapparent. The short-lived adult worms in the intestine can cause transient gastroenteritis, but the most severe signs and symptoms result from the migration and presence of the larvae in voluntary muscle. The disease is transmitted by eating infected meat, primarily from swine or wildlife sources, that has not been sufficiently cooked (or otherwise made safe). Although the prevalence of Trichinella infection in horses is low, consumption of raw or undercooked horsemeat is a well documented source of human trichinellosis (Boireau et al., 2000). Prevention of human infection is accomplished by meat inspection, by processing (cooking, freezing, or curing of meat), and by preventing the exposure of food animals to meat harbouring Trichinella larvae including uncooked food waste, rodents and other wildlife (Gajadhar et al., 2006; Gamble, 1997; Gamble et al., 2000). Game meats should always be considered a potential source of infection, and should be tested or properly cooked. Trichinella found in game
meats (mainly *T. nativa*, T-6 and to a lesser degree *T. britovi*) may be resistant to freezing and therefore untested, frozen game poses a public health risk.

Testing methods for the detection of *Trichinella* infection in pigs and other species either: (a) directly demonstrate the parasite in tissue samples; or (b) indirectly demonstrate the parasite by using immunological methods to detect specific antibodies to *Trichinella* spp. in blood, serum or tissue fluid samples (Gajadhar et al., 2009).

The risk of laboratory acquired infection for analysts is minimal if good laboratory practices are followed. Infection is acquired by the ingestion of muscle larvae in tissues or freed by artificial digestion. Naked larvae die quickly when exposed to the environment or commonly used disinfectants. Contaminated glassware and other surfaces should be cleaned with water at ≥85°C to lyse and remove all larvae. Laboratory waste, including sample remnants, should be treated by boiling, autoclaving, incineration or other suitable processes to kill larvae and prevent their re-introduction into the environment. This is critical when testing proficiency samples containing live larvae in a non-endemic region.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The only recommended procedures for the detection of *Trichinella* larvae in muscle tissues are digestion assays. A number of digestion assays are officially recognised in various countries for trade purposes. The International Commission on Trichinellosis (ICT) recommends several of these assays, which are documented standards in the European Union (EU), Canada and elsewhere (Canadian Food Inspection Agency, 2010; European Commission, 2005). Other methods are not recommended because of their lack of efficiency or reliability. Modern diagnostic assays should meet internationally accepted standards of quality assurance, which include scientifically derived validation data and a design that allows routine monitoring and documentation of critical control points. Although there is general consensus that the digestion assay is the best procedure, a universally accepted digestion test protocol for trade and food safety purposes is not yet available. The digestion assay recommended here is based on desirable innovations inherent in some digestion assays that are accepted for international trade purposes.

a) Recommended direct procedure for testing muscle tissue (the prescribed test for international trade)

*Sensitivity*: the sensitivity of direct testing methods depends on the amount of tissue examined and the site from which the sample was obtained. Direct methods will identify infected pigs, horses or other animals infected with *T. spiralis* as early as 17 days after exposure, coincident with the time that muscle larvae become infective for a new host. Direct methods are most sensitive on fresh samples. The number of larvae that can be recovered from samples declines unpredictably after prolonged storage, putrefaction and freezing. Samples tested for food safety related purposes should be stored at 4°C and tested as soon as possible, and certainly before putrefaction occurs. For wildlife larger samples (≥10 g) should be tested to compensate for a possible decrease in sensitivity due to unknown variation of predilection sites in these host species. Current methods for testing fresh samples for food safety or individual animal inspection by artificial digestion and employing a 1 g sample have a sensitivity of approximately three larvae/g of tissue, and testing of a 5 g sample increases sensitivity to 1 larva/g of tissue (Gamble et al., 2000; Nockler et al., 2000). Where large amounts of tissue (up to 100 g) are available for digestion, the sensitivity of this test is further increased.

*Sampling*: tests are usually conducted on carcass samples collected post-mortem. Muscle samples are taken from predilection sites, usually the diaphragm pillars or tongue of pigs, or tongue or masseter muscles of horses. For wildlife in which predilection sites are unknown, tongue (preferred), diaphragm or masseter should be taken. The sample sizes for carcass inspection testing are based on reliable detection of animals harbouring 1 lpg in tissue, but for surveillance purposes, a higher sensitivity is required to provide better disease prevalence data and to overcome sampling limitations such as those seen with wildlife. Surveillance samples should be ≥10 g and taken from predilection sites (if known). Samples of 100 g would allow detection of 0.01 lpg in the source tissue and if the sample was a predilection site, a low or negative result would indicate a low larval load in the rest of the carcass, with an associated low risk for transmission. For food safety testing or carcass inspection, individual samples of 100 g may be taken from one animal, or multiple samples of lesser amounts may be collected from a number of animals to make a 100 g pool. The size of the samples that make up the pool will determine the sensitivity of the method. The ICT recommends 5-g samples per pig for testing in endemic areas (Gamble et al., 2000). For testing horsemeat, a minimum of 5 g per carcass is required. For horses originating from endemic areas, or if horsemeat is consumed raw, a 10 g sample is recommended (Gamble et al., 2000).
Confirmatory testing of pooled digestion samples and serologically positive animals: When a pool of samples from different animals is digested and has a positive result, additional digestion tests should be used to retest animals individually to determine the identity of the infected animal(s). Animals that are positive on serological testing should have tissues tested by digestion to confirm infection status and to facilitate recovery of larvae and genotype identification.

- **Digestion and detection**
  
  i) Determine the volume of digestive solution required for the digestion (2000 ml of the solution for 100 g of meat, and 1000 ml for 50 g or less).

  ii) Digestive solution: Prepare the appropriate volume of HCl/water solution by combining the HCl with tap water (e.g. for 2.0 litres use 11 ml of 37% HCl or 16 ml of 25% HCl). Do not add pepsin to the solution at this time. This solution should be preheated to 45°C before use.

  iii) Remove as much fat and fascia as possible from each sample of meat.

  iv) Weigh the appropriate amount of trimmed meat from each sample. Cut each sample into 1–2 g pieces and pool with other samples into a 100-g amount.

  v) Place the pooled meat sample into a blender. Add 50–100 ml of the water/HCl solution for a 100 g sample pool.

  vi) Chop the meat in a blender until it is homogeneous (no chunks of meat should be present; the sample should be the consistency of pureed baby food). This is usually achieved with several 1–3-second pulses. Add approximately 100 ml of the prepared water/HCl solution and blend until the mixture is uniformly liquid. This may take 5–10 seconds (additional solution may be needed).

  vii) Sprinkle 10 g of pepsin (1:10,000 NF/1:12500 BP/2000 FIP; granular or equivalent amount of liquid pepsin) onto the homogenate, add about 200 ml of water/HCl solution, and blend for about 5 seconds.

  viii) Transfer the homogenised sample to a 3-litre beaker containing a stir bar. Add the remainder of the 2 litres of water/HCl solution by pouring the water/HCl into the blender and rinsing all residual homogenate into the 3-litre beaker. Rinse any adhering material from the blender lid into the beaker using 10–20 ml of digestive solution from a squirt bottle.

  ix) Place the beaker on a preheated magnetic stirrer hot plate or in an incubation chamber set at 45±2°C. Cover the beaker with aluminium foil. Activate the stirrer at a sufficiently high speed to create a deep vortex without splashing. Note: If the digest temperature at the beginning of digestion is not 45±2°C, the sample should be allowed to warm to this temperature before the timing of the digestion is started.

  x) Allow the digestion to proceed for 30 minutes. If the temperature of the digest has fallen below 45±2°C, additional digestion time may be required to complete the digestion. This can be determined by observing the digestion mixture. If pieces of undigested muscle tissue are present, the digestion should be continued for an additional 30 minutes or until the pieces are digested. Care should be taken to ensure that the digestion temperature range is not exceeded. Alternatively, the digestion may be performed at 37°C for a longer period of time.

  xi) Within 5 minutes of removal from the magnetic stirrer hot plate pour the digestion fluid through a 177–180-µm sieve and into a 2-litre separatory funnel. Rinse the beaker with room temperature tap water from a squirt bottle and pour this through the sieve into the 2-litre separatory funnel.

  xii) Rinse the sieve into the 2-litre separatory funnel by squirting a small volume of room temperature tap water through the top of the sieve. There should be no undigested pieces of muscle remaining on the sieve, although small remnants of fat, fascia and other tissues may be present. Allow the fluid in the separatory funnel to settle undisturbed for 30 minutes.

  xiii) Drain 40 ml of digestion fluid from the separatory funnel into a 50 ml conical tube or measuring cylinder (Pilsner flask) and allow to stand for 10 minutes.

  xiv) At the end of 10 minutes use a pipette to remove 30 ml of the upper part of the fluid (supernatant), leaving the bottom 10 ml in the tube (do not pour off the upper 30 ml, as this will disturb the sediment).

  xv) Gently swirl the remaining 10 ml of fluid and quickly transfer it into a gridded Petri dish or larval-counting basin. Rinse the tube or cylinder into the Petri dish twice using 5 ml of tap water each time. The layer of fluid in the petri dish should not be more than a few millimetres deep.

  xvi) Wait a minimum of 1 minute to allow larvae to settle to the bottom, then use a stereomicroscope at ×10–16 magnification to systematically examine each grid of the Petri dish for the presence of Trichinella larvae. The detection of any suspect larvae on the systematic examination must be confirmed by the identification of morphological details at a higher magnification such as ×40. If the sediment is cloudy or otherwise difficult to examine, it will require further clarification as described below.
Chapter 2.1.16. – Trichinellosis

xvii) Digests should be examined soon after they are ready. Under no circumstance should examination of
digests be postponed until the following day.

xviii) If digestes are not examined within 30 minutes of their preparation they may require clarification as
described below.

xix) Sample clarification: transfer the contents of the Petri dish into a 50 ml conical tube using a pipette.
Rinse the Petri dish thoroughly with tap water, adding the rinse water to the conical tube. Add
additional tap water to bring the volume to 45 ml. Let the tube settle undisturbed for 10 minutes.
At the end of 10 minutes use a pipette to withdraw the supernatant, leaving the bottom 10 ml (do not
pour off the supernatant, as this will disturb the sediment). Save the removed fluid for disposal or
decontamination after the sample has been read.
Repeat steps xv and xvi.

xx) In the event of a positive or doubtful result, a further sample should be collected from each carcass
making up the pooled sample. These should be tested individually or in successive smaller pools until
the individual infected animals are identified.

Identification of the larvae: first stage larvae, digested free from muscle cells, are approximately 1 mm in
length and 0.03 mm in width. The most distinguishing feature of Trichinella larvae is the stichosome, which
consists of a series of discoid cells lining the oesophagus and occupying the anterior half of the worm's
body. Trichinella larvae may appear coiled (when cold), motile (when warm) or C-shaped (when dead). In
case of doubt, larvae should be viewed at higher magnification and further tissues should be examined. If
the counts are high, appropriate dilutions must first be made.

Larvae recovered from muscle digestion may be stored in 70–75% ethanol (or 95% for long-term storage)
for subsequent genotyping by polymerase chain reaction (PCR) (see Section B.1.c).

Quality assurance: laboratories using artificial digestion methods should maintain a suitable quality
assurance system to ensure test sensitivity. Components of a quality assurance system for digestion testing
are described by the ICT (Gamble et al., 2000) and elsewhere (Gajadhar & Forbes, 2001; Gajadhar et al.,
2009) and should include regular use of proficiency testing (Forbes et al., 1998; 2005, Gajadhar et al.,
2009).

b) Other direct detection methods

i) The double separatory funnel method: this assay is recommended as an alternative to the commonly
used digestion procedure described above, and is approved by the EU for export use. The method was
designed to operate under strict conditions of quality control, minimise technical error, and has been
extensively validated for use on pork and horse meat (Forbes & Gajadhar, 1999; Forbes et al., 2008). It
includes a spin-bar digestion technique and sequential separatory funnels for sedimentation of the
larvae. The procedure has fewer steps, requires less time and seldom needs further clarification steps.
An incubation chamber equipped with transparent glass doors and set at 45°C is used to perform the
digestion. The digestion is conducted in 3 litres of digest fluid on a magnetic stirrer. Following digestion
the suspension is poured into a 4-litre separatory funnel through a 177–180-µm sieve, which is rinsed
thoroughly into the separatory funnel with tap water. The suspension is allowed to settle for 30 minutes
and 125 ml is drained into a 500-ml separatory funnel. The volume is increased to 500 ml by adding
375 ml of tap water, and the resultant suspension is allowed to settle for an additional 10 minutes.
Finally, 22–27 ml of sediment is drained into a Petri dish and observed for larvae as previously
described.

ii) The mechanically assisted pooled sample digestion method/sedimentation technique (Equivalent
method A, Regulation [EC] No. 2075/2005): this method uses a heated Stomacher blender for the
digestion phase, and a separatory funnel for sedimentation of the larvae (European Commission,
2005).

iii) Automatic digestion method for pooled samples of up to 35 g (Trichomatic 35): this method involves an
automated digestion chamber and a membrane filter for the recovery and examination of larvae
digestion and larval recovery are difficult to control in the automatic method and it is not recommended
by the ICT.

c) Other tests

i) Polymerase chain reaction: limited studies have shown that PCR can be used to detect the nucleic acid
of larvae in the musculature of infected animals. However, this method lacks sensitivity and is not
practical for routine testing of food animals. Identification of the species or genotype of Trichinella
recovered from muscle tissue is useful in understanding the epidemiology of the parasite in animals, in
assessing the relative risk of human exposure and to trace back the infection to the farm of origin. Specific primers have been developed that allow the identification of single larva collected from muscle tissues at the species and genotype level by PCR (Pozio & La Rosa, 2003). Requests for speciation or genotyping of Trichinella larvae can be made through the OIE Reference Laboratories in Rome, Italy or Saskatoon, Canada (see Table given in Part 4 of this Terrestrial Manual).

ii) Trichinoscopy: This method involves the compression of multiple 2 ×10 mm pieces of muscle tissue between two glass plates (compressorium) until they become translucent, followed by examination using a microscopic technique. There are good comparative data available indicating that trichinoscopy is not as sensitive as digestion assays, so it is not recommended by the ICT or EU for the routine examination of carcasses (European Commission, 2005; Gajadhar et al., 2009).

2. Serological tests

A variety of immunological assays have been described for the diagnosis of trichinellosis in domestic and wild animals (Gamble et al., 2004). Methods include immunofluorescence assay (IFA), immuno-electrotransfer blot (IETB), western blot, enzyme immunohistochemical assays, and enzyme-linked immunosorbent assays (ELISA). Except for the ELISA, these tests have not been standardised, and reagents are not available for routine use. Nevertheless, the ICT has provided a uniform set of recommendations for the development and use of serological tests for the detection of circulating antibodies (Gamble et al., 2004). The ELISA is the only immunological assay endorsed by the ICT. It is only approved as an epidemiological surveillance tool to detect anti-Trichinella antibodies in pigs; it is not reliable for the detection of Trichinella infection in individual animals.

Although other serological tests may have some practical applications, the ELISA is generally acknowledged as the test of choice based on economy, reliability, adaptability to good quality assurance practices, increasing body of validation data and good sensitivity and specificity when conducted under appropriate conditions. It is a useful tool for testing populations and is routinely used for surveillance programmes and disease outbreak investigations. Nevertheless, for reasons given below, the ELISA is not recommended for the testing of individual pigs, horses or other animals for food safety purposes.

a) Enzyme-linked immunosorbent assay (ELISA)

• Sensitivity and specificity

Infection levels as low as one larva/100 g of tissue are detectable by ELISA in pigs (Gamble et al., 2004). This high level of sensitivity makes serological testing by ELISA a useful method for detecting ongoing transmission of Trichinella infection at the farm or for more broadly based surveillance programmes. A disadvantage of serology for the detection of trichinellosis is the low rate of false-negative results observed in infected animals. This is primarily due to the lag time of the immune response following the ingestion of infective larvae. Detectable levels of antibody are not usually present in pigs until 3–5 weeks or more following exposure (Gamble, 1996; Gamble et al., 1996). For this reason, serological methods are not recommended for individual carcass testing. Serological responses in pigs persist for a long time after infection with no decline in titre; however, antibody has been reported to decline in horses within a few months following infection (Nockler et al., 2000). Serological tests may be of little practical use in horses as antibody titres eventually drop below diagnostic levels despite the presence of infective larvae in muscle (Hill et al., 2007; Pozio et al., 2002). Little is known of antibody responses to Trichinella infection in game animals and other wildlife, but high quality serum samples should be obtained to reduce the likelihood of false positive reactions. Currently, no validated serological assay is available for non-pig host species.

• Samples

The use of ELISA to detect the presence of parasite-specific antibodies provides a rapid method that can be performed on serum, blood or tissue fluid collected before or after slaughter (Gamble & Patrascu, 1996). The dilution used is different for serum than for tissue fluid (Nockler et al., 2005).

• Antigens

The specificity and sensitivity of ELISA is largely dependent on the quality of the antigen used in the test. Antigens that are specifically secreted from the stichocyte cells of living L1 larvae and bear the TSL-1 carbohydrate epitope are recognised by Trichinella-infected animals. The antigens recognised in worm ES products consist of a group of structurally related glycoproteins with molecular weights of 45–55 kDa (Ortega-Pierses et al., 1996). A synthetic carbohydrate antigen (Tyvelose) has also been used in ELISA. Studies in swine indicate that Tyvelose may be as good as ES antigen for surveillance testing in pigs however, the sensitivity of the ELISA using this synthetic antigen is lower than that using ES antigens (Forbes et al., 2004; Gamble et al., 1997). Antigen preparations have been developed that provide a high degree of specificity for Trichinella infection in pigs (Gamble et al., 1988). The T. spiralis ES antigens used in the ELISA are conserved in all species and genotypes of Trichinella (Ortega-Pierses et al., 1996), and therefore infection may be detected in pigs or other animals harbouring any of the twelve genotypes.
Chapter 2.1.16. – Trichinellosis

- **Antigen production**

  Diagnosis of *Trichinella* infection by ELISA can be accomplished by using stichosome antigens collected from the ES products of *Trichinella* larvae in culture (Gamble et al., 1988). For purposes of standardisation, it is recommended that *T. spiralis* be used for antigen production for food animal testing. However, it has been demonstrated that antigen prepared from any of the *Trichinella* species can be used for detection of antibodies in infected animals regardless of the infecting species (Kapel & Gamble, 2000). Parasites to be used for antigen preparation may be maintained by serial passage in mice, rats or guinea pigs. To prepare antigen for use in the ELISA (Gamble et al., 1988), *T. spiralis* (T-1) muscle-stage larvae are recovered from skinned, eviscerated, ground mouse or rat carcasses by digestion in 1% pepsin with 1% HCl for 30 minutes at 37°C (as described above). These larvae are washed (three times for 20 minutes each) in Dulbecco’s modified Eagle’s medium (DMEM) with penicillin (500 units/ml) and streptomycin (500 units/ml), and then placed (at a density of 5000 L1/ml) into DMEM supplemented with HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) (10 mM), glutamine (2 mM), pyruvate (1 mM), and penicillin (250 units/ml)/streptomycin (250 µg/ml) (complete DMEM) at 37°C in 10% CO₂ in air. Culture medium is recovered after 18–20 hours, worms are removed by filtration, and the fluid is concentrated under pressure with a 5000 Da molecular weight retention membrane. ES antigens thus recovered may be stored frozen for short periods at –20°C or for longer at –70°C; they consist of approximately 25 protein components as determined by SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis), many of which bear the diagnostic TSL-1 carbohydrate antigen epitope.

  Antigen purity is critical to the specificity of the ELISA. Steps should be taken to monitor growth of bacteria either visually, by phase microscopy, or by plating a sample of media. Cultures showing any bacterial growth should be discarded. Larvae should not be maintained longer than 18 hours; worm deterioration after this time contributes to leaking of somatic antigens that reduce test specificity. Antigen, produced as described, should have a 280:260 nm absorbance ratio of >1.0. The antigens obtained from *in-vitro* maintenance of *Trichinella* larvae, should be tested against a panel of known negative and positive sera before use.

- **Test procedure**

  An example of an ELISA for detecting *Trichinella* infection in pigs is given below. It is essential that all reagents used in the assay be standardised for optimal concentration to obtain reliable results. Typical values are indicated in the example.

i) Coat 96-well microtitre plates with 100 µl/well of *T. spiralis* ES antigens diluted to 5 µg/ml in coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6). Coating is performed for 60 minutes at 37°C or overnight at 4°C.

ii) Wash antigen-coated wells three times with wash buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 5.0% non-fat milk powder and 1.0% Triton X-100. Following each washing, plates are blotted dry.

iii) Dilute pig sera 1/50 or 1/100 in wash buffer. Alternative sources of antibodies that may be used in place of sera include whole blood or tissue fluids at the dilution of 1/5 or 1/10 (Nockler et al., 2005). Add 100 µl of diluted sera to antigen-coated wells. A known positive and known negative serum sample should be used on each plate at the same dilution as the test sera. Incubate at room temperature for 30 minutes.

iv) Wash wells three times as in step ii.

v) Add 100 µl/well of an affinity-purified rabbit anti-swine IgG–peroxidase conjugate at an appropriate dilution in wash buffer. Following the addition of the second antibody, incubate the plates for 30 minutes at room temperature.

vi) Wash wells three times as in step ii. Rinse once with distilled water.

vii) Add 100 µl of a suitable peroxidase substrate (e.g. 5'-aminosalicylic acid 0.8 mg/ml with 0.005% hydrogen peroxide, pH 5.6–6.0).

viii) After 5–15 minutes, read plates for colour density at 450 nm on an automated microplate reader. Values obtained in the ELISA four times that of normal serum pool controls are considered to be positive. Values three times higher than normal are classified as suspect.

Commercial adaptations of the ELISA are available. The manufacturer must validate the kit prior to licensure and the user should also evaluate the performance of the kit, prior to use, by using selected negative and positive reference samples.

The test should be conducted within an environment in which internationally accepted standards of quality management, such as ISO 17025, have been implemented.
There are currently no international standard reference sera to provide a common benchmark for *Trichinella* ELISAs. In addition to the use of international standard sera (when available), all commercial and in-house ELISAs should be evaluated against a bank of negative control sera that represents the population under test, and a group of positive animals that represents different stages of infection as per ICT guidelines.

C. REQUIREMENTS FOR VACCINES

There are no vaccines for *trichinellosis* in food animals or game.

REFERENCES


**CANADIAN FOOD INSPECTION AGENCY** (2010). Meat Hygiene Manual of Procedures, Chapter 5, Sampling and Testing, Section 5.5.2.7.6, Double Separatory Funnel.


Chapter 2.1.16. – Trichinellosis


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**NB:** There are OIE Reference Laboratories for Trichinellosis

(see Table in part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)).

Please contact the OIE Reference Laboratories for any further information on diagnostic tests and reagents for Trichinellosis.
CHAPTER 2.1.17.

TRYPANOSOMA EVANSI INFECTION (SURRA)

SUMMARY

Definition of the disease: Trypanosoma evansi causes a trypanosomosis known as ‘surra’. It affects a large number of wild and domesticated animal species in Africa, Asia, and Central and South America. The principal host species varies geographically, but camels, horses, buffalos and cattle are particularly affected, although other animals, including wildlife, are also susceptible. It is an arthropod-borne disease; several species of haematophagous flies, including Tabanids and Stomoxes, are implicated in transferring infection from host to host, acting as mechanical vectors. In Brazil, vampire bats are also implicated in a unique type of biological transmission.

Description of the disease: The general clinical signs of T. evansi infections: pyrexia directly associated with parasitaemia together with a progressive anaemia, loss of condition and lassitude are not sufficiently pathognomonic for diagnosis. Recurrent episodes of fever and parasitaemia occur during the course of the disease. Oedema, particularly of the lower parts of the body, urticarial plaques and petechial haemorrhages of the serous membranes are sometimes observed in horses. Abortions have been reported in buffalos and camels. Nervous signs are common in horses. The disease causes immunodeficiencies that may be of high impact when interfering with other diseases or vaccination campaigns (foot and mouth disease and haemorrhagic septicaemia for example).

Identification of the agent: The general clinical signs of T. evansi infection are not sufficiently pathognomonic for diagnosis. Laboratory methods for detecting the parasite are required. In early infection or acute cases, when the parasitaemia is high, examination of wet blood films, stained blood smears or lymph node materials might reveal the trypanosomes. In more chronic cases, or more generally when the parasitaemia is low, the examination of thick blood smears, as well as methods of parasite concentration and the inoculation of laboratory rodents are required. In apparently healthy carriers (animals without clinical signs), parasites are rarely observed and mouse inoculation gives the best results. Several primer pairs targeting the subgenus (Trypanozoon) or the species-specific (T. evansi) parasitic DNA sequences are available for diagnosis by polymerase chain reaction (PCR). PCR is more sensitive than parasitological examination, but it may give false-negative results when the parasitaemia is very low; in these cases, suspicion of potential carriers can only be confirmed by serological examination.

Serological tests: Infection gives rise to specific antibody responses and a variety of antibody detection tests have been introduced for laboratory and field use. Some have been partially validated, but await large-scale evaluation and standardisation. The most relevant are immunofluorescence test (IFAT), enzyme linked immunosorbent assays (ELISA) and card agglutination test (CATT/T. evansi). For field use, only CATT/T. evansi can be applied. Pen side tests are currently unavailable. Estimates of predictive values indicate that ELISA for detecting IgG is more likely to classify correctly uninfected animals, while the CATT is more likely to classify correctly truly infected animals. ELISA would thus be suitable for verifying the disease-free status of animals prior to movement or during quarantine. CATT can be used to target individual animals for treatment with trypanocidal drugs. For declaring a disease-free status, serial testing – CATT and ELISA followed by re-testing of suspect samples – is recommended, preferably completed by PCR. In areas where T. cruzi, T. equiperdum or tsetse-transmitted trypanosomes occur, cross-reactions may occur with any serological test employed.

Requirements for vaccines: No vaccines are available for the disease.
A. INTRODUCTION

Infection with Trypanosoma evansi causes a disease named surra in India and, amongst others, El Debab, El Gafar, Tabourit or MBori in North Africa, Mal de Caderas or Murrina in Latin America. The clinical signs of surra are indicative but are not sufficiently pathognomonic, thus, diagnosis must be confirmed by laboratory methods (Dia et al., 1997a). The disease in susceptible animals, including camels (dromedary and bactrian), horses, buffalo, cattle and pigs is manifested by pyrexia, directly associated with parasitaemia, together with a progressive anaemia, loss of condition and lassitude. Such recurrent episodes lead to intermittent fever (as high as 44°C in horses [Gill, 1977]) and parasitaemia during the course of the disease. Oedema, particularly of the lower parts of the body, rough coat in camels, urticarial plaques and petechial haemorrhages of the serous membranes are sometimes observed. In advanced cases, parasites invade the central nervous system (CNS), which can lead to nervous signs (progressive paralysis of the hind quarters and, exceptionally, paraplegia), especially in horses, but also in other host species before complete recumbency and death. Abortions have been reported in buffalos and camels (Gutierrez et al., 2005; Lohr et al., 1986) and there are indications that the disease causes immunodeficiency (Dargantes et al., 2005b; Onah et al., 1998).

There is considerable variation in the pathogenicity of different strains and the susceptibility of different host species. The disease may manifest as an acute or chronic form, and in the latter case may persist for several months, possibly years. The disease is often rapidly fatal in camels and horses, but may also be fatal in buffalo, cattle, llamas and dogs, however these host species may develop mild or subclinical infections. Wild animals such as deer, capybara and coati can become infected and ill (including death), but they may also constitute a reservoir. Animals subjected to stress – malnutrition, pregnancy, work – are more susceptible to disease.

Biologically T. evansi is very similar to T. equiperdum, the causative agent of dourine (Brun et al., 1998; Claes et al., 2003), and morphologically resembles the slender forms of the tsetse-transmitted species, T. brucei brucei, T. b. gambiense and T. b. rhodesiense. Most of the molecular characterisations indicate that various strains of T. evansi isolated from Asia, Africa and South America are very homogeneous and may have a single origin (Ventura et al., 2002), but other works suggest that T. evansi could have emerged from T. brucei in several instances (Jensen et al., 2008; Lai et al., 2008) Molecular characterisation using random amplified polymorphic DNA techniques and endonuclease fingerprinting showed that isolates of T. evansi and T. equiperdum formed a closely homogeneous group. The difficulties in differentiating T. equiperdum from the other Trypanozoon spp. have been stressed (Claes et al., 2005; Zablotskij et al., 2003), and the existence of T. equiperdum was even questioned.

Like all pathogenic trypanosomes, T. evansi is covered by a dense protein layer consisting of a single protein called the variable surface glycoprotein (VSG). This acts as a major immunogen and elicits the formation of specific antibodies. The parasites are able to evade the consequences of these immune reactions by switching the VSG, a phenomenon known as antigenic variation.

Clinical suspicion of surra can emerge from the field in case of fever and/or anaemia. Anaemia is a reliable indicator of trypanosome infection, but it is not in itself pathognomonic. On the other hand, animals with a mild subclinical infection can have parasitaemia without evidence of anaemia (Dargantes et al., 2005a).

In enzootic areas, routine diagnoses can be made using parasitological techniques, while serological surveys can be carried out preferably by ELISA. CATT can be used to target individual animals for treatment with trypanocidal drugs.

Where a definitive confirmation of the infection in suspected animals is needed (e.g. for importation into a disease-free area), mouse inoculation is the best test to apply. However, animal testing should be limited and used only if fully justified.

For declaring disease-free status, at the individual level, serial testing by CATT and ELISA at 40-day intervals is recommended. However the conditions for importation of animals from infected to non-infected areas should be defined, including the status of the exporting farm, the status of exported animals, the application of a diagnostic protocol, and possibly the preventive administration of curative treatments.

In areas where T. cruzi, T. equiperdum or tsetse-transmitted trypanosomoses are present, cross-reactions may occur with any serological test employed. In such conditions, the exact status of an animal regarding trypanosomosis cannot be established.

The OIE has developed international standard monographs for trypanocidal drugs.

- Zoonotic risk and biosafety requirements

Trypanosoma evansi is not known to have a zoonotic potential. It should be handled in the laboratory in accordance with the principles outlined in Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities.
B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The classical direct parasitological methods for the diagnosis of trypanosomosis, namely microscopic examination of blood or lymph node material, are not highly sensitive, but a number of techniques, including enrichment of the sample, rodent inoculation and DNA methods may increase the sensitivity. In regions where other *Trypanozoon* spp. occur in addition to *T. evansi*, specific identification by microscopy is not possible; molecular tools are then very useful for species specific diagnosis.

a) Direct microscopic examination

i) Blood sampling

*Trypanosoma evansi* is a parasite of the blood and tissues. As for other trypanosomes, it is recommended that blood for diagnosis be obtained from peripheral ear or tail vein, even if the jugular vein is most often preferred for practical reasons. However it should be realised that less than 50% of infected animals may be identified by examination of blood.

Peripheral blood is obtained by puncturing a small vein in the ear or tail. Deeper samples are taken from a larger vein by syringe. Cleanse an area of the ear margin or tip of the tail with alcohol and, when dry, puncture a vein with a suitable instrument (lancet, needle). Ensure that instruments are sterilised or use disposable instruments to avoid iatrogenic transmission of the infection by residual blood.

ii) Wet blood films

Place a small drop of blood (2–3 µl) on to a clean glass slide and place over it a cover-slip to spread the blood as a monolayer of cells. Examine by light microscopy (200×) to detect any motile trypanosomes. Improved visualisation can be obtained with dark-ground or phase-contrast microscopy (200–400×). The sensitivity of this method is low, approximately 10 trypanosomes per µl, which is frequent in early or acute infections only.

iii) Stained thick smears

Place a large drop of blood (10 µl) on the centre of a microscope slide and spread with a toothpick or the corner of another slide so that an area of approximately 1.0–1.25 cm in diameter is covered. Air-dry for 1 hour or longer, while protecting the slide from insects. Placing the slide in a horizontal position, stain the unfixed smear with Giemsa’s Stain (one drop of commercial Giemsa + 1 ml of phosphate-buffered saline, pH 7.2), for 25 minutes. After washing and drying, examine the smears by light microscopy at a magnification of 500× with oil immersion. The advantage of the thick smear technique is that it concentrates the drop of blood into a small area, and thus less time is required to detect the parasites, which are more visible owing to the haemolysis of the unfixed red cells. The disadvantage is that the trypanosomes may be damaged in the process, and the method is therefore not suited for species identification in case of mixed infections.

iv) Stained thin smears

Place a small drop of blood (3–5 µl) at one end of a clean microscope slide and draw out a thin film in the usual way. Air-dry briefly and fix in methyl alcohol for 1 minute and allow to dry. Stain the smears in Giemsa (one drop Giemsa + 1 ml PBS, pH 7.2) for 25 minutes. Pour off stain and wash the slide in tap water and dry. Nowadays, fast stains are most often used¹, which allow fixation and staining within a few seconds. Slides are then washed in tap water and dried. Examine at a magnification of 400–1000× with oil immersion. This technique permits detailed morphological studies and identification of the *Trypanosoma* species, but it is of a very low sensitivity (it can detect parasitaemia >500,000 trypanosomes/ml of blood).

v) Lymph node biopsies or oedema fluid

Samples are usually obtained from the prescapular or precrural (subiliac) lymph nodes. Select a suitable node by palpation and cleanse the site with alcohol. Puncture the node with a suitable gauge needle, and draw lymph node material into a syringe attached to the needle. Expel lymph on to a slide, cover with a cover-slip and examine as for the fresh blood preparations. Fixed thin or thick smears can also be stored for later examination. Similar examination can be done by collection of oedema fluid.

¹ For example Diff-Quick®, RAL555®
b) Concentration methods

In most hosts *T. evansi* can induce mild clinical or subclinical carrier state infections with low parasitaemia in which it is difficult to demonstrate the parasites. In these circumstances, concentration methods are necessary, as they increase the sensitivity of microscopic examination.

i) **Haematocrit centrifugation technique (also known as Woo’s technique, or HCT)**

Collect blood (70 µl) into two heparinised capillary tubes (75 × 1.5 mm). Close the wet end with plasticine and centrifuge at 3000 \( g \) for 5 minutes (generally 12,000 rpm in a haematocrit centrifuge machine). The capillary tube is examined and the value of the haematocrit is expressed as a percentage of packed red blood cells (RBCs) to total blood volume; this gives an indication on the anaemia of the animal. The capillary tube is then placed in a groove made with pieces of slide glued to a slide. Trypanosomes are large cells that concentrate at the junction between the buffy coat and the plasma, which is observed under the microscope (100–200×). Light conditions must be set to induce refringence of the cells to increase the visibility of the moving trypanosomes; this can be obtained by lowering the position of the light condenser or with intermediary positions of the turret light condenser. Specially designed reading chambers for HCT can be obtained at the OIE Reference Laboratory for Surra, at the Institute of Tropical Medicine (ITM)\(^2\). The fresher the sample, the better is the sensitivity as strong parasitic movements make trypanosomes more visible. This technique can detect around 50–200 trypanosomes/ml of blood (Desquesnes & Tresse, 1996). The buffy coat can also be collected in a microtube and frozen; also the sample can be prepared for polymerase chain reaction (PCR).

ii) **Dark-ground/phase-contrast buffy coat method (also known as Murray’s technique, or BCM)**

This technique is very similar to the previous one. Collect blood into heparinised capillary tubes and centrifuge as above. Scratch the tube with a glass-cutting diamond and break it 0.5 mm below the buffy coat layer – the upper part thus contains a small top layer of RBCs, the buffy coat (white blood cells and platelets) and some plasma.

Partially expel the contents of this piece on to a slide; avoid expelling more than 5–8 µl of plasma, but make sure the buffy coat has been expelled (the small disk of the buffy coat should be visible to the naked eye), press on a cover-slip to spread the buffy coat and examine by dark-ground, phase-contrast or similar microscopy under the previously described refringent conditions at a magnification of 200–500×. Trypanosomes are mostly present at the periphery of the thick buffy coat material.

Both the Woo and the Murray techniques allow anaemia to be estimated by measuring the packed cell volume and may be used in surveys of herds at risk. The value of the haematocrit can be used as an indicator (when <24% for example in cattle) to select a subset of samples to be submitted to the more expensive PCR analysis (Desquesnes et al., 1999).

iii) **Mini-anion exchange centrifugation technique**

When a blood sample from animals infected with salivarian trypanosomes is passed through an appropriate DEAE-cellulose (diethylamino-ethylcellulose, such as Whatman DE 52) anion-exchange column, the host blood cells, being more negatively charged than trypanosomes, can be adsorbed onto the anion-exchanger (in pH and ionic strength conditions adapted to the host species), while the trypanosomes are eluted, retaining viability and infectivity (Lanham & Godfrey, 1970). This technique is mostly used for the purification of parasites from the blood (for example, for parasite antigen preparation), but miniature systems have been developed, especially for diagnosis in humans (Lumsden et al., 1981). A simplified field method for detection of low parasitaemia has been developed (Sachs, 1984). The sensitivity of this technique can be increased by approximately tenfold by the use of buffy coat preparations rather than whole blood (Reid et al., 2001).

### Preparation of phosphate buffered saline glucose (PSG), pH 8

\[ \text{Na}_2\text{HPO}_4 \text{ anhydrous (13.48 g)}; \text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O (0.78 g)}; \text{NaCl (4.25 g)}; \text{distilled water (1 litre).} \]

Solutions of different ionic strength are made by diluting the stock PBS, pH 8, and adding glucose to maintain a suitable concentration. For blood of mice, domestic and wild ruminants and dog, add four parts PBS to six parts distilled water and adjust the final glucose concentration to 1%. For blood of pigs and rabbits, add three parts PBS to seven parts distilled water and adjust the final glucose concentration to 1.5%. The PBS/glucose solution (PSG) must be sterile (however, PBS must be autoclaved before adding glucose).

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Chapter 2.1.17. — Trypanosoma evansi infection (surra)

- **Equilibration of DEAE-cellulose**
  Suspend 500 g of DEAE-cellulose in 2 litres of distilled water. Mix for 20 minutes with a magnetic stirrer at low speed. Adjust the pH to 8 with phosphoric acid. Allow to settle for 30 minutes. Discard the supernatant fluid containing the finest granules. Repeat the procedure three times. Store the equilibrated concentrated suspension of DEAE-cellulose (slurry) at 4°C for a short period, or in small aliquots at –20°C for longer conservation.

- **Packing of equilibrated DEAE-cellulose**
  Place a 2 ml syringe without the plunger on a test-tube rack complete with a flexible tube that can be closed with a clamp to act as a tap. Put a disc of Whatman No. 41 filter paper at the bottom of the syringe and moisten by adding a few drops of PSG. Pour 2–2.5 ml of the slurry of equilibrated cellulose into the syringe and allow packing for 5 minutes before elution of the buffer. The height of the sediment should be approximately 3 cm. Wash and equilibrate the column with 2 ml of PSG without disturbing the surface.

- **Adsorption of blood eluate of the trypanosomes**
  Gently place 100–300 µl of heparinised blood (or preferably buffy coat) on the surface of the cellulose column; allow it penetrate the cellulose, but do not let the cellulose dry before pouring on the eluting buffer. Progressively add 1.5 ml of PSG and start collecting the eluate into a finely tapered Pasteur pipette with a sealed end. The cellulose column should remain wet throughout the procedure. Put the filled pipette, protected by a conical plastic pipette tip, in a tube and centrifuge at 525 g (or up to 1000 g) for 10 minutes. Examine the bottom of the pipette under the microscope (100× or 200×) using a special mounting device. Alternatively, the eluate could be collected into 50 ml plastic tubes, with conical bottoms, centrifuged at 1000 g and the sediment examined by dark-ground microscopy.

A similar method used in cattle, pig and goat is also referred to as the miniature anion exchange chromatography method (Gutierrez et al., 2004a; Reid et al., 2001; Sachs, 1984). In addition, large amounts of blood or buffy coat can be applied to large columns for preparation of antigen for indirect fluorescence antibody test (IFAT), card agglutination test (CATT) or enzyme linked immunosorbent assay (ELISA).

c) **Animal inoculation**
  Due to the increasing concern to eliminate the use of animals for biological testing, animal inoculation should be limited as far as possible and only used if fully justified. Laboratory animals may be used to reveal subclinical (non-patent) infections in domesticated animals. Trypanosoma evansi has a broad spectrum of infectivity for small rodents, and so rats and mice are often used. Rodent inoculation is not 100% sensitive (Monzon et al., 1990) but further improvement in its efficacy can be obtained by the use of buffy coat material. Such a procedure was able to detect as few as 1.25 T. evansi/ml blood (Reid et al., 2001). This technique is suitable when highly sensitive detection is required.

Inoculate heparinised blood intraperitoneally into rats (1–2 ml) or mice (0.25–0.5 ml). Inoculate a minimum of two animals. Bleed animals from the tail after every 48 hours to detect parasitaemia. The incubation period before appearance of the parasites and their virulence depends on the strain of trypanosomes, their concentration in the inoculum, and the strain of laboratory animal used; however in most cases it is very short (5 ± 2 days), but can extend to 2 weeks in rare cases (Monzon et al., 1990). Sensitivity of this in vivo culture system may be increased by use of immunosuppressed laboratory animals. Drugs such as cyclophosphamide or hydrocortisone acetate, X-ray irradiation, or splenectomy have been used for this purpose. Such a procedure is only justified when the detection of a potentially infected host is of high importance (for example, importation into a disease-free area).

d) **Detection of trypanosomal DNA**
  Detection of minute amounts of trypanosomal DNA is a possible mean of identifying animals with active infections as the parasitic DNA does not remain for more than 24–48 hours in the blood of the host after the trypanosomes are killed (Desquesnes, 1997b).

- **DNA probes**
  Specific DNA probes have been used to detect trypanosome DNA in infected blood or tissue but are not routinely applied as further evaluation needs to be made (Basagoudanavar et al., 2001; Reid et al., 2001; Viseshakul & Panyim, 1990). PCR techniques are generally preferred and are routinely used in some laboratories.

- **Polymerase chain reaction (PCR)**
  Polymerase chain reaction (PCR) based on DNA sequences of various taxonomic levels is used. The gold standard, to date, for detection of the Trypanozoon subgenus are the NRP or TBR primers (Masiga et al., 2001).
1992: Moser et al., 1989). Other primers have been published and are being evaluated; some of them are specific for Trypanozoon (Desquesnes et al., 2001; Holland et al., 2001; Wuyts et al., 1994) and others for T. evansi ± T. equiperdum (Artama et al., 1992; Claes et al., 2004; Panyim et al., 1993) (evaluation of the latter is very difficult because of the absence of collections of reference strains). To date, the most sensitive test is that of satellite DNA using TBR primers (Masiga et al., 1992); the sensitivity of the other primers is being compared under various conditions, including in laboratory rodents, but can only be validated with a sufficient batch of field samples from natural hosts. The use of TBR primers is recommended, at least in the first instance, and, if necessary, for example in areas and host species potentially infected with other Trypanozoon such as T. brucei brucei, species confirmation can be obtained with more specific primers such as TEPAN (Panyim et al., 1993) or TE2249/2250 (Artama et al., 1992). Other primers specific for RoTat (Claes et al., 2004; Verloo et al., 2001) or non-RoTat strains (Ngaira et al., 2005), and other techniques such as the loop-mediated isothermal amplification (LAMP) (Thekiso et al., 2005) and Taqman (Taylor et al., 2008), are under development but need to be further evaluated and validated.

DNA preparation is an important step that determines the success and the sensitivity of the PCR. It can be done on plain blood (generally collected with anticoagulant), or, preferably, on the buffy coat to increase the sensitivity of the test (Desquesnes & Davila, 2002; Majwa et al., 1994). Several classical techniques are available, such as Chelex preparation (Solano et al., 1999), commercial kits and the phenol–chloroform preparation (Maciel et al., 2009). Blood conserved 1/1 in 70% alcohol, or on dry filter paper can also be used (Desquesnes, 2004; Holland et al., 2002; Omanwar et al., 1999).

The sensitivity of the PCR being dependent on the amount of DNA available, it is proportional to the parasitaemia. PCR is thus more sensitive in highly susceptible hosts (camels, horses, dogs, etc.) than in hosts of mild or low susceptibility (cattle, buffalo, pigs, etc.). Using a suitable DNA preparation and the most sensitive primers available (TBR), PCR allows as little as 1–5 trypanosomes/ml of blood to be detected (Panyim et al., 1993; Penchenier et al., 1996), or only 10 per ml in buffaloes with a quantitative real-time PCR (Konnai et al., 2009).

PCR offers the sensitivity and specificity required for detection of trypanosome infection (Masiga et al., 1992; Wuyts et al., 1994; Wuyts et al., 1995), but it may give false-negative results. Experimental studies in sheep have shown that PCR can remain negative for long intervals during aparasitaemic periods (Bengaly et al., 2001), while in buffalo the diagnostic sensitivity of a PCR was only 78%, which is similar to mouse inoculation (Holland et al., 2001). Nevertheless, PCR is the most sensitive technique for detection of the infection.

f) Antigen detection

Circulating antigen detection in blood or serum is also a way to detect active infection. Several attempts to develop such tests have not yet reached a satisfactory level to be recommended for routine diagnosis (Desquesnes, 1996; Monzon, 2006; Morzaria et al., 1996).

2. Serological tests

Historically, different methods have been used to detect non-specific humoral antibodies present in cases of surra infection. These methods are biochemical tests including flocculation, formol-gel, mercuric chloride precipitation and thymol turbidity tests, and are considered to be outdated although the formol-test may still have some use in the field because it is simple to perform. These tests all depend on an increase in serum globulins as a result of infection, but this increase is not specific for T. evansi infection. Mercuric chloride must not be used because of its toxicity. The formol-gel test is the test of choice in camels but has not been validated in other species. It is carried out by adding two drops of concentrated formalin solution (40% formaldehyde [w/v]) to 1 ml of serum. The test is positive if the serum coagulates immediately and turns white. In negative reactions, the serum remains unchanged or coagulation may take up to 30 minutes to appear.

Similarly, many different methods have been used to detect specific humoral antibodies to trypanosomal antigens, such as direct or indirect agglutination tests, complement fixation test (CFT), IFAT (Desquesnes, 1997a; Uilenberg, 1998) and the trypanolysis test. The IFAT is still useful for small-scale surveys. The trypanolysis test is used for individual confirmation of positivity because of its high specificity. The other tests are no longer used because they have been replaced by the more easily standardised techniques of ELISA (Davison et al., 1999; Franke et al., 1994; Rae et al., 1989; Reid & Copeman, 2002; Reid & Copeman, 2003; Tuntasuvan et al., 1996) and CATT (Bajyan Songa & Hamers, 1988; Njiru et al., 2004). Attempts to develop new techniques such as latex agglutination tests have not been successful so far (Gutierrez et al., 2004b; Holland et al., 2005; Morzaria et al., 1996).
Evaluations of ELISA and CATT have been carried out in camels, horses, cattle, buffaloes and pigs (Desquesnes et al., 2009; Diall et al., 1994; Holland et al., 2005; Payne et al. 1991; Reid & Copeman, 2003; Verloo et al., 2000). Tests should preferably be carried out on plasma or serum, but the collection of samples can be simplified by using filter paper blood spots for later use in the ELISA, while for the CATT whole blood can be substituted for serum (Holland et al., 2002; Hopkins et al., 1998). It is vitally important that serological tests be validated and standardised if they are to be suitable for correctly identifying infected animals; cross evaluation in different laboratories is thus required. Standard criteria for interpreting the tests might have to be developed for each animal species and standardised at least at a regional level (Desquesnes, 1997c). It is also necessary to take into consideration the various Trypanosoma species and strains (RoTat versus non-RoTat for example) present in a given area.

a) Indirect immunofluorescent antibody test (IFAT)

Although the technique is not adapted to large-scale surveys, it is still useful to screen a small number of samples in laboratories that are carrying out the test for other purposes and/or that are not carrying out the ELISA. Cost of reagents is medium, around 0.5€/test, but the technique is time consuming.

- Test procedure

The antigen consists of dried blood smears containing from five to ten T. evansi per field at magnification 500×, collected from a highly parasitaemic mouse or rat (3–4 days post-infection). Smears are dried at room temperature for 1 hour and fixed with acetone (± ethanol) for 5 minutes. When kept dry, the fixed smears may be stored at –20°C for several months. Better results are obtained using purified trypanosomes separated from the rat’suffy coat on a DEAE-cellulose column (Lanham & Godfrey, 1970) using a mixture of 80% cold acetone and 0.25% formalin in a normal saline solution.

On testing, the slides are first subdivided into several circles of 5 mm diameter with nail varnish using mounting media (Teflon-coated multispot slides may also be used), then washed in PBS, pH 7.2, at room temperature for 10 minutes.

After washing, a positive and a negative control serum and field sera to be tested (diluted 1/50 in PBS), are added and allowed to react at 37°C for 30 minutes in a humid chamber. The slides are washed three successive times in PBS for 5 minutes each. A rabbit or goat anti-bovine IgG (for tests on bovine sera) conjugated to fluorescein isothiocyanate or other fluorescein-conjugated antiserum specific to the animal species tested is then added at a suitable dilution and left at 37°C for 30 minutes in a humid chamber. The slides are rewashed in PBS, mounted with 50% glycerol in PBS with immunofluorescence mounting media, and examined by fluorescence microscopy. The glycerol solution should be stored at 4°C and renewed every 2 weeks.

The fluorescein conjugate should be stored at –20°C in small aliquots to avoid repeated freezing and thawing. The tube should be shielded from light in some way, for example by wrapping in aluminium foil. The conjugate is diluted in PBS, pH 7.2, or in PBS containing Evans blue 1/1000 (w/v) as a counterstain to facilitate discrimination between positive (green) and negative (red) fluorescence. In general, monospecific anti-IgG (gamma-chain) conjugates give the most specific results.

The IFAT-T. evansi seroconversion can take 60–90 days (Jacquiet et al., 1993). Compared with the CATT, IFAT is more sensitive, probably because it can detect aparasitaemic animals, but specificity is lower (Dia et al., 1997b). In borderline cases, the interpretation is subjective and reproducibility has sometimes been questioned (Ferenc et al., 1990). For these reasons, ELISA is a more advisable technique.

b) Enzyme-linked immunosorbent assay (ELISA)

The principle of this technique is that specific antibodies to trypanosomes can be detected by enzyme-linked anti-immunoglobulins using solid-phase polystyrene plates coated with soluble antigen. The enzyme may be peroxidase, alkaline phosphatase or any other suitable enzyme. The enzyme conjugate binds to the antigen/antibody complex and then reacts with a suitable substrate to yield a characteristic colour change either of the substrate itself or of an added indicator (the chromogen).

The antigen for coating the plates is derived from the blood of a heavily parasitaemic rat. The trypanosomes are concentrated in the buffy coat by centrifugation and separated on a DEAE-cellulose column and washed three times by centrifugation in cold PSG, pH 8 (PBS with 1% glucose). The final pellet is suspended in cold PSG to a concentration of 5%, together with a protease inhibitor cocktail subjected to five freeze–thawing cycles, and ultrasonicated three times for 2 minutes on ice to ensure complete disintegration of the organisms. This preparation is centrifuged at 4°C and 14,000 g for 10 minutes. The supernatant is collected.
and the protein concentration estimated by UV readings at 260 and 280 nm (Warburg & Christian, 1942). The soluble antigen thus obtained can be stored in small aliquots at −80°C for several months. It can also be freeze-dried and stored at −20°C. Coating of the ELISA plate is generally made with 5 µg/ml protein concentration in coating buffer.

- **Test procedure**
  i) Dilute the soluble antigen at 5 µg/ml in freshly prepared 0.01 M carbonate/bicarbonate buffer, pH 9.6. Add 100 µl to each well of a 96-well microtitre plate and incubate overnight at 4°C or for 1 hour at 37°C. For this step immunoplates that ensure that the specific activities of the epitopes are preserved during binding to the plate surface, are preferred to other plates that may allow epitopes to be obscured or impaired due to the binding characteristics.
  ii) Remove antigen and add 150 µl of blocking buffer (BB: 0.01 M PBS containing 0.1% Tween 20 and 5% skim milk powder for 1 hour at 37°C. The quality of the skim milk is very critical; optimal skim milk concentration may vary from 0.5 to 7% depending on the skim milk origin. Bovine serum albumin may also be used as blocking agent.
  iii) Add test serum dilutions in BB (100 µl), in duplicate or triplicate. Include control negative and positive sera. Dilutions must be determined empirically, but are usually around 1/100–1/200. Incubate plates at 37°C for 30 minutes. Eject contents and wash five times with washing buffer (PBS-0.1% Tween 20).
  iv) Add a specific peroxidase conjugated species-specific anti-globulin (100 µl) appropriately diluted in BB (usually between 1/5000 and 1/20,000). If species-specific conjugates are not available, protein A or protein G conjugates can be used. Incubate the plates at 37°C for 30 minutes, eject contents and wash three times with washing buffer.
  v) For peroxidase conjugates a number of substrate/chromogen solutions can be used, consisting of hydrogen peroxide with a chromogen, such as tetramethylbenzidine (TMB), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) or ortho-diphenylenediamine (OPD). A suitable substrate/chromogen solution for peroxidase conjugates is 30% hydrogen peroxide (0.167 ml and 35 mg) in citrate buffer (100 ml), pH 6.0. The citrate buffer is made up as follows: Solution A (36.85 ml): (0.1 M citric acid [21.01 g/litre]); Solution B (65.15 ml): (0.2 M, Na 2HPO 4 [35.59 g/litre]); and distilled water (100 ml). Dissolve 10 mg TMB in 1 ml dimethyl sulphoxide and add to 99 ml of the citrate buffer. A number of these combinations are available commercially in ready-to-use formulations that remain stable at 4°C for up to 1 year. Add the substrate chromogen (100 µl) to the plates and incubate at room temperature for 20–30 minutes.
  vi) Read the plates or stop the reaction by adding 50 µl 1 M sulphuric acid. Read the absorbance of each well at 450 nm for TMB chromogen. Other chromogens may require the use of a different wavelength. All tests should include three known high and medium positive control sera, three low and medium negative control sera, and a buffer control. Results are expressed in relative percentage of positivity (RPP) based on the optical densities of the control samples (Desquesnes, 1997c; Desquesnes et al., 2009).

A large variety of other test procedures exists, for example, using purified native antigen (Verloo et al., 1998) or, more recently, using recombinant antigens (Tran et al., 2009). For closely related animal species, cross-reacting reagents may often be used (e.g. anti-bovine immunoglobulin for buffalos) and the use of monospecific anti-IgG conjugates is generally recommended. However, when specific conjugates are not available, nonspecific proteins able to fix on the Fc fragment of the immunoglobulins can be used, such as protein A (for detection of IgG) or protein G (for detection of IgM). Protein A conjugate has been validated for use in camels (Desquesnes et al., 2009).

There is a number of methods that can be used to determine a cut-off point to discriminate between positive and negative results. The simplest method is to base the cut-off on visual inspection of the test results from known positive and negative populations (Desquesnes, 1997c). These results are likely to show some overlap. The operator can choose the most appropriate point to modify the false-positive or false-negative results depending on the required application of the assay. An alternative is to base the cut-off on the mean + 2 standard deviations (SD) or + 3 SD values from a large sample of negative animals. Finally, if no suitable negative/positive samples are available, a cut-off can be based on the analysis of the data from animals in an endemic situation (Greiner et al., 1994). If a bimodal distribution separates infected from uninfected animals, then an appropriate value can be selected. The ELISA is likely to correctly identify uninfected animals (while the CATT would correctly qualify infected ones). A new ELISA/RoTat 1.2 based on the VSG from a *Trypanosoma evansi* RoTat 1.2 clone – a predominant antigen in *Trypanosoma evansi* (Verloo et al., 2001) – was successfully used in the field in Vietnam (Holland et al., 2002; Verloo et al., 2000); protocols are available.

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4 For example Polysorp Nunc® immunoplates
5 For example: ref. 190-12865, Wako Pure Chemical Industries Ltd, Osaka, Japan.
from the OIE Reference Laboratory at ITM (Antwerp) for use in equines, camelidae and water buffaloes. Another test based on invariant surface glycoprotein has recently been developed at the ITM (Tran et al., 2009) and should proceed to inter-laboratory evaluation.

The VSGs may be too specific to be used as antigen in a universal test (see below RoTat versus non-RoTat parasites), while the ELISA using soluble antigens is not strain specific and this qualifies it as a universal test. Soluble antigens from whole lysate of *T. evansi* are able to detect immunoglobulins directed against *T. evansi* strains present in various host species and geographical areas (Laha & Sasmal, 2008); they can also detect infections in heterologous systems owing to strong cross reactions with *T. vivax*, *T. congolense* and even *T. cruzi*. *Trypanosoma evansi* soluble antigen must then be considered as a universal reagent for detection of *T. evansi*, but consideration must be given to species specificity in multispecies areas. The cost of reagents is low, around 0.1€/test, and the technique is fast, allowing 500–1000 samples to be tested a day by experienced technicians.

c) Card agglutination tests

It is well known that certain predominant variable antigen types (VATs) are expressed in common in different strains of salivarian trypanosomes from different areas. This finding was used as a basis for a test for the diagnosis of *T. evansi*, the card agglutination test – CATT/*T. evansi*. The test makes use of fixed and stained trypanosomes of a defined VAT known as RoTat 1.2. Both variable and invariable surface antigens take part in the agglutination reaction. The CATT is available in kit form from the OIE Reference Laboratory ITM. It consists of lyophilised stained parasites ('antigen'), PBS, pH 7.4, plastic-coated cards, spatulas, positive and negative control sera and a rotator. The lyophilised antigen can be stored at 2–8°C for up to 1 year. Reconstituted antigen can be stored at 2–8°C for 1 week, but preferably should be used within 8 hours when kept at 37°C.

For screening, dilute test sera 1/4 or 1/8 in PBS on to circles inscribed on the plastic cards. Add 45 µl of the prepared antigen suspension (previously well shaken to homogenise the parasite suspension) onto circles inscribed on the plastic cards. Add 25 µl of each test serum. Mix and spread the reagents with a spatula and rotate the card for 5 minutes using the rotator provided in the kit (or at 70 rpm on a classical rotor agitator). Compare the pattern of agglutination with the illustrations of different reactions provided in the kit. Blue granular deposits reveal a positive reaction visible to the naked eye. The cost of reagents is medium, around 0.5€/test, around 200 tests can be carried out a day by one technician.

As the CATT principally detects IgM (agglutinating pentavalent immunoglobulins the half-life of which is short), it is suitable for detection of early infections or late infections with recent circulation of parasites in the blood, and can detect active infections with a high positive predictive value. The CATT is more likely to classify correctly truly infected animals, it can be used to target individual animals for treatment with trypanocidal drugs.

An alternative test format (LATEX/*T. evansi*) using latex beads coated with native RoTat 1.2 VSG is currently under evaluation.

d) Immune trypanolysis test

Immune trypanolysis test detects specific 'trypanolytic' antibodies directed against a given parasitic strain able to induce trypanolysis in the presence of complement. It is performed with *T. evansi* variable antigen type RoTat 1.2 and may therefore be positive only with hosts that produce trypanolytic immunoglobulins directed against RoTat 1.2 VAT (Van Meirvenne et al., 1995). Sera are tested at a 1/4 dilution. Live trypanosomes are incubated for 60 minutes with test serum in the presence of guinea-pig serum as the source of complement. When variant specific antibodies are present in the serum, lysis of the RoTat 1.2 trypanosomes occurs. The sample is considered positive for the presence of anti-RoTat 1.2 antibodies when 50% or more of the trypanosomes are lysed. This test requires the growth of trypanosomes in rodents and is thus costly. At present, it is mostly used to confirm samples suspected to be positive using other tests. It can be carried out at the ITM, Antwerp, on request. The cost of the test is very high (250€/test).

### 3. Test applications

Like the majority of biological tests, the methods described above are limited both in terms of sensitivity and specificity. Moreover, test performances and parameters are highly variable, depending on the host species or the geographical area in which the host occurs. To date, there is no common test (parasitological, serological or even molecular) that is capable of distinguishing *T. evansi* from the other *Trypanozoon* species or sub-species. The final diagnosis of surra will depend on epizoootiological information and laboratory results and observations. For these reasons, a number of test combinations adapted to the different circumstances relevant to a particular host...
species are currently recommended. These are guidelines that should be helpful in achieving the correct diagnoses.

**a) Recommended methods**

i) **Microscopic examination:** Microscopic observation (×400–1000 in oil immersion) of a Giemsa-stained thin blood smear from the host, or from a mouse inoculation test, allows identification of the subgenus *Trypanozoon* based on morphology and morphometry of parasites. When fresh samples are available, HCT or BCM must be used to increase the sensitivity.

ii) **PCR-TBR:** DNA must be prepared from blood with a commercial kit or phenol chloroform method, using a buffy coat obtained by 8000 g centrifugation of 0.5 ml of blood. PCR is carried out as described above, with TBR primers (Masiga *et al.*, 1992). Result is positive for *Trypanozoon* when a 177 bp product is visible on the agarose gel.

iii) **ELISA T. evansi:** Serum or plasma samples are tested in ELISA-*T. evansi* (soluble antigens from whole *T. evansi* lysate) as described above. A sample is positive when its RPP is > than the cut off value established for the host species (appropriate conjugates are defined for each species; see (d) below).

iv) **CATT/T. evansi:** Serum or plasma are diluted 1:4 and tested as described by the manufacturer. Positive samples are samples presenting results = or > to one + (doubtful samples are considered as negative samples).

**b) Equids**

An equid is negative to surra if it is negative to ELISA-*T. evansi* (anti-horse IgG whole molecule), CATT/*T. evansi*, PCR-TBR and microscopic examination.

An animal is considered to be infected by *Trypanozoon* spp. if it is positive to PCR-TBR and/or if *Trypanozoon* parasites are observed by microscopic examination.

An animal is considered as seropositive to surra if it is positive to ELISA-*T. evansi* and/or CATT/*T. evansi*; in this case, the animal should be tested for CFT-dourine, and, if it is positive for CFT-dourine it is also considered as seropositive to dourine; if it is negative to CFT-dourine, it is considered as seropositive to surra only.

**c) Camelids**

An animal is negative to surra if it is negative to ELISA-*T. evansi* (protein A conjugate), CATT/*T. evansi*, PCR-TBR and microscopic examination.

An animal is considered as infected by *Trypanozoon* spp. if it is positive to PCR-TBR and/or microscopic examination.

An animal is considered as seropositive to surra if it is positive to ELISA-*T. evansi* and/or CATT/*T. evansi*.

**d) Other host species**

An animal is negative to surra if it is negative to ELISA-*T. evansi* (conjugate see below), CATT/*T. evansi*, PCR-TBR and microscopic examination.

An animal is considered as infected by *Trypanozoon* spp. if it is positive to PCR-TBR and/or microscopic examination.

An animal is considered as seropositive to surra if it is positive to ELISA-*T. evansi* and/or CATT/*T. evansi*.

Conjugates to be used for each host species: Cattle and buffalo: anti-bovine IgG whole molecule; Pig and elephant: Protein G conjugate; Camels: Protein A conjugate; Rat: anti-rat IgG whole molecule. Conjugates remain to be defined for other host species.

**C. REQUIREMENTS FOR VACCINES**

No vaccines are available for this disease.
REFERENCES


Chapter 2.1.17. — Trypanosoma evansi infection (surra)


Chapter 2.1.17. — Trypanosoma evansi infection (surra)


Chapter 2.1.17. — Trypanosoma evansi infection (surra)


Chapter 2.1.17. – Trypanosoma evansi infection (surra)


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NB: There are OIE Reference Laboratories for Trypanosoma evansi infection (surra) (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).

Please contact the OIE Reference Laboratories for any further information on diagnostic tests and reagents for surra.
CHAPTER 2.1.18.

TULAREMIA

SUMMARY

Tularemia is a zoonosis caused by Francisella tularensis. The causative bacterium is a Gram-negative coccoid rod, 0.2–0.5 µm × 0.7–1.0 µm, non-motile and non-spore-forming organism that is an obligate aerobe with optimal growth at 37°C. It is oxidase-negative, weakly catalase-positive and cysteine is required for growth. It occurs naturally in lagomorphs (rabbits and hares), and in rodents, especially microtine rodents (such as voles, vole rats and muskrats), and beavers. A wide range of other mammals and several species of birds have also been reported to be infected. Among domestic animals, the cat seems to be able to act as a carrier of the bacterium.

Two types of F. tularensis are recognised on the basis of cultural characteristics, epidemiology, and virulence in some hosts. Tularemia is largely confined to the Northern Hemisphere and is not normally found in the tropics or the Southern Hemisphere. Francisella tularensis subsp. tularensis (Type A) is associated with lagomorphs in North America. It is transmitted primarily by ticks and biting flies, is highly virulent for humans and domestic rabbits, and most of the isolates ferment glycerol. Francisella tularensis subsp. palaearctica (Type B) occurs mainly in aquatic rodents (beavers, muskrats) in northern North America, and in hares and small rodents in Eurasia. It may be water- or arthropod-borne, is less virulent to humans and rabbits, and does not ferment glycerol. In addition to vector transmission, tularemia may be spread contact with infected animals or environmental fomites by inhalation, or by ingestion of the poorly cooked flesh of infected animals or contaminated water.

The disease is characterised by fever, depression and septicaemia. In humans, there may be ulcers or abscesses at the site of inoculation (this is rarely seen in animals), and swelling of the regional lymph nodes. On post-mortem examination, lesions may include caseous necrosis of lymph nodes and multiple greyish-white foci of necrosis in the spleen, liver, bone marrow and lungs. The spleen is usually enlarged.

It is important to understand that there is a high risk of direct infection of humans by direct contact with this organism. Special precautions, including the wearing of gloves, masks and eyeshields, are therefore recommended when handling infective materials. The facility should meet the requirements for Containment Group 3 pathogens (see Chapter 1.1.3 Biosafety and Biosecurity in the veterinary microbiological laboratory and animal facilities).

Identification of the agent: The bacterium can be demonstrated in impression smears or in fixed specimens of organs, such as liver, spleen, bone marrow, kidney and lung, as well as in blood smears. Immunological methods, such as the fluorescent antibody test (FAT) are the most reliable way to identify the bacterium. With Gram staining, the bacteria appear as very small punctiform Gram-negative rods, often difficult to distinguish as bacteria. They can also be stained with May–Grunwald–Giemsa or phenol thionin.

The organism is highly fastidious. For growth it is necessary to use Francis medium, McCoy and Chapin medium, or Modified Thayer-Martin agar. The colonies are small, round and transparent, and do not appear before 48 hours incubation at 37°C. On Francis medium, the colonies may be confluent and have a milky appearance. If transportation is necessary, samples should be inoculated into sterile nutrient broth and stored at 4–10°C for a few hours or at −70°C if transit is likely to be prolonged.

In the past mice or guinea-pigs were experimentally inoculated with infected tissue material or with cultures to aid in the diagnosis of tularemia. Animal inoculation has been replaced by polymerase
chain reaction (PCR) protocols for the identification of F. tularensis. The FAT demonstrates F. tularensis in pathological specimens.

**Serological tests:** Serological tests are useful diagnostic aids in human infection, but are of limited value in the more susceptible animal species that usually die before developing antibodies. Epidemiological surveys can be conducted in domestic animals in relatively resistant species that survive the infection, such as sheep, cattle, pigs, dogs, cats and wild ungulates, as these species develop antibodies. Also relatively resistant species of rodents and lagomorphs can also be used in epidemiological surveys. Serological surveys can and are conducted in a number of wildlife species, such as moose, that are exposed to F. tularensis.

**Requirements for vaccines and diagnostic biologicals:** A live attenuated vaccine strain is available for vaccination of humans at high risk of exposure to virulent F. tularensis. However, the vaccine is only available for restricted use. The outcome of vaccination can be estimated by the demonstration of specific antibodies and the ability of lymphocytes to proliferate in the presence of F. tularensis antigen.

### A. INTRODUCTION

Tularemia is a zoonosis caused by *Francisella tularensis*. It occurs naturally in lagomorphs (rabbits and hares) and rodents, especially microtine rodents such as voles, vole rats and muskrats, as well as in beavers. In addition, a wide variety of other mammals, birds, amphibians and invertebrates have been reported to be infected (Pearson, 1998; Phahler-Jung, 1989). Tularemia occurs endemically in the Northern Hemisphere. The disease can occur as epizootic outbreaks in many countries in North America and Europe, while it occurs only as sporadic cases in some other countries in Europe and Asia. It is rarely reported from the tropics or the Southern Hemisphere. Several epizootic outbreaks have been reported as a result of importation of subclinically infected lagomorphs.

The clinically most relevant two types of *F. tularensis* are recognised on the basis of culture characteristics, epidemiology, and virulence. *Francisella tularensis* subsp. *tularensis* (Type A) is mainly associated with lagomorphs in North America. It is primarily transmitted by ticks or biting flies, or by direct contact with infected lagomorphs. It is highly virulent for humans and domestic rabbits, and most isolates ferment glycerol. *Francisella tularensis* subsp. *palaearctica* (Type B) occurs mainly in aquatic rodents (beavers, muskrats) and voles in northern North America, and in lagomorphs (hares) and rodents in Eurasia. It is primarily transmitted by direct contact or by mosquitoes, but may be transmitted through inhalation or through infected water or food. It is less virulent for humans and domestic rabbits, and does not ferment glycerol (Bell, 1980; Ellis et al., 2002; Markowitz et al., 1985; Mollaret & Bourdin, 1972; Morner & Addison, 2001; Sandström et al., 1992).

In sensitive animals, clinical signs of severe depression are followed by a fatal septicaemia. The course of the disease is approximately 2–10 days in susceptible species, and animals are usually dead when presented for diagnosis. Most domestic species do not usually manifest signs of tularemia infection, but they do develop specific antibodies to the organism following infection. Outbreaks with high mortality caused by the Type A organism have occurred in sheep (Bell, 1980; Morner & Addison, 2001). Among domestic animals, the cat has been reported to be able to act as a carrier of the bacterium (Eliasson et al., 2002) and the disease is occasionally spread from cats to humans (Feldman, 2003).

At necropsy, animals that have died from acute tularemia are usually in good body condition. There are signs of septicaemia characterised by whitish foci of necrosis randomly distributed in the liver, bone marrow and spleen. In addition, the spleen is usually enlarged. Necrotic foci vary in size, and in some cases may be barely visible to the naked eye. The lungs are usually congested and oedematous, and there may be areas of consolidation and fibrinous pneumonia or pleuritis. Fibrin may be present in the abdominal cavity. Foci of caseous necrosis are often present in one or more lymph node(s). The lymph nodes that are most often affected are those in the abdominal and pleural cavities and lymph nodes draining the extremities. In less sensitive species, the histological picture can resemble that of tuberculosis with chronic granulomas in liver, spleen, lungs and kidneys.

There is a high risk of human infection from *F. tularensis*, as the infective dose is extremely low and infected animals excrete bacteria in urine and faeces. Infection can occur by simple contact. Suitable precautions, such as the wearing of gloves, masks and eyeshields during any manipulation of pathological specimens or cultures, must be taken in order to avoid human infection. The facility should meet at least the requirements for Containment Group 3 pathogens as outlined in Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities*. Countries lacking access to such a specialised national or regional laboratory
should send specimens to the OIE Reference Laboratory. Experimentally inoculated animals and their excreta are especially hazardous to humans.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Francisella tularensis can be demonstrated in smear preparations or in histological sections. It can also be identified by culture or animal inoculation. However, F. tularensis may be difficult to isolate from dead animals and carcasses due to overgrowth of other bacteria. As the post-mortem picture is variable, diagnosis is sometimes difficult and immunological or immunohistochemical methods are preferable, although reagents may be difficult to obtain. It can sometimes be recommended, therefore, that fixed specimens be analysed at laboratories equipped with proper reagents or methods.

a) Smear preparations

Smear preparations are made on microscope slides as impression smears of organs such as the liver, spleen, bone marrow, kidney, lung or blood. The bacteria are abundant in such smears, but may be overlooked because of their very small size (0.2–0.7 µm). The bacteria can be demonstrated by direct or indirect fluorescent antibody staining. This is a safe, rapid and specific diagnostic tool (Karlsson et al., 1970; Morner, 1981; Morner et al., 1988).

Gram staining of smears reveals a scattering of small, punctiform Gram-negative bacteria near the limit of visibility. The use of oil microscopy increases the visibility of the bacteria. The bacteria may be difficult to distinguish from precipitates of stain.

b) Histological sections

Bacteria can be demonstrated in sections using immunohistochemical methods, such as the fluorescent antibody test (FAT) (Morner, 1981). The test is normally performed on specimens from liver, spleen or bone marrow, fixed in neutral buffered formalin and paraffin embedded. Slides are treated with rabbit anti-tularemia serum, washed and thereafter treated with sheep fluorescein-isothiocyanate-conjugated anti-rabbit serum. The samples are examined under a fluorescence microscope. Large numbers of bacteria can be seen in necrotic lesions and in the blood.

c) Culture

Francisella tularensis will not grow on ordinary media, although an occasional strain can sometimes, on initial isolation, grow on blood agar. Incubation is at 37°C in ambient air or in 5% CO₂. Heart blood, liver, spleen and bone marrow from moribund animals should be used for culture. It is necessary to use special culture media, such as:

i) Francis medium: Peptone agar containing 0.1% cystine (or cysteine) and 1% glucose, to which is added, before solidification, 8–10% defibrinated rabbit, horse or human blood.

ii) McCoy and Chapin medium: This consists of 60 g egg yolk and 40 ml normal saline solution, carefully mixed and coagulated by heating to 75°C.

iii) Modified Thayer–Martin agar: Glucose cysteine agar (GCA)-medium base supplemented with haemoglobin and Iso VitaleX.

Media can be stored for up to 8–10 days at 4°C. Colonies that form on McCoy medium are small, prominent, round and transparent. A more abundant growth is obtained on Francis medium and modified Thayer–Martin agar, with confluent colonies that have a milky appearance and a mucoid consistency. On either medium, colonies do not appear until after 48 hours’ incubation at 37°C.

iv) GCA agar with thiamine (BBL): When used with added blood, the medium is commonly referred to as GBCA and can be substituted for the original, noncommercial medium described by Down et al. (1947). Suspend 58 g of the dry material in 1 litre of distilled or demineralised water, and mix thoroughly. Heat with frequent agitation and boil for 1 minute. Dispense into tubes and sterilise by autoclaving at 118–121°C for 15 minutes.

For larger volumes (up to several litres) of culture medium, autoclave at the same temperatures for 30 minutes. Cool to 45–48°C. Aseptically add 25 ml of packed human blood cells or 50 ml of defibrinated rabbit or sheep blood. Mix thoroughly and pour into plates. Incubate at 37°C for 24 hours before use to decrease surface moisture and to test for sterility (Down et al., 1947).
The following selective medium can be used in addition to the non-selective media: **Cystine heart agar (DIFCO)** with 5% rabbit blood, and penicillin (100,000 units), Polymyxin B sulphate (100,000 units), and cycloheximide (0.1 ml of a 1% stock solution) per litre.

Differential criteria for the identification of *F. tularensis* include absence of growth on ordinary media, distinctive cellular morphology, and specific fluorescent antibody and slide agglutination reactions. The bacteria are nonmotile, nonsporulating, bipolar staining, and of uniform appearance in 24-hour cultures, but pleomorphic in older cultures.

*Francisella tularensis* can be identified in stained smears, by agglutination with tularemia hyperimmune antiserum, or by animal inoculation. In areas of North America where both types of *F. tularensis* may occur, Type A may be distinguished from Type B by the fact that most Type A ferment glycerol.

The bacteria can also be identified by hybridisation with probes specific to the 16S rRNA of *F. tularensis*, *F. tularensis* Type A, and *F. tularensis* Type B (Forsman *et al*., 1990), or by polymerase chain reaction (PCR) with primers targeting specific regions of the 16 rDNA molecules. The PCR will allow identification at the genus, species and subspecies level (Forsman *et al*., 1995).

d) **Capillary tube precipitation test on pathological samples**

Tissues, such as spleen, liver or bone marrow, are ground with sterile sand in three-to-five times their volume of normal saline. The suspension is transferred to a tube and two volumes of ethyl ether are added. After shaking, the mixture is allowed to stand for 4–5 hours at room temperature. It is again shaken and then allowed to stand overnight.

The aqueous phase is drawn off and centrifuged at 2000 *g* for 30 minutes. The supernatant fluid, containing the antigen, is drawn off and distributed into capillary tubes to which tularemia antiserum is added.

The tubes are incubated at 37°C for 3 hours, then kept at 4°C overnight. A positive result is the formation of a ring of precipitate.

e) **Animal inoculation**

Animal inoculation is extremely hazardous and is only recommended for agent identification in cases when culture is negative and agent identification is needed for epidemiological reasons. It should only be undertaken where proper biosafety facilities and cages are available (see chapter 1.1.3). PCR techniques should be used for identification of *F. tularensis*.

Laboratory animals (preferably mice) are inoculated with culture material to confirm the nature of an isolate. Pathological specimens may be inoculated for the direct detection of *F. tularensis*. Inoculated mice usually die before lesions can form.

Intraperitoneal injection is sufficient for passage of pure cultures. All routes of administration in mice, such as subcutaneous, percutaneous, or intravenous, will lead to an infection that is invariably fatal within 2–10 days.

f) **Molecular techniques**

The PCR has recently been developed for the diagnosis of *F. tularensis* and is an excellent method for identifying the agent in wound specimens from humans (Johansson *et al*., 2000).

A real-time PCR has proven to be a highly sensitive and specific assay and is a major diagnostic improvement, as all other methods for the specific identification of *F. tularensis* subsp. *tularensis* are very time-consuming (Tamoso *et al*., 2007).

A DNA microarray has also been developed that is capable of distinguishing the highly virulent subspecies *F. tularensis* subsp. *tularensis* and the moderately virulent subspecies *F. tularensis* subsp. *holarctica* (Broekhuijsen *et al*., 2003).

2. **Serological tests**

Serology is currently carried out for diagnosis of tularemia in humans, but is of limited value in sensitive animal species, which usually die before specific antibodies can develop. Serology may be employed, either on sera or on lung extracts (Morner *et al*., 1988), in epidemiological surveys of animals that are resistant to infection, such as sheep, cattle, pigs, moose, dogs or birds (Morner *et al*., 1988; Phahler-Jung, 1989). As there is no antigenic difference between Type A and Type B, the less virulent *F. tularensis palaeartica* could be used as antigen in all serological tests.
Chapter 2.1.18. — Tularemia

a) Tube agglutination

The most commonly used serological test is the tube agglutination test. The antigen is a culture of *F. tularensis* on Francis medium. The culture is harvested after 5–6 days. Younger cultures yield a poorer antigen. The colonies are suspended in 96% alcohol, giving a thick suspension that can be stored for 1–7 days at room temperature. The sediment is washed with normal saline and resuspended in an equal volume of normal saline. Crystal violet powder is added to a final concentration of 0.25%. The bacteria are stained by adding crystal violet and incubating at 37°C for at least 24 hours and at most 7 days.

After the supernatant fluid has been discarded, the deposit is suspended in normal saline with or without thimerosal (merthiolate) at a final concentration of 1/10,000, or formaldehyde at a final concentration of 0.5%. The suspension is calibrated with positive and negative sera, and adjusted by adding normal saline to provide an antigen that when tested on a slide gives readily visible stained agglutination reactions against a clear fluid background.

The test is performed in tubes containing a fixed amount of antigen (0.9 ml) and different dilutions of serum commencing with 1/10, 1/20, 1/40, etc. The results are read after 20 minutes of shaking, or after 1 hour in a water bath at 37°C followed by overnight storage at room temperature. The agglutinated sediment is visible to the naked eye or, preferably, by using a hand lens. The positive tubes are those that have a clear supernatant fluid. Possible cross-reactions with *Brucella abortus*, *B. melitensis* and *Legionella* sp. have to be taken into consideration.

b) Enzyme-linked immunosorbenet assay

Another serological test, the enzyme-linked immunosorbenet assay (ELISA), also allows an early diagnosis of tularemia (Carlsson *et al*., 1979). This method is now widely used for clinical purposes. Different antigens, whole bacteria as well as subcellular components (Fulop *et al*., 1991), have been used as recall antigens against immunoglobulines IgA, IgM and IgG; 2 weeks after the onset of tularemia, specific antibodies can be detected in the serum. IgM is sustained for a long period and cannot be used as an indication of a recent infection (Bevanger *et al*., 1994). For routine diagnosis, whole heat-killed (65°C for 30 minutes) bacteria can be used as antigen. Bacteria can be coated to plastic plates, using the usual procedures (Carlsson *et al*., 1979) followed by serial dilutions of serum to be tested. Positive reactions can be visualised by anti-antibodies labelled with enzyme. The test should also be read in a photometer with positive and negative sera as controls.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Vaccines have been produced with the aim of protecting humans, but as all vaccine development involves testing in animals, it is clear that some of the vaccines could be used to protect animals. However, in most countries, there is no vaccine licensed for use in animals.

Before 1940, attempts to vaccinate against tularemia were performed by use of whole killed bacteria or bacterial extracts. None of these vaccines induced protection against highly virulent strains of *F. tularensis*. Instead, viable attenuated vaccines were developed. Attenuation was performed by repeated culture of bacteria on various media with or without antiserum. Such live attenuated vaccines have been used in mass vaccinations of people in the former Soviet Union since 1946, either as monocultures or as a mixture of strains.

An attenuated live vaccine strain of *F. tularensis* biovar palaearctica is available and can be used for restricted vaccination of individuals at high risk (Sandström, 1994).

REFERENCES


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CHAPTER 2.1.19.
VESICULAR STOMATITIS

SUMMARY

Vesicular stomatitis (VS) is a vesicular disease of horses, cattle and pigs caused by vesiculoviruses of the family Rhabdoviridae. This disease is clinically indistinguishable from foot and mouth disease (FMD), vesicular exanthema of swine (VES), or swine vesicular disease (SVD) when horses are not involved. Sheep, goats and many other wild species can be infected. Humans are also susceptible. The disease is limited to the Americas; however, it was previously described in France and in South Africa.

Virus is transmitted directly by the transcutaneous or transmucosal route and has been isolated from sandflies and mosquitoes. Experimental transmission has been shown from black flies to both pigs and cattle. There is seasonal variation in the occurrence of VS: it disappears at the end of the rainy season in tropical areas, and at the first frosts in temperate zones. There is also some evidence that it could be a plant virus and that animals are the end of the epidemiological chain. The pathogenesis of the disease is unclear, and it has been observed that the humoral-specific antibodies do not always prevent infection with VS serogroup viruses.

Although VS may be suspected when horses are involved as well as pigs and cattle, prompt differential diagnosis is essential because the clinical signs of VS are indistinguishable from FMD when cattle and pigs are affected, and from SVD or VES when only pigs are affected.

Identification of the agent: Virus can be readily isolated by the inoculation of several tissue culture systems, unweaned mice or embryonated chicken eggs. Viral RNA can be detected from epithelial tissue and vesicular fluid by conventional and real-time reverse transcriptase polymerase chain reaction (PCR). Viral antigen can be identified by an indirect sandwich enzyme-linked immunosorbent assay (IS-ELISA) – this is the least expensive and most rapid test. The complement fixation (CF) test is also a good alternative. The virus neutralisation (VN) test may be used, but it is elaborate and time-consuming.

Serological tests: Convalescent animals develop serotype-specific antibodies within 4–8 days of infection that are demonstrated by a liquid-phase blocking ELISA (LP-ELISA), a competitive ELISA (C-ELISA) and VN. Other described tests are CF, agar gel immunodiffusion and counter immunoelectrophoresis.

Requirements for vaccines: Inactivated virus vaccines with aluminium hydroxide or oil as adjuvants have been tested in the United States of America and in Colombia, respectively. Both vaccines generated high levels of specific antibodies in the sera of vaccinated cattle. However, it is not yet clear if serum antibodies would prevent the disease. An attenuated virus vaccine has been used in the field with unknown efficacy.

A. INTRODUCTION

Vesicular stomatitis (VS) was described in the United States of America (USA) by Oltsky et al. (1926) and Cotton (1927) as a vesicular disease of horses, and subsequently of cattle and pigs. Vesicles are caused by virus on the tongue, lips, buccal mucosa, teats and in the coronary band epithelium of cattle, horses, pigs, and many other species of domestic and wild animals. Natural disease in sheep and goats is rare, although both species can be experimentally infected. Mixed infections of foot and mouth disease (FMD) and VS viruses have occurred in the same herds of cattle and can be induced experimentally. Many species of laboratory animals are also susceptible. The disease is limited to the Americas; however, it was described in France (1915 and 1917) and in South Africa (1886 and 1897) (Hanson, 1952).
Influenza-like signs, normally without vesicles, have been observed in humans who are in contact with animals with VS or who handle infective virus. All manipulations involving virus, including infective materials from animals, should be undertaken with using proper biosafety procedures.

There are two distinct immunological classes of vesicular stomatitis virus (VSV) that have been recognised: New Jersey (NJ) and Indiana (IND). Both viruses are members of the genus Vesiculovirus, family Rhabdoviridae and have been extensively studied at the molecular level. Several other closely related rhabdoviruses have been isolated from sick animals over the past decades. There are three subtypes of the IND serogroup based on serological relationships: IND-1 IND-2 and IND-3; they are also known as classical IND virus (VSV), cocal virus (COCV), and alagoas virus (VSAV), respectively (Federer et al., 1987). Strains of the serotype NJ and subtype IND-1 are endemic in livestock in areas of southern Mexico, Central America, Venezuela, Colombia, Ecuador and Peru, with VSV NJ causing the vast majority (>80%) of the clinical cases. Sporadic activity of NJ and IND-1 VSV has been reported in northern Mexico and western United States. IND-2 has only been isolated in Argentina and Brazil and only from horses (Salto-Argentina/63, Maipú-Argentina/86, Rancharia-Brazil/66, Riberao-Brazil/79) (Alonso et al., 1991; Alonso Fernandez & Sondahl, 1985). Cattle living together with the affected horses did not develop antibodies against VSV (Alonso et al., 1991). The IND-3 subtype, (Alagoas-Brazil/64), has been identified, sporadically only in Brazil and only in horses until 1977. However, in 1977 the IND-3 serotype (Espinosa-Brazil/77 strain) was first isolated from cattle in Brazil; it has been observed that this serotype affects cattle to a lesser degree than horses (Alonso et al., 1991; Alonso Fernandez & Sondahl, 1985). This finding confirms the first descriptions, in 1926 and 1927 (Cotton, 1927; Olitsky et al., 1926), of the NJ and IND serotypes in horses, and subsequently in cattle and pigs; this same predilection has been observed in other VS outbreaks.

The mechanism of transmission of the virus is unclear. The viruses have been isolated from sandflies, mosquitoes, and other insects (Comer et al., 1992; Francy et al., 1988; Mason, 1978). Experimental transmission of VS NJ has been demonstrated to occur from black flies (Simulium vittatum) to domestic swine and cattle (Mead et al., 2004; 2009) There are also hypotheses that the VS virus is a plant virus present in pasture (Mason, 1978) and that animals are the end of the epidemiological chain and, in special circumstances, the virus could undergo an adaptation process to infect animals, followed by direct transmission between susceptible animals. During the 1982 epizootic in western USA, there were a number of cases where there was direct transmission from animal to animal (Sellers & Maarouf, 1990). While VS is not diagnosed in livestock every year in the USA, it is considered to be endemic in feral pigs on Ossabaw Island, Georgia (Boring & Smith, 1962).

The incidence of disease can vary widely among affected herds. Usually 10–15% of the animals show clinical signs. Clinical cases are mainly seen in adult animals. Cattle and horses under 1 year of age are rarely affected. Mortality is close to zero in both species. However, high mortality rates in pigs affected by the NJ virus have been observed. Sick animals recover in about 2 weeks. The most common complications of economic importance are mastitis and loss of production in dairy herds (Lauerman et al., 1962). Both NJ and IND-1 serotypes in the 1995, 1997 and 1998 US outbreaks primarily caused clinical disease in horses. Although some clinical signs were observed in cattle, the primary finding in cattle was seroconversion.

### B. DIAGNOSTIC TECHNIQUES

VS cannot be reliably clinically differentiated from the other vesicular diseases, such as foot and mouth disease (FMD), vesicular exanthema of swine (VES), and swine vesicular disease (SVD) when horses are not involved. An early laboratory diagnosis of any suspected VS case is therefore a matter of urgency.

The sample collection and technology used for the diagnosis of VS must be in concordance with the methodology used for the diagnosis of FMD, VES and SVD, in order to facilitate the differential diagnosis of these vesicular diseases. Note: VS serogroup viruses can be human pathogens and appropriate precautions should be taken when working with potentially infected tissues or virus (see Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

Vesicle fluid, epithelium covering unruptured vesicles, epithelial flaps of freshly ruptured vesicles, or swabs of the ruptured vesicles are the best diagnostic samples. These samples can be collected from mouth lesions, as well as from the feet and any other sites of vesicle development. It is recommended that animals should be sedated before samples are collected to avoid injury to helpers and for reasons of animal welfare. Samples from all species should be placed in containers of Tris-buffered tryptose broth with phenol red, pH 7.6. If complement fixation (CF) is to be carried out for antigen detection, samples from all species can be collected in glycerol/phosphate buffer, pH 7.2–7.6. (Note: glycerol is toxic to virus and decreases the sensitivity of virus isolation; it is therefore only recommended for collection of samples for CF test.) Samples should be kept refrigerated and if they can arrive at the laboratory within 48 hours after collection, they should be sent refrigerated. If samples are sent frozen with dry ice, precautions should be taken to protect the sample from contact with any CO₂. There are special packaging requirements for shipping samples with dry ice (see Chapter 1.1.1 Collection and shipment of diagnostic specimens, for further information on shipping of diagnostic samples.)
When epithelial tissue is not available from cattle, samples of oesophageal–pharyngeal (OP) fluid can be collected by means of a probang (sputum) cup. In pigs, throat swabs can be taken for submission to a laboratory for virus isolation. This material should be sent to the laboratory refrigerated in Tris-buffered tryptose broth. If the samples will be in shipment for more than 48 hours after collection, they should be sent frozen with dry ice as described previously. Probang samples for isolation of virus should not be treated with solvents such as chloroform. Virus can be isolated from oral and nasal specimens up to 7 days post-infection.

When it is not possible to collect samples for identification of the agent, serum samples from recovered animals can be used for detecting and quantifying specific antibodies. Paired sera from the same animals, collected 1–2 weeks apart, are preferred for checking the change in antibody titre.

Specific reagents for VS diagnosis are not commercially available and each laboratory must produce its own or obtain them from a Reference Laboratory. The two OIE Reference Laboratories for vesicular stomatitis (see Table given in Part 4 of this Terrestrial Manual), and the Institute for Animal Health¹, produce and distribute diagnostic reagents on request.

1. Identification of the agent

For identification of VS serogroup viruses and the differential diagnosis of vesicular diseases, clarified suspensions of field samples suspected to contain virus should be submitted for immunological testing. For virus isolation, the same samples are inoculated into appropriate cell cultures. The inoculation of African green monkey kidney (Vero), baby hamster kidney (BHK-21) and IB-RS-2 cell cultures with the same sample permits differentiation of the vesicular diseases: VS serogroup viruses cause a cytopathic effect (CPE) in all three cell lines; FMD virus causes a CPE in BHK-21 and in IB-RS-2, while SVD virus causes a CPE in IB-RS-2 only. Many other cell lines, as well as most primary cell cultures of animal origin, are susceptible to VS serogroup viruses.

Virus replicates and can be isolated in 8–10-day-old chicken embryos by inoculation into the allantoic sac, in 2- to 7-day-old unweaned mice by inoculation using any route, or in 3-week-old mice by intracerebral inoculation. In all three cases, virus causes death in between 2 and 5 days after inoculation.

The most susceptible route for horses and cattle is intradermal lingual administration. Pigs are inoculated in the coronary band or on the snout. Vesicular lesions may be observed in the epithelial tissues of the mouth, teats and feet, 2–4 days after inoculation. The presence of secondary vesicles after inoculation of cattle and horses depends mainly on the VS virus isolate used. The snout is normally affected in pigs.

If a CPE develops in the cultures, the suspension fluids can be used for identification of the agent by different immunological tests and the cell culture can be stained with VS-specific fluorescent antibody conjugate and viral antigen detected by enzyme-linked immunosorbent assay (ELISA), complement fixation (CF) test or polymerase chain reaction (PCR). Similar tests can be performed on homogenate suspensions of the dissected musculoskeletal tissues of dead mice and chicken embryos and with suspensions of epithelial samples. The brain tissue from mice is an excellent source of virus.

Due to the different morphological characteristics of the rhabdovirus (VS serogroup viruses), picornavirus (FMD virus and SVD virus), calicivirus (VES) and the large number of virus particles present in vesicular fluids and epithelial tissues, electron microscopy can be a useful diagnostic tool for differentiating the virus family involved.

The preferred immunological methods for the identification of the viral antigens in the laboratory are the ELISA (Alonso et al., 1991; Ferris & Donaldson, 1988), the CF test (Alonso et al., 1991; Jenny et al., 1958) and fluorescent antibody staining. The virus neutralisation (VN) test, with known positive antisera against the VS virus NJ and IND serotypes, may be used in tissue cultures, unweaned mice or embryonated eggs, but it is more time-consuming.

a) Virus isolation

i) Inoculate cell culture in Leighton tubes and 25 cm² flasks with the clarified suspension of tissues or vesicular fluid.

ii) Incubate inoculated cell cultures at 37°C for 1 hour.

iii) Discard inoculum and wash cell cultures three times with cell culture medium and replace with cell culture medium containing 2.5% fetal bovine serum (FBS).

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Chapter 2.1.19. – Vesicular stomatitis

iv) Incubate Leighton tube cell cultures at 33–35°C and observe for CPE.

v) After 18–24 hours of incubation, the cover-slip from one Leighton tube culture per specimen inoculated is stained with New Jersey and Indiana VS virus-specific fluorescent antibody (FA) conjugate.

vi) Remaining Leighton tube cultures and 25 cm² flask cultures are incubated at 35–37°C for 6 more days and observed daily for CPE.

vii) At 7 days post-inoculation, the remaining Leighton tube cover-slips are stained with FA conjugate.

viii) If CPE is observed and the FA staining is negative, a second passage as is made, as described above, using the cells from the 25 cm² flask. Note: First passage cultures with significant CPE may yield false-negative immunofluorescence results. Serial tenfold dilutions may be prepared and inoculated to provide distinct plaques of fluorescing cells.

ix) Interpretation of the results: If no fluorescence is observed and no CPE evident in the flask culture, the sample is negative for virus isolation. If specific fluorescence is observed, the sample is positive for virus isolation.

x) Alternatively cell culture in flasks can be inoculated with field samples, incubated at 35–37°C for 48 hours and observed daily for CPE. If no CPE is observed after 48 hours, the flask cultures are frozen and thawed and a sample of the supernatant is inoculated into fresh cell culture. Up to three passages are made, of 48 hours each. To detect the presence of VSV antigen, clarified supernatants of each passage are tested by ELSA or CF test.

b) Enzyme-linked immunosorbent assay

The indirect sandwich ELISA (IS-ELISA) (Alonso et al., 1991; Ferris & Donaldson, 1988) is currently the diagnostic method of choice for identification of viral serotypes of VS and other vesicular diseases. Specifically, the ELISA procedure with a set of polyvalent rabbit/guinea-pig antisera, prepared against virions of the representative strains of the three subtypes of the IND serotype, identifies all strains of the VS virus IND serotype (Alonso et al., 1991). For detection of VS virus NJ strains, a monovalent set of rabbit/guinea-pig antisera is suitable (Alonso et al., 1991; Ferris & Donaldson, 1988).

• Test procedure

  i) Solid phase: ELISA plates are coated either for 1 hour at 37°C or overnight at 4°C with rabbit antisera and normal rabbit serum (as described in Alonso et al., 1991 and Allende et al., 1992), and optimally diluted in carbonate/bicarbonate buffer, pH 9.6. Subsequently, the plates are washed once with phosphate buffered saline (PBS) and blocked for 1 hour at room temperature with 1% ovalbumin in PBS. The plates are used immediately or are washed three times and stored at −20°C for future use.

  ii) Test samples: Antigen suspensions of test samples (10–20% epithelial tissue suspension, musculoskeletal tissue of chicken embryo or mice in PBS or undiluted clarified cell culture supernatant fluid) are deposited in the corresponding wells and the plates are incubated for 1 hour at 37°C on an orbital shaker.

  iii) Detector: Monovalent and polyvalent guinea-pig antisera to VS virus NJ and IND serotypes, respectively, that are homologous to coated rabbit serum and that have been diluted appropriately in PBS containing 0.05% Tween 20, 1% ovalbumin, 2% normal rabbit serum, and 2% normal bovine serum (PBSTB) are added to the corresponding wells and left to react for 30–60 minutes at 37°C on an orbital shaker.

  iv) Conjugate: Peroxidase/rabbit or goat IgG anti-guinea-pig Ig conjugate, diluted in PBSTB, is added and left to react for 30–60 minutes at 37°C on an orbital shaker.

  v) Substrate: H₂O₂-activated substrate is added and left to react at room temperature for 15 minutes, followed by the addition of sulphuric acid to stop the reaction. Absorbance values are measured using an ELISA reader.

Throughout the test, 50 µl reagent volumes are used. The plates are washed five times between each stage with PBS containing 0.05% Tween 20. Controls for the reagents used are included.

vi) Interpretation of the results: An antiserum giving an absorbance more than 20% greater than the other antisera, negative serum and controls is considered to be positive for the corresponding virus subtype.

c) Complement fixation test

The ELISA is preferable to the CF test because it is more sensitive and it is not affected by pro- or anti-complementary factors. When ELISA reagents are not available, however, the CF test may be performed. The CF test in U-bottomed microtitre plates, using the reagents titrated by CF50% test, is described.
Chapter 2.1.19. – Vesicular stomatitis

Test procedure

i) **Antisera:** Guinea-pig monovalent anti-NJ VS virus and polyvalent anti-IND VS virus, diluted in veronal buffer (VB) at a dilution containing 2.5 CFU₅₀ (50% complement fixation units) against homologous virus, are deposited in plate wells. Those antisera are the detectors used in ELISA.

ii) **Test samples:** The antigen suspension of test samples, prepared as described for IS-ELISA, is added to the wells with serum.

iii) **Complement:** 4 CHU₅₀ (50% complement haemolytic units) are added to the serum and antigen. (An alternative is to use 7.5, 10 and 20 CHU₅₀ with the goal of reaching 4 CHU₅₀ in the test.) The mixture of antisera, test samples and complement is incubated at 37°C for 30 minutes.

iv) **Haemolytic system:** A suspension of sheep red blood cells (SRBC) in VB, sensitised with 10 HU₅₀ (50% haemolytic units) of rabbit anti-SRBC serum, is added to the wells. The haemolytic system has an absorbance of 0.66 read at 545 nm, in the proportion of two volumes of haemolytic system + three volumes of distilled water. The mixture is incubated for 30 minutes at 37°C. Subsequently, the plates are centrifuged and the reaction is observed visually.

Volumes of 25 µl for antisera, test samples and complement, and 50 µl of haemolytic system, are required. Appropriate controls for the antisera, antigens, complement and haemolytic system are included.

It is possible to perform the CF50% test in tubes (Alonso et al., 1991) using reagent volumes eight times greater than those indicated for the CF in microtitre plates. With the CF50% test, the reaction can be expressed as absorbance read spectrophotometrically at 545 nm.

v) **Interpretation of the results:** When controls are as expected, samples with haemolysis <20% for one antiserum in comparison with the other antiserum and controls are considered to be positive for the corresponding type.

Field samples that are negative on the ELISA or CF test should be inoculated into cell culture or unweaned mice. If there is no evidence of viral infection after three passages, the specimen is considered to be negative for virus.

d) **Nucleic acid recognition methods**

The PCR can be used to amplify small genomic areas of the VS virus (Hofner et al., 1994; Rodriguez et al., 1993; Wilson et al., 2009). This technique will detect the presence of virus RNA in tissue and vesicular fluid samples and cell culture, but cannot determine if the virus is infectious. In general, PCR techniques have not been routinely used for screening diagnostic cases for viruses causing VS.

2. **Serological tests**

For the identification and quantification of specific antibodies in serum, the ELISA and the VN test are preferable. The CF test may be used for quantification of early antibodies. Antibody can usually be detected between 5 and 8 days post-infection; the length of time antibody persists has not been accurately determined for the three tests but is thought to be relatively short for the CF and for extended periods for the VN and ELISA (Katz et al., 1997).

a) **Enzyme-linked immunosorbert assay (a prescribed test for international trade)**

The liquid-phase blocking ELISA (LP-ELISA) is a method for the detection and quantification of antibodies to VS serogroup viruses. The use of viral glycoproteins as antigen is recommended because they are not infectious, allow the detection of neutralising antibodies, and give fewer false-positive results than the VN (Allende et al., 1992).

• **Test procedure**

i) **Solid phase:** As described above in Section B.1.a for the IS-ELISA.

ii) **Liquid phase:** Duplicate, twofold dilution series of each test serum, starting at 1/4, are prepared in U-bottomed microtitre plates. An equal volume of VS virus NJ or IND glycoprotein, in a dilution providing 70% reaction, is added to each well and the plates are incubated for 1 hour at 37°C. 50 µl of these mixtures is then transferred to the ELISA plates with the solid phase and left to react for 30 minutes at 37°C on an orbital shaker.

iii) **Detector, conjugate and substrate:** The same reagents and methods are used as those indicated for the IS-ELISA.
iv) **Interpretation of the results:** 50% end-point titres are expressed in log_{10} in reference to the 50% reduction of negative serum control, according to the Spearmann–Kärber method. Titres of >1.0 (1/10) are considered to be positive.

### Competitive enzyme-linked immunosorbent assay (a prescribed test for international trade)

A competitive ELISA for detection of antibodies has also been developed. The procedure described here is based on a procedure described by Afshar *et al.* (1993). It uses vesicular stomatitis NJ and IND-1 recombinant antigens as described by Katz *et al.* (1995).

#### Test procedure

i) **Solid phase:** Antigens are diluted in carbonate/bicarbonate buffer, pH 9.6, and 75 µl is added to each well of a 96-well ELISA plate. The plates are incubated overnight at 4°C; coated plates can be frozen at −70°C for up to 30 days. The plates are thawed, antigen is decanted, and 100 µl of blocking solution (5% nonfat dry milk powder solution in PBS [for example, 5 g dry milk powder dissolved in 95 ml PBS]) is added. The plates are then incubated at 25°C for 15–30 minutes and blocking solution is decanted. The plates are washed three times with PBS/0.05% Tween 20 solution.

ii) **Liquid phase:** 50 µl of serum diluted 1/8 in 1% nonfat dry milk in PBS is added to each of the duplicate wells for each sample. A positive and negative control serum for each serotype should be included on each ELISA plate. The plates are incubated at 37°C for 30 minutes. Without washing, 50 µl of polyclonal ascites fluid is added to each well and plates are incubated at 37°C for 30 minutes.

iii) **Detector:** The plates are washed three times, and 50 µl of goat anti-mouse horseradish-peroxidase conjugate diluted in 1% nonfat dry milk with 10% normal goat serum is added to each well. The plates are incubated at 37°C for 30 minutes, washed three times, and 50 µl of tetramethyl-benzidine (TMB) substrate solution is added to each well. The plates are incubated at 25°C for 5–10 minutes and then 50 µl of 0.05 M sulphuric acid is added to each well. The plates are read at 450 nm and the optical density of the diluent control wells must be > 1.0.

iv) **Interpretation of the results:** A sample is positive if the absorbance is ≤50% of the absorbance of the diluent control. Note that horses naturally infected with New Jersey virus have been known to test positive by this assay for at least 5 years following infection.

### Virus neutralisation (a prescribed test for international trade)

The VN test is carried out in tissue culture microtitre plates with flat-bottomed wells using inactivated serum as test sample, 1000 TCID_{50} (50% tissue culture infective dose) of VS NJ or IND virus, and Vero M cells, or preformed monolayer (Allende *et al.*, 1992) or a suspension IB-RS-2 cells to test for the presence of unneutralised virus.

#### Test procedure

i) **Virus:** VS NJ or IND virus is grown in Vero cell monolayers and stored in liquid nitrogen or frozen at −70°C.

ii) **Test samples:** Sera are inactivated at 56°C for 30 minutes before testing. Positive and negative control standard sera are included in the test.

iii) **Virus neutralisation:** Sera are diluted in a twofold or four-fold dilution series across the plates, starting from 1/4 dilution. Two rows of wells are used per serum. The same volume of NJ or IND VS virus suspension containing about 1000 TCID_{50}/25 µl is added and incubated at 37°C for 60 minutes to allow neutralisation to take place. Subsequently, 50 µl of the mixtures is deposited on preformed cell monolayers in microtitre plates or 150 µl of 300,000/ml IB-RS-2 or Vero cell suspension is added to each well with the serum/virus mixtures. The plates are covered with loosely fitting lids and incubated for 48–72 hours at 37°C in an atmosphere of 5% CO_{2} or sealed with pressure-sensitive tape and incubated in a normal atmosphere. (It has been determined that a virus titre of 1000 TCID_{50} will decrease the nonspecific reactions and maintain a high test sensitivity.)

iv) **Interpretation of the results:** Wells without CPE are considered to be positive. End-point titres of test serum titres are determined by the Spearmann–Kärber method when the virus titres are between 750 and 1330 TCID_{50} and when titres of positive and negative standard sera are within twofold of their mean values as estimated from previous titration. The 100% neutralisation titres of each serum are expressed at log 10. Sera with values of 1/32 or greater are considered to be positive for antibodies against VSV. Note that horses naturally infected with New Jersey virus have been known to test positive by this test method for at least 5 years following infection. In an alternative protocol, the end-point titre of the test serum is determined when the virus doses are between 10^{2.55}±0.5/100 µl and when titres of positive and negative standard sera are within twofold of their mean values as estimated from
the previous titration. The 50% neutralisation titre of each serum is expressed as log 10. Sera with values of 1.3 (1/20) or greater are considered to be positive for VS antibodies (Allende et al., 1992).

c) Complement fixation (a prescribed test for international trade)

A detailed description of this test is given in Section B.1.b. This is modified as follows. The CF test may be used for quantification of early antibodies, mostly IgM. For this purpose, twofold serum dilutions are mixed with 2 CFU50 of known antigen and with 5% normal bovine or calf sera included in 4 CHU50 of complement. The mixture is incubated for 3 hours at 37°C or overnight at 4°C. Subsequently, the haemolytic system is added followed by incubation for 30 minutes at 37°C. The serum titre is the highest dilution in which no haemolysis is observed. Titres of 1/5 or greater are considered to be positive. This CF has low sensitivity and is frequently affected by anticomplementary or nonspecific factors.

C. REQUIREMENTS FOR VACCINES

1. Background

a) Rationale and intended use of the product

Vesicular stomatitis virus infections can have significant impacts on the health and production aspects of animals, resulting in considerable economic losses for producers. Reduced feed intake caused by oral lesions can result in weight loss and delays to market. Lesions on the feet can cause temporary locomotor problems affecting the ability of an animal to obtain food and water, and permanent foot problems that result in the animal being culled. Lesions of the mammary gland can impact the ability of the dam to nurse her offspring and for harvesting milk for sale. Animals may be culled if mammary or teat lesions are severe. Where vaccination is practised, vaccine is used to reduce the severity of clinical signs and the economic impacts of the disease.

Attenuated virus vaccines have been tested in the field in the USA, Panama, Guatemala, Peru and Venezuela (Lauerman et al., 1962; Mason, 1978) with unknown efficacy. Killed vaccines for the Indiana and New Jersey serotypes are manufactured in Colombia and Venezuela (2002 OIE vaccine survey).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed

i) Biological characteristics

Identity of the seed and the source of the serum used in growth and passage of the virus should be well documented, including the source and passage history of the organism.

ii) Quality criteria (sterility, purity, freedom from extraneous agents)

The purity of the seed and cells to be used for vaccine production must be demonstrated. The master seed virus (MSV) should be free from adventitious agents, bacteria, or Mycoplasma, using tests known to be sensitive for detection of these microorganisms. The test aliquot should be representative of a titre adequate for vaccine production, but not such a high titre that hyperimmune antisera are unable to neutralise seed virus during purity testing. Seed virus is neutralised with monospecific antiserum or monoclonal antibody against the seed virus and the virus/antibody mixture is cultured on several types of cell line monolayers. A cell line highly permissive for bovine viral diarrhoea virus, types 1 and 2, is recommended as one of the cell lines chosen for evaluation of the MSV. Bovine viral diarrhoea virus is a potential contaminant introduced through the use of fetal bovine serum in cell culture systems.

Cultures are subpassaged at 7-day intervals for a total of at least 14 days, then tested for adventitious viruses that may have infected the cells or seed during previous passages.

b) Method of manufacture

i) Procedure

Once the vaccine is shown to be efficacious, and the proposed conditions for production are acceptable to regulatory authorities, approval may be granted to manufacture vaccine. Virus seed can be grown in cell culture. Selection of a cell type for culture is dependent on the degree of virus
adaptation, growth in medium, and viral yield in the specific culture system. Vaccine products should be limited to the number of passages from the MSV that can be demonstrated to be effective. Generally, large-scale monolayer or suspension cell systems are operated under strict temperature-controlled, aseptic conditions and defined production methods, to assure lot-to-lot consistency. Dose of virus used to inoculate cell culture should be kept to a minimum to reduce the potential for viral defective interfering particles. When the virus has reached its appropriate titre, as determined by CPE, fluorescent antibody assay, or other approved technique, the virus is clarified, filtered, and inactivated (for killed vaccines).

ii) Requirements for substrates and media

Cell cultures should be demonstrated free of adventitious viruses. All animal origin products used in the production and maintenance of cells (i.e. trypsin, fetal bovine sera) and growth of virus should be free of adventitious agents, with special attention paid to the presence of bovine viral diarrhoea virus.

iii) In-process controls

Cell cultures should be checked macroscopically for abnormalities or signs of contamination and discarded if unsatisfactory. Virus concentration can be assessed using antigenic mass or infectivity assays.

An inactivation kinetics study should be conducted using the approved inactivating agent on a viral lot with a titre greater than the maximum production titre and grown using the approved production method. This study should demonstrate that the inactivation method is adequate to assure complete inactivation of virus. Samples taken at regular timed intervals during inactivation, then inoculated on to a susceptible cell line, should indicate a linear and complete loss of titre by the end of the inactivation process.

During production, antigen content is measured to establish that minimum bulk titres have been achieved. Antigen content is generally measured before inactivation (if killed vaccine) and prior to further processing.

iv) Final product batch tests

Vaccine candidates should be shown to be pure, safe, potent, and efficacious.

Sterility and purity

During production, tests for bacteria, Mycoplasma, and fungal contamination should be conducted on both inactivated and live vaccine harvest lots and confirmed on the completed product (see Chapter 1.1.7 Tests for sterility and freedom from contamination of biological materials).

Safety

Final container samples of completed product from inactivated vaccines should be tested.

Batch potency

Relative potency can be used to determine antigen content in final product. It is necessary to confirm the sensitivity, specificity, reproducibility, and ruggedness of such assays.

c) Requirements for authorisation

i) Safety requirements

Target and non-target animal safety

Final product may be evaluated in the host animal using two animals of the minimum age recommended for use, according to the instructions given on the label; the animals are observed for 21 days. Field safety studies conducted on vaccinates, in at least three divergent geographical areas, with at least 300 animals per area, are also recommended.

For killed and modified live virus (MLV) vaccines product safety will be based on an absence of adverse reactions such as shock, abscesses at site of inoculation, etc. In the specific case of MLV vaccines, it would not be expected to see clinical signs. If clinical signs of vesicular stomatitis virus are observed, use of the vaccine should be reconsidered. Residual virus should be evaluated for prior to mixing the antigen with adjuvant. Initial safety is evaluated in a few animals for 21 days under close observation to assess for gross safety issues. If the vaccine passes this first safety test, the vaccine is used in the field in a larger number of animals to evaluate if subtle safety issues are present: adverse reactions/swelling, abscesses, shock, etc.

Reversion-to-virulence for attenuated/live vaccines

Reversion to virulence for live viral vaccines is often demonstrated by back passage through susceptible species. Virus is isolated from the vaccinated animal and the isolated virus is then used to inoculate additional animals. Sequential passage through animals should show that animals remain clinically healthy with no demonstration of typical vesicular stomatitis lesions.
Chapter 2.1.19. – Vesicular stomatitis

Environmental consideration

Inactivated vesicular stomatitis vaccines probably present no special danger to the user, although accidental inoculation may result in an adverse reaction caused by the adjuvant and secondary components of the vaccine. Modified live virus vaccines may pose a hazard to the user depending on the level of inactivation of the virus.

Preservatives should be avoided if possible, and where not possible, should be limited to the lowest concentration possible. Vaccine bottles, syringes, and needles may pose an environmental hazard for vaccines using adjuvants or preservatives and for modified live virus vaccines. Instructions for disposal should be included within the vaccine packaging information and based on current environmental regulations in the country of use.

ii) Efficacy requirements

For animal production

Virus(es) used in vaccine production should be antigenically relevant to virus(es) circulating in the field. A vaccination/challenge study in the species for which the vaccine will be used will indicate the degree of protection afforded by the vaccine. Species used in vaccination/challenge studies should be free of antibodies against vesicular stomatitis. Vaccination/challenge studies should be conducted using virus produced by the intended production method, at the maximum viral passage permitted, and using an experimental animal model. It is necessary to confirm the sensitivity, specificity, reproducibility, statistical significance and confidence level of such experimental model.

Antibody levels after vaccination measured in vitro could be used to assess vaccine efficacy provided a statistically significant correlation study has been made. For vaccines containing more than one virus (for example, New Jersey and Indiana-1), the efficacy of the different components of these vaccines must each be established independently and then as a combination in case interference between different viruses exists.

The duration of immunity and recommended frequency of vaccination of a vaccine should be determined before a product is approved. Initially, such information is acquired directly using host animal vaccination/challenge studies. The period of demonstrated protection, as measured by the ability of vaccinates to withstand challenge in a valid test, can be incorporated into claims found on the vaccine label.

If the vaccine is to be used in horses, swine, cattle, or other ruminants destined for market and intended for human consumption, a withdrawal time consistent with the adjuvant used (generally 21 days) should be established by such means as histopathological examination submitted to the appropriate food safety regulatory authorities.

For control

The same principles apply as for animal production usage. In addition, it should be noted that antibody responses in vaccinated animals may not be differentiated from animals exposed to field virus. Therefore, vaccinated animals will need to be clearly identified if serological methods will be used in conjunction with compatible clinical signs to assess field virus exposure.

iii) Stability

Vaccines should be stored at 4–8°C, with minimal exposure to light. The shelf life should be determined by use of the approved potency test (Section C.5.b) over the proposed period of viability.

3. Vaccines based on biotechnology

a) Vaccines available and their advantages

None

b) Special requirements for biotechnological vaccines, if any

None

REFERENCES


* * *

**NB:** There are OIE Reference Laboratories for Vesicular stomatitis (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for vesicular stomatitis. 


**CHAPTER 2.1.20.**

**WEST NILE FEVER**

**SUMMARY**

West Nile virus (WNV) is a member of the genus Flavivirus in the family Flaviviridae. The arbovirus is maintained in nature by cycling through birds and mosquitoes; numerous avian and mosquito species support virus replication. For many avian species, WNV infection causes no overt signs while other birds, such as American crows (Corvus brachyrhynchos) and Blue Jays (Cyanocitta cristata), often succumb to fatal systemic illness. Among mammals, clinical disease is primarily exhibited in horses and humans.

Clinical signs of WNV infection in horses arise from viral-induced encephalitis or encephalomyelitis. Infections are dependent on mosquito transmission and are seasonal in temperate climates, peaking in the early autumn in the Northern Hemisphere. Affected horses frequently demonstrate mild to severe ataxia. Signs can range from slight incoordination to recumbency. Some horses exhibit weakness, muscle fasciculation, and cranial nerve deficits. Fever is not a consistently recognised feature of the disease in horses.

**Identification of the agent:** Bird tissues generally contain higher concentrations of virus than equine tissues. Brain and spinal cord are the preferred tissues for virus isolation from horses. In birds, kidney, heart, brain, liver or intestine can yield virus isolates. Cell cultures (using, for example, rabbit kidney or Vero cells) are used most commonly for virus isolation. WNV is cytopathic in susceptible culture systems. Viral nucleic acid and viral antigens can be demonstrated in tissues of infected animals by reverse-transcription polymerase chain reaction (RT-PCR) and immuno-histochemistry, respectively. The most sensitive method for identifying WNV in equine tissues is a nested format of the RT-PCR procedure.

**Serological tests:** Antibody can be identified in equine serum by IgM capture enzyme-linked immunosorbent assay (IgM capture ELISA), haemagglutination inhibition (HI), IgG ELISA or plaque reduction neutralisation (PRN). The ELISA and PRN methods are most commonly used for identifying antibody against WNV in avian serum. In some serological assays, antibody cross-reactions with related flaviviruses, such as St Louis encephalitis virus, Japanese encephalitis virus, or tick-borne encephalitis (TBE) virus may be encountered.

**Requirements for vaccines and diagnostic biologicals:** A formalin-inactivated WNV vaccine derived from tissue culture, WNV live canarypoxvirus vectored vaccine, a WNV DNA vaccine and a chimeric vaccine are licensed for use in horses.

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**A. INTRODUCTION**

West Nile virus (WNV) is a zoonotic mosquito-transmitted arbovirus belonging to the genus *Flavivirus* in the family *Flaviviridae* (Smithburn et al., 1940). The genus *Flavivirus* also includes Japanese encephalitis virus (see chapter 2.1.7), St Louis encephalitis virus, Murray Valley virus, Usutu virus, and Kunjin virus, among others (Burke & Monath, 2001). WNV has a wide geographical range that includes portions of Europe, Asia, Africa, Australia (Kunjin virus) and in North, Central and South America. Migratory birds are thought to be primarily responsible for virus dispersal, including reintroduction of WNV from endemic areas into regions that experience sporadic outbreaks (Burke & Monath, 2001). WNV is maintained in a mosquito–bird–mosquito transmission cycle, whereas humans and horses are considered dead end hosts. Genetic analysis of WN isolates separates strains into two clades. Lineage 1 isolates are found in northern and central Africa, Israel, Europe, India, Australia (Kunjin virus) and in North and Central America, and Columbia and Argentina in South America (Morales et al., 2006). Lineage 2 strains are endemic in central and southern Africa and Madagascar, with co-circulation of both virus lineages in central Africa (Berthet et al., 1997; Burt et al., 2002). There has been a recent report of lineage 2 from Hungary.
While recent human and equine outbreaks have been due to lineage 1 viruses, strains from each lineage have been implicated in human and animal disease.

WNV was recognised as a human pathogen in Africa during the first half of the 20th century. Although several WNV fever epidemics were described, encephalitis as a consequence of human WN infection was rarely encountered prior to 1996, but since then, outbreaks of human West Nile encephalitis have been reported from Romania, Russia, Israel, North America, France, and Tunisia (Bin et al., 2001; Del Giudice et al., 2004; Hayes, 2001; Hubalek & Halouzka, 1999; Zeller & Schuffenecker, 2004). During the 1960s, West Nile viral encephalitis of horses was reported from Egypt and France (Panthier et al., 1966; Schmidt & El Mansoury, 1983). Since 1998, outbreaks of equine WNV encephalitis have been reported from France, Italy, Canada, United States of America, Israel and Morocco (Cantile et al., 2000; Hayes et al., 2005; Murgue et al., 2001; Ostlund et al., 2000). In the Western Hemisphere, the virus range has dramatically expanded from a discrete region along the East Coast of New York State to include the contiguous States of the United States of America (USA), Canada, Mexico, the Caribbean islands, Central America, Argentina, Columbia and Venezuela (Davis et al., 2005; Morales et al., 2006; Ostlund et al., 2000; USDA, 2010). Other than in the United States and Canada, the introduction of West Nile virus in the Western Hemisphere has not been characterised by large disease outbreaks or significant mortality in any species, possibly because of exposure to indigenous flaviviruses circulating in these regions.

The fleeting viraemia of low virus titre precedes clinical onset (Bunning et al., 2002; Schmidt & El Mansoury, 1963). WN viral encephalitis occurs in only a small per cent of infected horses; the majority of infected horses do not display clinical signs (Ostlund et al., 2000). The disease in horses is frequently characterised by mild to severe ataxia. Additionally, horses may exhibit weakness, muscle fasciculation and cranial nerve deficits (Cantile et al., 2000; Ostlund et al., 2000; 2001; Snook et al., 2001). Fever is an inconsistently recognised feature. Treatment is supportive and signs may resolve or progress to terminal recumbency. The mortality rate is approximately one in three clinically affected horses. Differential diagnoses in horses include other arboviral encephalidites (e.g. eastern, western or Venezuelan equine encephalomyelitis, Japanese encephalitis), equine protozoal myelitis (Sarcocystis neurona), equine herpesvirus-1, Borna disease and rabies.

Most species of birds can become infected with WNV; the clinical outcome of infection is variable. Chickens and turkeys, are resistant to disease, Outbreaks of fatal neurologic disease have been reported in zoo birds in the USA and in domestic geese in Israel and Canada (Austin et al., 2004). WNV has been associated with sporadic disease in small numbers of other species, including squirrels, chipmunks, bats, dogs, cats, white-tailed deer, reindeer, sheep, alpacas, alligator and a harbour seal during intense periods of local viral activity. Most human infections occur by natural transmission from mosquitoes, but laboratory acquired infections have been reported. In clinically suspicious cases, diagnostic specimens from all animals, particularly birds, should be handled at containment level 3 following appropriate laboratory procedures (see Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities) (Richmond & McKinney, 1999). There has been confirmed transmission of WNV in humans by blood transfusion, organ transfer and breast milk.

Due to the occurrence of inapparent WNV infections, diagnostic criteria must include a combination of clinical assessment and laboratory tests.

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

a) **Culture**

Specimens for virus isolation include brain and spinal cord from encephalitic horses (Ostlund et al., 2000; 2001); a variety of bird tissues including brain, heart or liver may be used with success (Steele et al., 2000); WHV has been isolated from kidney but the tissue may be toxic to cell culture. In general, virus isolates are obtained more easily from avian species. Virus may be propagated in susceptible cell cultures, such as rabbit kidney (RK-13) and African green monkey kidney (Vero) cells, or embryonating chicken eggs. Intracerebral inoculations of newborn mice are less likely to yield virus isolates from mammalian tissues than cell culture methods. More than one cell culture passage may be required to observe cytopathic effect (CPE). Confirmation of WNV isolates is achieved by indirect fluorescent antibody staining of infected cultures or nucleic acid detection methods (see below).

b) **Immunological methods**

Immunohistochemical (IHC) staining of formalin-fixed avian tissues is a reliable method for identification of WNV infection in birds. Brain, heart, kidney, spleen, liver, intestine, and lung are often IHC-positive tissues in infected birds. The success rate of IHC detection in positive birds is enhanced by the examination of multiple tissues. The specificity of identification (e.g. flavivirus specific or WNV specific) depends on the selection of
detector antibody. The brain and spinal cord tissues of horses with WN viral encephalitis are inconsistently positive in IHC tests; approximately 50% of equine encephalitis cases yield false-negative results. Failure to identify WNV antigen in equine central nervous system does not rule out infection.

c) **Nucleic acid recognition methods**

Nucleic acid detection by reverse-transcription polymerase chain reaction (RT-PCR) significantly enhances the identification of WNV-infected tissues, particularly when a nested PCR approach is applied to fresh, unfixed, equine brain and spinal cord specimens (Johnson et al., 2001). The RT-nested PCR method to detect WNV nucleic acid encoding a portion of the E gene is described below. This method was developed using a 1999 North American isolate and has been successful in detecting WNV RNA in animal tissues during recent North American outbreaks. St Louis encephalitis virus is not detected by this method. Lineage 1 West Nile viruses from China (People’s Rep. of), France, Egypt, Israel, Italy, Kenya, Mexico and Russia demonstrate a highly conserved nucleotide sequence in the target region, regardless of species of origin (Lanciotti et al., 2000). Analysis of sequence information for the Uganda 1937 Lineage 2 strain (GenBank M12294) in the region targeted by the PCR primers indicate that amplification of lineage 2 strains of WNV would not be expected. Other viruses from the Japanese encephalitis serogroup have not been examined. Non-nested methods, including real-time PCR, pose less risk of laboratory cross-contamination and may be applied successfully to avian tissue samples (Lanciotti et al., 2000). A real-time RT-PCR has been described for the detection of WNV nucleic acid (Tewari et al., 2004). In order to standardise WNV molecular techniques a proficiency study based on formalin-fixed tissues was developed, administered and reported (Niedrig et al., 2006). Tissues selected for PCR are the same as those selected for virus isolation attempts.

- **Reverse-transcription nested polymerase chain reaction (RT-nPCR) procedure**

The RT-nPCR described here includes several procedures: extraction of RNA, reverse transcription to generate DNA from RNA and first stage PCR, second stage PCR using ‘nested’ primers and, finally, detection of the appropriately sized amplicon by gel electrophoresis. WNV E gene protein regions of 445 bp (base pairs) and 248 bp are amplified in the first-stage and nested procedures, respectively. The kits and reagents described below are provided as examples. Equivalent products may be available from other sources. Extreme care in handling all materials and inclusion of proper controls are essential to ensure accurate results. The precautions to be taken have been covered in Chapter 1.1.5 Principles and methods of validation of diagnostic assays for infectious diseases. Duplicate samples of each diagnostic specimen should be processed and tested to increase confidence in test results. Use appropriate precautions when handling hazardous reagents such as ethidium bromide.

- **Extraction of viral RNA**

From 50 to 100 mg of tissue, extract total RNA using Trizol® reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer’s instructions. Also extract total RNA from WNV control stock virus containing 10–100 tissue culture infective dose (TCID$_{50}$) per 100 µl volume.

- **Reverse transcription and first stage PCR**

First stage primers:
1401: 5‘-ACC-AAC-TAC-TGT-GGA-GTC-3’
1845: 5‘-TTC-CAT-CTT-CAC-TCT-ACA-CT-3’

i) Suspend extracted RNA samples in 12 µl RNase-free water.

ii) Incubate at 70°C for 10 minutes.

iii) Add 2 µl of each RNA sample to 48 µl of RT-PCR mixture containing a final composition of:
10 mM Tris/HCl, pH 8.3
50 mM KCl
2.0 mM MgCl$_2$
0.8 mM deoxynucleoside triphosphate (dNTP) pool
25 units M-MLV (Moloney murine leukaemia virus) RT
1.25 units RNase inhibitor
1.25 units AmpliTaq Gold™ (Applied Biosystems, Foster City, CA, USA)
37.5 pmol of the first stage primers.

Include ‘no RNA’ controls using 2 µl RNase-free water in place of denatured RNA.

iv) Incubate reaction tubes at 45°C for 45 minutes.

v) Incubate reaction tubes at 95°C for 11 minutes.

vi) PCR amplification through 35 cycles:
Denaturation at 95°C for 30 seconds,
Suggestions for second stage (nested) PCR

- For each sample and control, add 1.5 µl of the first-stage amplification product to 48.5 µl of PCR mixture with a final composition of:
  - 10 mM Tris/HCl, pH 8.3
  - 50 mM KCl
  - 2.0 mM MgCl₂
  - 0.8 mM deoxynucleoside triphosphate (dNTP) pool
  - 1.25 units AmpliTaq Gold™ (Applied Biosystems, Foster City, CA, USA)
  - 37.5 pmol each of the nested primers.

- Incubate reactions tubes at 95°C for 11 minutes.

- PCR amplification through 35 cycles:
  - Denaturation at 95°C for 30 seconds,
  - Primer annealing at 55°C for 45 seconds,
  - Primer extension at 72°C for 60 seconds (for the 35th cycle, primer extension at 72°C for 5 minutes).

- Hold samples at 4°C or –20°C until electrophoresis.

- Analysis of PCR products by gel electrophoresis
  - Prepare a 2.5% NuSieve® 3/1 (FMC Bioproducts, Rockland, Maine, USA) agarose solution in 0.045 mM Tris/borate, pH 8.6, 1.5 mM EDTA (ethylene diamine tetra-acetic acid) (1 × TBE buffer). Boil the agarose on a hotplate or in a microwave oven until completely dissolved. Cool the agarose to 45–55°C. Add 5.0 µl ethidium bromide solution (10 mg/ml) per 100 ml warm agarose and pour agarose gel with comb. Allow to solidify then remove comb.

- Add 30 µl ethidium bromide solution (10 mg/ml) per 600 ml 1 × TBE tank buffer. Position gel in apparatus and fill buffer tanks.

- Mix 15 µl of each sample and control with 5 µl gel loading solution (e.g. Sigma product G-2526, St Louis, MO, USA). Include 100 bp DNA ladder (e.g. Life Technologies, Grand Island, NY, USA product 15268-019, range 100–1500 bp) in at least one gel well. Load samples into agar wells and electrophorese at 65–75 volts until the gel loading dye has travelled approximately two-thirds the length of the gel.

- Visualise and photograph gel under ultraviolet illumination.

- Interpretation of the test

For the PCR test to be valid, the positive controls must show the appropriate size band (248 bp). The 'no RNA' controls should have no bands. Samples are considered to be positive if there is a band of the same size as the positive control. Duplicate samples should both show the same reaction. If there is a disparity, the test should be repeated, starting with extraction from tissue. If further validation is required, the final nested PCR product can be sequenced and compared with the published sequences of WNV from GenBank.

2. Serological tests

Antibody can be identified in equine serum by IgM capture enzyme-linked immunosorbent assay (IgM capture ELISA), hemagglutination inhibition (HI), IgG ELISA or plaque reduction neutralisation (PRN) (Beaty et al., 1989; Hayes, 1989). The IgM capture ELISA described below is particularly useful to detect antibodies resulting from recent natural exposure to WNV. Equine WNV-specific IgM antibodies are usually detectable from 7–10 days post-infection to 1–2 months post-infection. Most horses with WNV encephalitis test positive in the IgM capture ELISA at the time that clinical signs are first observed. WNV neutralising antibodies are detectable in equine serum by 2 weeks post-infection and can persist for more than 1 year. The HI and PRN methods are most commonly used for identifying WNV antibody in avian serum. In some serological assays, antibody cross-reactions with related flaviviruses, such as St Louis encephalitis virus or Japanese encephalitis virus, will be encountered. The PRN test is the most specific among WNV serological tests; when needed, serum antibody titres against related flaviviruses can be tested in parallel. Finally, WN vaccination history must be considered in
interpretation of serology results, particularly in the PRN test and IgG ELISA. IgM capture ELISA may be used to
test avian or other species provided that species-specific capture antibody is available (e.g. anti-chicken IgM).
The PRN test is applicable to any species, including birds.

a) Equine IgM capture ELISA

WNV and negative control antigens for the IgM capture ELISA may be prepared from mouse brain (see
chapter 2.5.5), tissue culture or recombinant cell lines (Davis et al., 2001). Commercial sources of WNV
testing reagents are available in North America. Characterised equine control serum, although not an
international standard, can be obtained from the National Veterinary Services Laboratories, Ames, Iowa,
USA. Virus and negative control antigens should be prepared in parallel for use in the ELISA. Antigen
preparations must be titrated with control sera to optimise sensitivity and specificity of the assay. Equine
serum samples are tested at a dilution of 1/400 and equine cerebrospinal fluid samples are tested at a
dilution of 1/2 in the assay. To ensure specificity, each serum sample is tested for reactivity with both virus
antigen and control antigen.

- Test procedure
  i) Coat flat-bottom 96-well ELISA plates (e.g. Immulon 2HB, Dynex Technologies, Chantilly, VA, USA)
     with 100 µl/well anti-equine IgM diluted in 0.5 M carbonate buffer, pH 9.6, according to the
     manufacturer’s suggested dilution for use as a capture antibody.
  ii) Incubate plates overnight at 4°C in a humid chamber. Coated plates may be stored for several weeks.
  iii) Prior to use, wash plates twice with 200–300 µl/well 0.01 M phosphate buffered saline, pH 7.2,
       containing 0.05% Tween 20 (PBST).
  iv) Block plates by adding 300 µl/well freshly prepared 5% nonfat dry milk in PBST and incubate
      60 minutes at room temperature. After incubation, remove blocking solution and wash plates three
times with PBST.
  v) Test and control sera are diluted 1/400 (cerebrospinal fluid is diluted 1/2) in PBST and 50 µl/well of
     each sample is added to duplicate sets of wells (total of four wells per sample) on the plate. Include
     control positive and negative sera prepared in the same manner as samples.
  vi) Cover the plates and incubate 75 minutes at 37°C in a humid chamber.
  vii) Remove serum and wash plates three times in PBST.
  viii) Dilute virus and negative control antigens in PBST and add 50 µl of virus antigen to one set of wells per
       test and control sera and add 50 µl normal antigen to the second set of wells per test and control sera.
  ix) Cover the plates and incubate overnight at 4°C in a humid chamber.
  x) Remove antigens from the wells and wash the plates three times in PBST.
  xi) Dilute horseradish peroxidase conjugated anti-Flavivirus monoclonal antibody\(^1\) in PBST according to
      manufacturer’s directions and add 50 µl per well.
  xii) Cover the plates and incubate at 37°C for 60 minutes.
  xiii) Remove conjugate and wash plates six times in PBST.
  xiv) Add 50 µl/well freshly prepared ABTS (2,2’-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) substrate
       with hydrogen peroxide (0.1%) and incubate at room temperature for 30 minutes.
  xv) Measure absorbance at 405 nm. A test sample is considered to be positive if the absorbance of the
       test sample in wells containing virus antigen is at least twice the absorbance of negative control serum
       in wells containing virus antigen and at least twice the absorbance of the sample tested in parallel in
       wells containing control antigen.

b) Plaque reduction neutralisation (applicable to serum from any species)

The PRN test is performed in Vero cell cultures in either 25 cm\(^2\) flasks or 6-well plates. The sera can be
screened at a 1/10 and 1/100 final dilution or may be titrated to establish an endpoint. A description of the
test as performed in 25 cm\(^2\) flasks using 100 plaque-forming units (PFU) of virus is as follows.

Prior to testing, serum is heat inactivated at 56°C for 30 minutes and diluted (e.g. 1/5 and 1/50) in media.
Virus (200 pfu per 0.1 ml) working dilution is prepared in media containing 10% guinea-pig complement.
Equal volumes of virus and serum are mixed and incubated at 37°C for 75 minutes before inoculation of
0.1 ml on to confluent cell culture monolayers. The inoculum is adsorbed for 1 hour at 37°C, followed by the

\(^1\) Available from the Centers for Disease Control and Prevention, Biological Reference Reagents, 1600 Clifton Road NE,
Mail Stop C21, Atlanta, Georgia, 30333, USA.
addition of 4.0 ml of primary overlay medium. The primary overlay medium consists of two solutions that are prepared separately. Solution I contains 2 × Earle’s Basic Salts Solution without phenol red, 4% fetal bovine serum, 100 µg/ml gentamicin and 0.45% sodium bicarbonate. Solution II consists of 2% Noble agar that is sterilised and maintained at 47°C. Equal volumes of solutions I and II are adjusted to 47°C and mixed together just before use. The test is incubated for 72 hours at 37°C. A second 4.0 ml overlay prepared as above, but also containing 0.003% neutral red is applied to each flask. Following a further overnight incubation at 37°C, the number of virus plaques per flask is assessed. Endpoint titres are based on 90% reduction compared with the virus control flasks, which should have about 100 plaques.

Standard microneutralisation or microtitre plaque reduction neutralisation assays may be more suitable when small volumes of samples are available (Weingartl et al., 2003).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

In February 2003, the United States Department of Agriculture (USDA) issued a license for a formalin-inactivated WNV vaccine derived from tissue culture for use in horses. This was followed in December 2003, by a USDA licensed live canarypoxvirus vectored WNV vaccine for use in horses. In 2004, an inactivated human cell line-derived WNV vaccine developed by Crucell NV (the Netherlands) and Kimron Veterinary Institute (Israel) obtained a market authorisation in Israel as a veterinary vaccine for geese. In July 2005, the USDA issued the first fully licensed WNV DNA vaccine for animals in the USA. The vaccine contains genes for two WNV proteins, and therefore, does not contain any whole WNV, live or killed. In late 2006, a chimeric vaccine, based on a yellow fever virus vector, was licensed by USDA for use in horses. These vaccines have demonstrated sufficient efficacy and safety in adequately vaccinated horses. Vaccination may be helpful in preventing neurological signs associated with WNV infection.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

The isolate of WNV used for vaccine production must be accompanied by documentation describing its origin and passage history. The isolate must be safe in host animals at the intended age of vaccination and provide protection after challenge.

b) Method of culture

The WNV should be propagated in cell lines known to support the growth of WNV. Cell lines should be free from extraneous viruses, bacteria, fungi, and mycoplasma. Viral propagation should not exceed five passages from the master seed virus (MSV), unless further passages prove to provide protection in the host animal.

c) Validation as a vaccine

The MSV should be free from bacteria, fungi and mycoplasma. The MSV must be tested for and be free of extraneous viruses, including equine herpesvirus, equine adenovirus, equine viral arteritis virus, bovine viral diarrhoea virus, reovirus, and rabies virus by the fluorescent antibody technique. The MSV must be free from extraneous virus by CPE and haemadsorption on the Vero cell line and an embryonic equine cell type.

In an immunogenicity trial, the MSV at the highest passage level intended for production must protect susceptible horses against a virulent challenge strain. A statistically significant number of vaccinated horses must be protected from viraemia when compared with the controls. Field trial studies should be conducted to determine the safety of the vaccine.

2. Method of manufacture

The susceptible cell line is seeded into suitable vessels. Minimal essential medium, supplemented with fetal bovine serum (FBS), is used as the medium for production. Incubation is at 37°C.

Cell cultures are inoculated directly with WN working virus stock, which is generally from 1 to 4 passages from the MSV. Inoculated cultures are incubated for 1–8 days before harvesting the culture medium. During incubation, the cultures are observed daily for CPE and bacterial contamination.
Killed virus vaccines are chemically inactivated with either formalin or binary ethylenimine and mixed with a suitable adjuvant.

The DNA vaccine expression cassette is amplified in *Escherichia coli* using a plasmid vector cutting out plasmid backbone and purified for formulation into a vaccine.

3. **In-process control**

Production lots of WNV must be titrated in tissue culture for standardisation of the product. Low-titred lots may be concentrated or blended with higher-titred lots to achieve the correct titre.

Production lots of DNA are quantified by analytical methods and characterised before standardisation and blending at the correct DNA content. The highest level of lipopolysaccharide (LPS) contamination of the DNA vaccine is 100 EU/dose (EU = endotoxin units).

4. **Batch control**

Final container samples are tested for purity, safety and potency.

a) **Purity**

Samples are examined for bacterial and fungal contamination. To test for bacteria, ten vessels, each containing 120 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The ten vessels are incubated at 30–35°C for 14 days and observed for bacterial growth. To test for fungi, ten vessels, each containing 40 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The vessels are incubated at 20–25°C for 14 days and observed for fungal growth.

b) **Safety**

Safety tests can be conducted in a combination of guinea-pigs, mice or horses. Field safety studies should be conducted before the vaccine receives final approval. Generally, two serials should be used, in three different geographical locations, and a minimum of 600 animals. About one-third of the animals should be at the minimum age recommended for vaccination (correlated to efficacy). If the final product is a modified-live vaccine, additional safety testing of the MSV is required to demonstrate a lack of virulence.

c) **Potency**

Killed virus vaccines may use host animal or laboratory animal vaccination/serology tests or vaccination/challenge tests to determine potency of the final product. Parallel-line assays using ELISA antigen-quantifying techniques to compare a standard with the final product are acceptable in determining the relative potency of a product. The standard should be shown to be protective in the host animal (USDA, 1998). Live viral products are titred in cell cultures to determine the potency of the final product. The final release potency titre should include an additional 0.7 log₁₀ for test variability and 0.5 log₁₀ for end-of-dating stability than the minimum protective dose established in the immunogenicity trial.

DNA vaccines are tested for bioactivity and DNA content using parallel-line direct quantification methods that compare a standard preparation to the final product.

d) **Duration of immunity**

Duration of immunity studies are conducted before the vaccine receives final approval. The duration should be for the length of the mosquito season in the infected areas. For animals at higher risk and in infected areas with year-round mosquito activity, more frequent vaccine boosters may be advised.

e) **Stability**

All vaccines are initially given 24 months before expiry. Real-time stability studies are conducted to confirm the appropriateness of all expiration dating.

f) **Preservatives**

Antibiotics are added during production, generally gentamicin sulphate or neomycin not to exceed 30 µg/ml.
Chapter 2.1.20. — West Nile fever

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

REFERENCES


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**NB:** There are OIE Reference Laboratories for West Nile fever (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for West Nile fever.
**SECTION 2.2.**

**APINAE**

**INTRODUCTORY NOTE ON BEE DISEASES**

*Bees are insects that are closely related to ants and wasps. There are many thousands of species of bee, most of which are not social insects, living solitary lives. The honey bee, *Apis* species, lives as a colony, which is a family of social insects. There are many species, subspecies, races and subraces of honey bees that are adapted to their environment.*

Two species are important for bee keeping – the western honey bee *Apis mellifera*, and the eastern honey bee *A. cerana*. The Africanised bee, which is found in South and Central America and some states of the United States of America, is a cross between two subspecies of the western honey bee, the European bees and the South African bee. *Apis cerana* is important in South and South-East Asia. The colonies are small and docile, but the honey yields are low. In a suitable climate, the western honey bee, *A. mellifera*, is sometimes preferred for its greater honey production.

It is thought that all bees are susceptible to the known diseases of bees, but different races may have varying susceptibility. For example, *A. cerana* is less susceptible to varroosis. When sampling a colony of bees for diagnosis of diseases, live bees must first be killed with diethyl ether or in a deep freezer (−20°C) overnight. Bees may also be killed by submersion in 70% ethyl alcohol, e.g., when collected for diagnosis of acariosis (*Acarapis*). Larval and pupal smears must be made when testing for brood diseases or a piece of comb containing brood showing visible signs of disease may be sent to the laboratory.

* *
CHAPTER 2.2.1.

ACARAPISISOSIS OF HONEY BEES

SUMMARY

Acarapisosis or acariosis or acarine disease is a disease of the adult honey bee Apis mellifera L. and other Apis species. It is caused by the Tarsonemid mite, known as the tracheal mite, Acarapis woodi (Rennie). The mite is approximately 150 µm in size, and is an internal parasite of the respiratory system, living and reproducing mainly in the large prothoracic trachea of the bee. Sometimes they are also found in the head, thoracic and abdominal air sacs. Mites feed on the haemolymph of their host.

The pathogenic effects found in infected bees depend on the number of parasites within the trachea and are attributable both to mechanical injuries and to physiological disorders consequent to the obstruction of air ducts, lesions in the tracheal walls, and the depletion of haemolymph. As the parasite population increases, the tracheal walls, normally white and translucent, become opaque and discoloured with blotchy black areas, probably due to melanin crusts.

The mortality rate may range from moderate to high. Early manifestations of infection normally go unnoticed, and only when infection is heavy does it become apparent. This is usually in the early spring. The infection spreads by direct contact. Generally, only newly hatched bees under 10 days old are susceptible. Reproduction occurs within the tracheae of adult bees, where female mites may lay 8–20 eggs. There are 2–4 times as many females as males. Development takes 11–12 days for males and 14–15 days for females.

Identification of the agent: The parasites are demonstrated only by laboratory methods and under the microscope. The mites need to be observed inside the tracheae or removed from them to be observed microscopically. Several techniques are available for demonstrating the mites, such as dissection, grinding and staining.

The thoraces of suspect bees are dissected to expose the trachea. Each trachea is examined under a dissecting microscope (×18–20), where the mites will be seen through the transparent wall as small oval bodies.

Alternatively, larger samples of suspect bees can be ground or homogenised in water, followed by coarse filtration of the suspension, and centrifugation. The deposit is treated with undiluted lactic acid for 10 minutes. This is then mounted for microscopic examination.

The parasites may be stained by histological techniques so that they can be observed within the bee trachea. The tracheae are separated out, cleared with 8% potassium hydroxide, and stained with 1% methylene blue. This is the best method for large numbers of samples.

Serological tests: Serological tests are not available.

Requirements for vaccines and diagnostic biologicals: There are no biological products available. Menthol crystals or oil patties made with vegetable oil (not animal fat) and white granulated sugar will keep mite levels under control.

A. INTRODUCTION

Acarapisosis is a disease of the adult honey bee Apis mellifera L. and other Apis species, caused by the microscopic Tarsonemid mite Acarapis woodi (Rennie). The mite is approximately 150 µm in size and is an
internal parasite of the respiratory system (Figure 1). These tracheal mites enter, live and reproduce mainly in the large prothoracic tracheae of all bees, feeding on the haemolymph of their host (Figure 2). Sometimes they are also found in the head, thoracic and abdominal air sacs (Giordani, 1965; Wilson et al., 1997).

![Fig. 1. Acarapis woodi (Rennie). Top: Adult male, Centre: Adult Female, Bottom: Egg.](image)

![Fig. 2. Main thoracic tracheae of a honey bee where Acarapis is commonly found; light infestations are near the spiracle opening.](image)

The pathogenic effects on individual bees depend on the numbers of parasites within the tracheae and are attributable both to mechanical injuries and to physiological disorders consequent to the obstruction of the air
ducts, lesions in the tracheal walls, and to the depletion of haemolymph. As the parasite population increases, the tracheal walls, which are normally whitish and translucent, become opaque and discoloured with blotchy black areas, probably due to melanin crusts (Giordani, 1964).

The mortality rate may range from moderate to high. Early signs of infection normally go unnoticed, except for a slow dwindling in the colony size. Only when infection is heavy does it become apparent. This is generally in the early spring after the winter clustering period when the mites have bred and multiplied undisturbed into the longer-living winter bees. This applies mainly to the Northern Hemisphere where there are seasonal variations in the reproduction of bees.

Infection spreads from one bee to another by direct contact. Generally, only newly hatched bees under 10 days old, are susceptible. Attempts to rear A. woodi on artificial and synthetic diets have been unsuccessful, while culturing them on immature stages of the honey bee itself has been only partially successful (Giordani, 1970). The life span of the mites in dead bees is approximately 1 week. Reproduction occurs within the tracheae of adult bees, where female mites may lay 8–20 eggs. There are 2–4 times as many females as males; development takes 11–12 days for males and 14–15 days for females.

There are no reliable clinical signs for the diagnosis of acarapisosis as the signs of infection are not specific and the bees behave in much the same way as do bees affected by other diseases or disorders. They crawl around in the front of the hive and climb blades of grass, unable to fly. Dysentery may be present.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Acarapisosis can be detected only in the laboratory using microscopic examination or an enzyme-linked immunosorbent assay (ELISA). There is no reliable method for detection of very low levels of infection. The number of bees sampled determines the detection threshold of the method. It has been shown that a 1 to 2% rate of infection can be detected by sampling 50 bees. Sequential sampling data are available (Frazier et al., 2000; Tomasko et al., 1993). The best time to take bee samples is in the early spring or late autumn (Northern hemisphere), when Acarapis populations are high. Visualisation of mites is easier in older bees, which have more mites. Samples of queens, drones or workers can be used, but Acarapis prefer drones.

a) Dissection (Giordani, 1974)

A sample of 50 bees (see above) is collected at random from the suspected colony. These are mainly bees crawling and unable to fly, found within about 3 metres of the front of the hive. This is preferable to random collection from within the colony. The bees may be living, dying, or dead. Live bees must first be killed with ethyl alcohol or in a deep freezer (–20°C); bees must not have been dead for over 2–3 days unless kept at 4°C for up to 4 weeks or –20°C for several months. They may be preserved indefinitely in a preservative such as Oudemann solution: glacial acetic acid (80 ml); glycerol (50 ml); 70% ethanol (870 ml).

- Test procedure: direct preparation (Ritter, 1996; Wilson et al., 1997)
  i) Remove the abdomen at the thorax of the bees (see Figure 3).
  ii) Pick up the thorax with the beginning of the head and examine it under the binocular magnifying glass at 20–30-fold magnification.
  iii) Remove the pleural sclerite of the first thoracic segment with the first pair of legs, by means of a pair of tweezers. In the circular opening the main strains of the thoracic tracheae and the branches of the head tracheae can be seen.
  iv) By means of a fine pair of tweezers, remove the thoracic tergite of the first thoracic segment and part of the second thoracic tergite. After removing the overlying musculature, the two thoracic tracheae are exposed. Positive diagnosis consists of either the presence of melanisation of one or both tracheae or, in light infestation, of the presence of oval translucent bodies (eggs etc.) easily seen within the tracheae.
  v) For further microscopic examination (e.g. confirmation of light infestation), remove the tracheae and put them onto a slide, with a drop of water. Under the microscope at 100-fold magnification the adult mites as well as their individual stages of development can be recognised.
Chapter 2.2.1. – Acarapisosis of honey bees

Fig. 3. Preparation of bees to reveal Acarapis woodi in the first thoracic pair of tracheae.

• Test procedure: maceration (Ritter, 1996)

i) Lay and secure bees on their backs or hold with thumb and first finger.

ii) Remove the heads and forelegs using a small forceps and remove the collar surrounding the neck opening to expose the tracheae (Figure 4). Check the tracheae nearest to the spiracle (as mites enter through the spiracle) to see light infestations. Heavy infestations are easily visible as shadows or dark objects in clear to dark brown tracheae. Old and heavy infestations will make the tracheae brown to black.

iii) Cut through the thorax in front of the middle pair of legs and the base of the forewings with a sharp razorblade. These thin disks can be further treated to clear muscle tissue.

iv) Macerate either by gentle heating in an 8% solution of potassium hydroxide for approximately 20 minutes or by leaving them to stand overnight without heating.

v) Examine the first pair of tracheae, which are covered by muscle tissue, under a dissecting microscope at a magnification of ×18–20, or transfer the tracheae to another slide, add glycerin or water and observe at higher magnification.

vi) Mites are easily seen through the transparent wall as small, oval bodies.

Fig. 4. Left: front view of bee thorax with head removed and collar intact. Right: Collar removed and tracheae exposed to spiracle openings.

This is the simplest and most reliable technique for the laboratory diagnosis of acarapisosis, allowing the detection of early infections and enabling the infection rate to be established. Even light infections can be detected by using a dissecting microscope with this technique. Only in very exceptional instances will it be necessary to employ higher magnifications in order to make a diagnosis. However, this is a demanding technique, especially when a large number of acarapisosis diagnoses have to be made. If it is necessary only to distinguish between heavily infected and lightly or non-infected colonies, dissection can be stopped at step ii and the colour of the tracheae observed.
b) Grinding (Colin et al., 1979)

A sample of about 200 bees is collected at random from the suspect colony. The wings and legs of each bee are removed from the thorax, and the bodies are pooled in a 100 ml container that has been one-quarter filled with water. This suspension is homogenised three times, each time for several seconds, in a homogeniser at 10,000 rpm with the addition of more water. The resulting suspension is strained through a sieve (mesh 0.8 mm) and the sieve is rinsed with water to a final volume of approximately 50 ml. The filtrate is centrifuged at 1500 \( g \) for 5 minutes and the supernatant fluid is discarded. A few drops of undiluted lactic acid solution are added to the debris of the deposit, which will contain the mites. This is left for 10 minutes to allow the muscle fibres to dissolve, and is then mounted under a cover-slip for microscopic examination. This technique is quicker than dissection, but may be less accurate. External mites \( A. \) externus, \( A. \) vagans and \( A. \) dorsalis, all of which are morphologically similar to \( A. \) woodi, are often found on the thorax of healthy bees and can very easily be mistaken for \( A. \) woodi (Table 1). It seems, however, that they do not cause any serious threat to bees or beekeeping. This method should therefore only be chosen if all that is required is a rough estimation of the degree of infection in a region. It is not suitable for determining a first outbreak.

\[
\text{Table 1. Differential diagnosis of Acarapis species (Ritter, 1996)}
\]

<table>
<thead>
<tr>
<th>Character</th>
<th>( A. ) dorsalis</th>
<th>( A. ) externus</th>
<th>( A. ) woodi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch of the coxal plate</td>
<td>Deep</td>
<td>Short</td>
<td>Flat</td>
</tr>
<tr>
<td>Space between stigmata</td>
<td>16.7 ( \mu m )</td>
<td>16.8 ( \mu m )</td>
<td>13.9 ( \mu m )</td>
</tr>
<tr>
<td>Length of tarsal limb (IV leg pair)</td>
<td>7.6 ( \mu m )</td>
<td>11.4 ( \mu m )</td>
<td>7.5 ( \mu m )</td>
</tr>
</tbody>
</table>

c) Staining (Peng & Nasr, 1985)

The mites and trachea can be stained specifically, rendering them easily visible by microscopy.

- **Test procedure**
  1. Remove the head and forelegs.
  2. Make a transverse cut through the membranous areas behind the forelegs.
  3. Make a second transverse cut in front of the middle pair of legs at the base of the forewings.
  4. To clear the sections (1–1.5 mm thick), place them in an 8% solution of potassium hydroxide.
  5. Stir gently and heat near to boiling point for approximately 10 minutes until the soft internal tissues are dissolved and cleared, leaving the chitinous tissues intact.
  6. Retrieve sections by filtration and wash with tap water.
  7. Stain and mount the sections.
  8. Examine for mites by low-power microscopy.

Permanent mounts are prepared by the usual histological techniques.

Cationic stains are the most suitable and specific as they stain the mites intensely but the tracheae only weakly. A solution of 1% aqueous methylene blue is the most suitable, prepared by dissolving the methylene blue first and then adding sodium chloride to make a 0.85% NaCl solution.

- **Test procedure**
  1. Stain in 1% aqueous methylene blue.
  2. Differentiate sections in distilled water for 2–5 minutes.
  3. Rinse the sections in 70% alcohol.

When kept in 95% ethanol, the mites will retain the stain for 6 hours (Bancroft & Stevens, 1982). It is essential with this technique to macerate the tissues effectively in the potassium hydroxide solution. Using this method, it is possible to process a large number of samples rapidly and conveniently.
d) Enzyme-linked immunosorbent assay

An ELISA for trachea mites has been developed (Grant et al., 1993; Ragsdale & Furgala, 1987; Ragsdale & Kjer, 1989). This test may produce false-positive results, and is therefore only recommended for survey examinations. Another method is the visualisation of guanine, a nitrogenous waste product of mites (Mozes-Koch & Gerson, 1997).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available. Menthol crystals (50 g for a two story colony) control mites if left in the colony for 28 days, providing the ambient temperature is at least 18°C. The optimum temperature range for the vapours to work is 27–29°C. Small cakes made with vegetable shortening (e.g. margarine, not animal fat) and white granulated sugar will keep mite levels to 10%. The cake (about 100 g in weight) should be placed on the top bars of the frames in the brood nest in the autumn and early spring (Sammataro & Needham, 1996). Formic acid may be used to treat infected colonies (Hood & McCreadie, 2001).

Some races of bees, such as Buckfast bees (Brother, 1968) and some hygienic strains, are less susceptible to attack by *Acarapis*.

ACKNOWLEDGEMENTS

Illustrations by Diana Sammataro and Wolfgang Ritter are reproduced with their permission.


REFERENCES


* * *

NB: There are OIE Reference Laboratories for Bee diseases (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)). Please contact the OIE Reference Laboratories for any further information on diagnostic tests and reagents for bee diseases.
CHAPTER 2.2.2.

AMERICAN FOULBROOD OF HONEY BEES

SUMMARY

American foulbrood (AFB) affects the larval stage of the honey bee Apis mellifera and other Apis spp., and occurs throughout the world. Paenibacillus larvae, the causative organism, is a bacterium that can produce over one billion spores in each infected larva. The spores are extremely resistant to heat and chemical agents, and can survive for many years in scales (from diseased dead brood), hive products and equipment. Only the spores are capable of inducing the disease.

Identification of the agent: Combs of infected colonies have a mottled appearance due to a mixture of healthy capped brood, uncapped cells containing the remains of diseased larvae, and empty cells. This is not a characteristic of AFB only. Cell cappings of a diseased larva appear moist and darkened, becoming concave and possibly punctured as infection progresses. The larval or pupal colour changes to creamy brown and then to a dark brown with a ropy appearance when drawn out. In some cases the larval remains are rather watery. The diseased brood eventually dries out to form characteristic brittle scales that adhere tightly to the lower sides of the cell. The formation of a pupal tongue is one of the most characteristic but rarely seen signs of the disease and precedes the formation of the scales.

Diagnosis of AFB is based on identification of the pathogenic agent and the presence of clinical signs. The analyst can rely on a broad range of samples. However, in practice, the samples of choice will depend on whether it concerns a suspicious or diseased honey bee colony/apiary, or analysis in the context of an AFB monitoring/prevention programme. Some of the identification methods require a previous culturing step, while others can be performed directly on collected samples. Four solid culture media are recommended: PLA (Paenibacillus larvae agar), MYPGP agar, BHIT agar and Columbia sheep blood agar. Two polymerase chain reaction (PCR) protocols are described in this chapter. The first protocol can be used for rapid confirmation of clinical AFB and for identification of bacterial colonies after a cultivation step. The second protocol is a so-called nested PCR that also permits direct analysis of spore solutions. The biochemical profiling of P. larvae is based on the catalase test, the production of acid from carbohydrates and the hydrolysis of casein. Further, antibody-based techniques and the microscopic identification of the pathogenic agent are described.

Serological tests: There are no serological tests available.

Requirements for vaccines and diagnostic biologicals: Monoclonal and polyclonal antibodies produced for the development of diagnostic tests should be sufficiently specific.

A. INTRODUCTION

American foulbrood (AFB) is an infectious disease of the larval stage of the honey bee Apis mellifera and other Apis spp., and occurs throughout the world where such bees are kept. Paenibacillus larvae, the causative organism, is a Gram-positive bacterium that can produce over one billion spores in each infected larva. The bacterium is a round-ended, straight and sometimes curved rod, which varies greatly in size (0.5 µm wide by 1.5 to 6 µm long), occurring singly and in chains and filaments; some strains are motile. The sporangia are often sparse in vitro, and the ellipsoidal, central to subterminal spores, which may swell the sporangia, are often found free (Heyndrickx et al., 1996). The spores are extremely heat stable and resistant to chemical agents. Only spores are capable of inducing the disease.

The infection can be transmitted to larvae by nurse bees or by spores remaining at the base of a brood cell. Although the larval stages of worker bees, drones and queens are susceptible to infection, infected queens and...
drone larvae are rarely seen under natural conditions. The susceptibility of larvae to AFB disease decreases with increasing age (Woodrow, 1941); larvae cannot be infected later than 53 hours after the egg has hatched. The mean infective dose (LD_{50}= spore dose at which 50% of the larvae are killed) needed to initiate infection, though very variable, is 8.49 spores in 24–48 hour-old bee larvae (Hansen & Brødsgaard, 1999). Exchanging combs containing the remains of diseased brood is the most common way of spreading the disease from colony to colony. In addition, feeding or robbing of spore-laden honey or bee bread, package bees and the introduction of queens from infected colonies can also spread the disease. Wax contaminated with the spores of *P. larvae*, which are used in the production of combs foundation, can also spread the disease. The early detection of AFB helps to prevent further spread.

### B. DIAGNOSTIC TECHNIQUES

1. **Identification of the agent**

Diagnosis of AFB is based on identification of the pathogenic agent only. The analyst can rely on a broad range of samples. However, in practice, the samples of choice will depend on whether it concerns a suspicious or diseased honey bee colony/apiary, or analysis in the context of an AFB monitoring/prevention programme. An initial overview of clinical signs of the disease will be provided in this chapter, followed by identification methods that require a previous culturing step, or that can be performed directly on collected samples. The techniques involved are microbiological characterisation, the polymerase chain reaction (PCR), biochemical profiling, antibody-based techniques and microscopy. The analyst should be aware of differences in sensitivity between the presented approaches and should select the most appropriate for a given situation.

![Fig. 1. Progression of the disease: (a) Point of infection. (b) Larval development to the prepupal stage. (c) Cell contents reduced and capping is drawn inwards or is punctured. (d) Cell contents become glutinous. (e) Residual scale tightly adherent to bottom of cell.]()

#### a) Epizootology and clinical signs

Spores of *P. larvae* can survive in bee products (honey, wax, dry larval scales) and in the environment for 3 to 10 years and purified spores can survive even more than 70 years (Rudenko, 1987).

The clinical signs of AFB are very diverse and depend on the genotype involved, the stage of the disease and the strength of the bee colony (and possibly its resistance to AFB). Larvae can be killed rapidly at an early age when they are curled at the base of uncapped brood cells. Adult worker bees will remove these dead larvae leaving only an empty cell (Brødsgaard *et al.*, 2000). Other larvae will die later on in their development, when they are in an upright position, filling most of the brood cell. Often the larvae or pupae will die after brood cell capping.

In severely infected colonies, the combs have a mottled appearance caused by a pattern of healthy capped brood, uncapped cells containing the remains of diseased larvae, and empty cells. The capping of a cell that contains a diseased larva appears moist and darkened and becomes concave and punctured as the infection progresses. Also, the larva or pupa changes colour, first to a creamy and eventually to a dark brown. The larvae can become glutinous in consistency and can be drawn out as threads when a probe is
inserted into the larval remains and removed from the cell (match-stick test). This is probably the best-known technique for field diagnosis of the disease, but in some cases the larval remains are rather watery, resulting in a negative match-stick test. Finally, 1 month or more after the larva becomes ropy, the remains of the diseased brood dry out to form typical hard, dark scales that are brittle and adhere strongly to the lower sides of the cell (Figure 1). If death occurs in the pupal stage, the pupal tongue protrudes from the pupal head, extending to the top of the brood cell or may angle back towards the bottom of the cell. The protruding tongue is one of the most characteristic signs of the disease, although it is rarely seen (Figure 2). The tongue may persist also on the dried scale. European foulbrood needs to be taken into consideration as a differential diagnosis.

![Fig. 2. Clinical American foulbrood (a-c) and Gram staining (d): (a) Combs have mottled appearance. (b) A matchstick draws out the brown, semi-fluid larval remains in a ropy thread. (c) The formation of a pupal tongue is a very characteristic sign, but rarely seen. (d) Microscopic examination reveals Gram-positive rods, occurring singly and in chains.](image)

### b) Selection of samples

#### i) Collection of samples from a suspicious or diseased colony/apiary

While maintaining their colonies, beekeepers often find brood combs with signs of disease. In this case a brood sample can be collected for diagnosis. The brood is sampled by cutting out a piece comb of about 20 cm² in size, containing as much of the dead or discoloured brood as possible. An experienced person can collect infected larval/pupal remains directly from the cells with a sterile swab, significantly reducing the sample size and facilitating packaging and sample transportation to the laboratory (see below). When microscopic examination is the method of choice, smears of the remains of diseased larvae can also be made at the apiary (Hornitzky & Wilson, 1989). After air-drying they can be forwarded to the laboratory.

Every bee colony in the vicinity of such a clinical case of AFB should be considered as suspicious and a broad range of samples should be taken for confirmation. Apart from brood samples, food stores (honey [Ritter & Kiefer, 1995; von der Ohe & Dustmann, 1997], pollen [Gochnauer & Corner, 1987] and royal jelly), adult workers (Lindström & Fries, 2005) and wax debris (Titera & Haklova, 2003) can be used to detect the presence of *P. larvae* spores. Honey samples can be collected from cells close to the brood with separate disposable spoons to prevent cross-contamination between samples; however, honey may have been sitting in the comb for months at the time of sampling. Adult bees can be shaken or brushed from the combs of the brood chamber or the honey supers into a plastic bag or container. For the most reliable picture of the actual situation, bees from the brood nest (and not the honey supers) should be analysed. Wax debris can be collected at the hive bottom all year round.
Chapter 2.2.2. — American foulbrood of honey bees

ii) **Samples for AFB monitoring/prevention programmes**

To prevent the propagation of diseased brood, honey, adult bee and debris samples can be used to detect AFB in colonies where no clinical signs are observed. Routine collection of samples from colonies or from harvested honey can be used as part of an operational or regional AFB detection programme.

Microscopic examination of smears from larvae with no clinical signs is far less sensitive at detecting spores in colonies compared with bacteriological or PCR-based methods. In fact, bacteriological and PCR-based methods will often detect spores in colonies that never develop clinical signs of AFB. High numbers of spores cultured from honey and bee samples using bacteriological methods, however, can often predict the presence of clinical AFB signs at colony, apiary and operational levels.

b) **Packaging and transportation of samples to the laboratory**

Brood comb should be wrapped in a paper bag, paper towel or newspaper and placed in a wooden or heavy cardboard box for transport. Swabs with larval remains can be put into appropriate test tubes with a cap. Holders for microscope slides are commercially available. Adult bees can be kept frozen or submerged in 70% ethanol during transportation, although dried bees are adequate. Food supplies can be put into a test tube or a suitable pot, or wrapped in a plastic bag together with the spoon. Leaking and cross-contamination of the samples must be prevented. If possible, fresh material for laboratory tests should be sent refrigerated.

c) **Sample preparation**

i) **Samples for cultivation**

In general, an aqueous solution containing *P. larvae* spores should be prepared for further analysis. This spore suspension is heat-shocked at 80°C for 10 minutes or 95–96°C for 3–5 minutes in order to kill other spore-forming microorganisms.

Larval/pupal remains from brood comb are collected with a sterile swab and suspended in 5–10 ml of sterile water or physiological solution (phosphate buffered saline or 0.9% NaCl) in a test tube.

Honey samples to be examined for spores are heated to 45–50°C and shaken to distribute any spores that may be present. Dilution with an equal volume (25 ml) of water permits easier handling. The diluted honey is transferred into 44 mm width dialysis tubing that has been tied at one end. The open end is tied after filling. The tubes are submerged in running water for 18 hours or in a water bath with 3–4 water changes over the same time period. After dialysis, the contents are centrifuged at 2000 g for 20 minutes. The supernatant liquid is discarded leaving approximately 1 ml (or less) of residue in each sample. The residue is then resuspended in 9 ml of water (Shimanuki & Knox, 1988).

Honey can also be prepared for cultivation without the dialysis step, however this requires longer (30 minutes) and faster (3000 g) centrifugation. Likewise, the volume in which the deposit is finally resuspended can be much smaller (200 µl) in order to improve the sensitivity of the test (de Graaf et al., 2001).

Direct plating of diluted honey (Ritter & Kiefer, 1995) is widely used, but its sensitivity is inferior to that of the centrifugation method as only a fraction of the total volume will be plated out. Whatever the method of choice is, when honey is analysed quantitatively and threshold values are set, the methodology that was used to establish these values should always be strictly followed.

An aqueous filtrate of pollen can be made by thoroughly dispersing 1 g of pollen in 10 ml final volume sterile distilled water and filtering it through Whatman No. 1 paper (Gochnauw & Corner, 1987).

When adult bees are dispatched in ethanol, the latter should be decanted and replaced by sterile water or physiological solution before crushing.

Debris and bee wax (1.5 g) should be dissolved in an organic solvent (10 ml): toluene (Titera & Haklova, 2003), chloroform (Kostecki, 1969) or diethyl ether (Ritter, 2003). The liquid part (2 ml) is then diluted in physiological solution (6 ml). After shaking roughly, this suspension can immediately be plated out (no heat-shock) (Titera & Haklova, 2003). In another protocol, bee wax is first diluted in water (wax/water 1/10) and heated up to 90°C for 6 minutes. After cooling down, the organic solvent is added (organic solvent/water 1/9) and the mixture is shaken carefully. After 2 minutes standing time, a deposit of a watery solution containing *P. larvae* spores forms (Ritter, 2003).

ii) **Samples for PCR**

Cell/spore suspensions and suspensions containing only spores have to be differentiated, the latter requiring a more complex DNA extraction step (except for the nested PCR).

If the PCR is aimed at identifying bacterial colonies (= cell/spore suspension) after a cultivation step, the pre-treatment is as follows: one colony is suspended in 50 µl of distilled water and heated to 95°C for 15 minutes. Following centrifugation at 5000 g for 5 minutes, 1–5 µl of the supernatant is used as template DNA in a PCR 50 µl mixture (Dobbelaere et al., 2001b).
For rapid confirmation of clinical AFB, the samples should be prepared as follows: the remains of two diseased honey bee larvae (= cell/spore suspension) are suspended in 1 ml of sterile distilled water and mixed thoroughly. 100 µl of this suspension is diluted with 900 µl distilled water. This dilution is vortexed and 100 µl of it is used to extract DNA by heating and centrifugation (see above) (Dobbelaere et al., 2001b).

All aqueous solutions resulting from the sampling of honey, adult bees, debris, bee wax, pollen and royal jelly should be considered as a spore suspension. Here, the extraction of DNA demands another approach. Indeed, spore suspensions are centrifuged at 6000 g and 4°C for 30 minutes. Next, the pellet is subjected to microwave treatment for 5 minutes at maximum power to break the spores, and the released DNA is suspended in 30 µl of 10 mM Tris/HCl, pH 8.0, containing 1 mM EDTA (Piccini et al., 2002).

When spores are to be detected from honey, DNA is serially diluted with sterile distilled water to eliminate PCR inhibition caused by honey (Piccini et al., 2002). Another DNA extraction method, based on lysozyme and proteinase K treatment, has been described (Bakonyi et al., 2003).

Good results can also be obtained by incubating a pelleted spore suspension in MYPGP broth at 37°C for 2–24 hours. Thereafter, the suspension is centrifuged at 14,500 g for 5 minutes, washed with sterile distilled water and resuspended in 200 µl of sterile distilled water. This short incubation step causes spores to germinate, making them sensitive for DNA preparation by heat treatment again (see above) (Lauro et al., 2003).

When the nested PCR is chosen, the spore solution should only be boiled at 100°C for 10 minutes and thereafter centrifuged at 14,500 g for 2 minutes. The supernatant can immediately serve as template DNA sample in the nested PCR reaction (Lauro et al., 2003).

d) Culture

Several media for cultivating *P. larvae* have been described but best results were obtained with PLA (Paenibacillus larvae agar) (Schuch et al., 2001), MYPGP agar (the abbreviation refers to its constituents: Mueller-Hinton broth, yeast extract, potassium phosphate, glucose and pyruvate) (Dingmann & Stahly, 1983), BHIT agar (Brain-Heart Infusion medium supplemented with thiamine) (Gochnauer, 1973) and CSA (Columbia sheep blood agar). The formulations of the first two media are as follows:

- **PLA**

  This selective medium combines three different media to comprise the base, to which is added antibiotics and egg yolk supplements (Schuch et al., 2001). Equal quantities (100 ml) of sterile, molten Bacillus cereus selective agar base (Oxoid CM617), trypticase soy agar (Merck 5458) and supplemented nutrient agar (SNA) are combined and mixed. SNA is composed of (per litre): nutrient agar 23 g, yeast extract 6 g, meat extract 3 g, NaCl 10 g, Na2HPO4 2 g; final pH is 7.4 ± 0.2. All solid media are sterilised at 121°C/15 minutes. Nalidixic acid stock solution (Horitzky & Clark, 1991) is prepared by dissolving 0.1 g in 2 ml of 0.1 N NaOH and diluting to 100 ml with 0.01 M phosphate buffer (pH 7.2). Pipemidic acid stock (Alippi, 1995) is prepared by dissolving 0.2 g in 2 ml of 0.1 N NaOH and then diluting to 100 ml with the same phosphate buffer. Both antibiotic solutions are filter sterilised.

  After the three molten media are combined, 3 ml of stock nalidixic acid, 3 ml of stock pipemidic acid, and 30 ml of 50% egg-yolk suspension (Gordon et al., 1973) is added to form the PLA medium. The PLA medium is poured (20 ml) into sterile Petri dishes and plates are dried before use (45–50°C for 15 minutes).

  - **MYPGP agar**

    MYPGP agar is composed of (per litre): Mueller-Hinton broth (Oxoid CM0405) 10 g, yeast extract 15 g, K2PO4 3 g, glucose 2 g, Na-pyruvate 1 g and agar 20 g (Dingmann & Stahly, 1983). Addition of nalidixic acid and pipemidic acid is as above.

  If cultivation of *P. larvae* is hampered by the occurrence of fungi, the addition 16.8 µg/ml medium of amphotericin B (Sigma) works very well.

  A sterile cotton swab is used to transfer a portion of the sample on to the surface of the solid medium. For a quantitative evaluation, it is recommended to spread a fixed volume of the suspension on the solid agar with a sterile scraper or pipette rather than using cotton swabs.

  Inoculated plates are best incubated at 34–37°C for 2–4 days in an atmosphere of 5–10% CO2 in air, although aerobic incubation will do as well.
e) Identification

i) Colony morphology

Samples from clinically diseased larvae will result in confluentely grown plates after 2–4 days, leading to a subculturing step in order to isolate colonies.

On PLA, colonies of P. larvae are small, pale green to yellow (= the same colour as the medium), with a slightly opaque and rough surface; sometimes the centre is raised.

On MYPGP agar, colonies are small, regular, mostly rough, flat or raised and whitish to beige coloured.

On Columbia sheep blood agar, colonies are small, regular, glossy, butyrous and greyish.

Paenibacillus larvae colonies with orange to red pigmentation have been described (Genersch et al., 2005; Neuendorf et al., 2004).

It is advised to run P. larvae reference strains in parallel, for instance LMG 9820 (other designation: ATCC 9545, DSM 7030) for the non-pigmented variant and DSM 16115 or DSM 16116 for the pigmented genotype.

A proven positive brood or honey sample can serve as a positive control for the entire examination.

Colony morphology is not conclusive but might serve to select the bacterial colonies for further identification.

ii) Polymerase chain reaction

PCR reactions are set up as 50 µl mixtures containing 5 µl template DNA (see sample preparation), 50 pmol forward (AFB-F) and reverse primer (AFB-R; primer sequences are given below), 10 nmol of each deoxynucleoside triphosphate and 1–2.5 U of Taq polymerase, in the appropriate PCR buffer (provided together with Taq polymerase) containing 2 mM MgCl₂ (Dobbelaere et al., 2001b with modifications). Reducing the volume of the PCR mixtures to 25 µl is possible. Amplification of a specific DNA fragment occurs in a thermocycler under the following PCR conditions: a 95°C (1–15 minutes) step; 30 cycles of 93°C (1 minute), 55°C (30 seconds), and 72°C (1 minute); and a final cycle of 72°C (5 minutes).

Nested PCR comprises an external and an internal amplification step (Lauro et al., 2003). The external amplification is performed using primers PleF and PleR (see below). Each 50 µl PCR reaction contains: 10 µl template DNA (see sample preparation), 1 × PCR buffer (with 1.5 mM MgCl₂), 0.5 µM PleF primer, 0.5 µM PleR primer, 0.2 mM of each dNTP, additional 0.75 mM MgCl₂, 1.25 U Taq polymerase. A ‘touchdown’ PCR protocol was performed in which annealing is lowered by 0.5°C/cycle, from 69 to 59°C, for a total of 20 cycles with each annealing step lasting 30 seconds. Twenty more cycles are then performed with the annealing temperature at 59°C for 30 seconds. Denaturation steps are all executed at 94°C (for 30 seconds) and extensions at 72°C (for 45 seconds). Following this, a final extension at 72°C for 5 minutes is performed, and then the reaction is cooled at 4°C. Internal amplification is performed using primers PliF and PliR (see below). Each 50 µl PCR reaction contains 1 µl of the external PCR amplification, 1 × PCR buffer (with 1.5 mM MgCl₂), 0.5 µM PliF primer, 0.5 µM PliR primer, 0.2 mM of each dNTP, additional 1 mM MgCl₂, 1.25 U Taq polymerase. Cycling conditions are: 94°C (30 seconds), 59°C (30 seconds), 72°C (45 seconds) for a total of 30 cycles followed by 5 minutes at 72°C and then the reaction is cooled at 4°C.

The molecular weights of the PCR products are determined by electrophoresis in a 0.8% agarose gel and staining with ethidium bromide.

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<th>Specificity level</th>
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<td>(Lauro et al., 2003)</td>
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<tr>
<td></td>
<td>PliF</td>
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<tr>
<td></td>
<td>PliR</td>
<td>5'-TCA-GTT-ATA-GGC-CAG-AAA-GC-3'</td>
<td></td>
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</tr>
</tbody>
</table>

iii) Biochemical tests

Paenibacillus larvae can be also be identified by its biochemical profile. The bacteria are catalase negative or weak delayed positive, they have a typical carbohydrate acidification profile with acid from glucose and trehalose, not from arabinose and xylose, and they can hydrolyse casein or milk. Some strains of P. larvae can change the biochemical signs.
• **Catalase test**
  A drop of 3% hydrogen peroxide is placed on an actively growing culture on solid medium. Most aerobic bacteria break down the peroxide to water and oxygen, producing a bubbly foam, but *P. larvae* is negative or weak delayed positive for this reaction (Haynes, 1972). When Colombia sheep blood agar is used for cultivation, the test cannot be done on the solid medium, as the presence of sheep blood will cause a false-positive reaction. In this case, colonies should be transferred to a clean microscope slide for the execution of the test. Here the evaluation of the test occurs as above with the naked eye.

• **Production of acid from carbohydrates** *(Gordon et al., 1973)*
  Bacteria are grown in J-broth (per litre: yeast extract 15 g, tryptone 5 g and K₂HPO₄ 3 g) in which 0.5% of the test substrate, separately sterilised in aqueous solution, is substituted for the glucose. The carbohydrates used are L (+)-arabinose, D (+)-glucose, D (+)-xylose and D (+)-trehalose. The cultures are tested at 14 days by aseptically removing one ml or less to a spot plate, mixing the sample with a drop of 0.04% alcoholic bromocresol purple, and observing the colour of the indicator. *Paenibacillus larvae* produces acid aerobically from glucose and trehalose. No acid is produced from arabinose and xylose (Alippi, 1992).

The use of commercial kits, such as API 50 CHB *(Carpana et al., 1995)*, BBL CRYSTAL *(Dobbelaere et al., 2001a)* and Biolog system *(Neuendorf et al., 2004)* for the biochemical characterisation of *P. larvae* can be taken into consideration.

• **Hydrolysis of casein** *(Schuch et al., 2001)*
  Casein hydrolysis is assayed using milk agar plus thiamine (per litre: agar 20 g, yeast extract 10 g; sterilised at 121°C/15 minutes). Add to each 70 ml cooled medium 30 ml of UHT (ultra-heat treated) skimmed milk and 1.5 ml filter sterilised 0.1% thiamine solution. Plates are streaked and examined after 5 days of incubation at 36 ± 1°C. *Paenibacillus larvae* hydrolyses casein, hence zones of clearing are observed around bacterial colonies.

iv) **Antibody-based techniques**
Different antibody-based techniques have been developed for the diagnosis of AFB. Most of them rely on polyclonal rabbit serum developed against pure cultures of *P. larvae*. They can be used for identification of bacterial colonies resulting from a culturing step or for direct examination of suspicious larval remains.

In an immunodiffusion test the antibodies interact with the bacterial antigen during a double diffusion process, leaving precipitation marks behind *(Peng & Peng, 1979)*. In the fluorescent antibody technique these antibodies are conjugated with a fluorochrome dye. The resulting fluorescent antibody reacts with a bacterial smear on a slide. Any excess antiserum is washed off and the smear is examined by fluorescence microscopy. *Paenibacillus larvae* stains can be recognised specifically as brightly fluorescing bacteria on a dark background *(Otte, 1973; Toshkov et al., 1970; Zhavnenko, 1971)*. An enzyme-linked immunosorbent assay using a monoclonal antibody specific to *P. larvae* exists *(Olsen et al., 1990)*. A lateral flow device for rapid confirmation of AFB has been commercialised.

v) **Microscopy**
Two microscopic techniques are commonly used. Gram staining is often done on smears of bacteria from isolated bacterial colonies. *Paenibacillus larvae* is Gram positive. Carbol fuchsin staining is done on larval smears and can confirm clinical AFB based on spore morphology. These techniques are outlined below:

• **Gram staining of bacteria**
  Flood (cover completely) the entire slide with crystal violet. Let the crystal violet stand for about 60 seconds. When the time has elapsed, wash the slide for 5 seconds with water. The specimen should appear blue-violet when observed with the naked eye. Now, flood the slide with the iodine solution. Let it stand for about a minute as well. When the time has expired, rinse the slide with water for 5 seconds and immediately proceed. At this point, the specimen should still be blue-violet. This step involves addition of the decolouriser, ethanol. This step is somewhat subjective because using too much decolouriser could result in a false Gram (−) result. Likewise, not using enough decolouriser may yield a false Gram (+) result. To be safe, add the ethanol drop-wise until the blue-violet colour is no longer emitted from the specimen. As in the previous steps, rinse with the water for 5 seconds. The final step involves applying the counter-stain, safranin. Flood the slide with the dye and let this stand for about a minute to allow the bacteria to incorporate the safranin. Gram-positive cells will incorporate little or no counter-stain and will remain blue-violet in appearance. Gram-negative bacteria, however, take on a pink colour and are easily distinguishable from the Gram positives. Again, rinse with water for
5 seconds to remove any excess of dye. Blot the slide gently with bibulous paper or allow it to air dry before viewing it under the microscope.

- **Carbol fuchsin staining of larval smears (Hornitzky & Wilson, 1989)**

Heat-fix smears. Flood the slides with 0.2% carbol fuchsin for 30 seconds. Wash off the stain and allow to air dry or gently blot dry with absorbent material. Examine under the microscope for *P. larvae* spores, which are about 1.3 × 0.6 µm, ellipsoidal and thick rimmed.

2. **Serological tests**

No serological tests are available.

**C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS**

1. **Antibody production**

VITA diagnostic kit for the early detection of AFB was developed by the Central Science Laboratory Pocket Diagnostic (UK).

When monoclonal or polyclonal *P. larvae*-specific antibodies are produced for the development of a diagnostic test, no cross-reactivity may occur with closely related bacteria or bacteria that commonly occur in beehives, for example against *Paenibacillus alvei*, often found in late phase European foulbrood.

- **Acknowledgement**

Illustrations by Karl Weiss, extracted from Bienen-Pathologie, 1984, are reproduced with the kind permission of the author and Ehrenwirth-Verlag, Munich (Germany). Photographs are from the Central Science Laboratory, York (UK) and the Informatiecentrum voor Bijenteelt, Ghent (Belgium) and published with kind permission of respectively Ruth Waite and Frans J. Jacobs.


**REFERENCES**


* * *

NB: There are OIE Reference Laboratories for Bee diseases (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the OIE Reference Laboratories for any further information on diagnostic tests and reagents for bee diseases.
CHAPTER 2.2.3.
EUROPEAN FOULBROOD OF HONEY BEES

SUMMARY

The causal organism of European foulbrood of honey bees is the bacterium Melissococcus plutonius. The identification of its presence by the observation of signs of disease in the field is unreliable. The most usual and obvious sign is the death of larvae shortly before they are due to be sealed in their cells, but this may be for reasons other than European foulbrood. Most infected colonies display few visible signs, which themselves often quickly abate spontaneously before the end of each active season. Infection remains enzootic within individual colonies because of mechanical contamination of the honeycombs by the durable organism. Recurrences of disease can therefore be expected in subsequent years.

Identification of the agent: Examination, by high-power microscopy, of suitable preparations of larval remains for the presence of numerous lanceolate cocci is adequate for most practical purposes, especially when it is done by experienced individuals.

Traditionally the diagnosis of European foulbrood is done by isolating and identifying the causative organism. This can be differentiated quite readily from all other bacteria associated with bees by its fastidious cultural requirements.

The isolated bacterium can be identified and differentiated by means of simple tube agglutination tests. A polymerase chain reaction and a hemi-nested polymerase chain reaction are also available. The latter permits direct analysis of larvae, adult bees and honey bee products.

Serological tests: No tests for detecting antibodies in bees are available.

Requirements for vaccines and diagnostic biologicals: There are no biological products available.

A. INTRODUCTION

Bee larvae usually die of European foulbrood 1–2 days before being sealed in their cells, or sometimes shortly afterwards, and always before transformation to pupae. The disease is caused by Melissococcus plutonius and occurs mostly during the period when colonies are growing quickly. Most sick larvae become displaced from their coiled position in the bottom of their cells before they die. Many are quickly detected and removed by nurse bees, leaving empty cells scattered randomly among the remaining brood. Some infected larvae survive, successfully pupate and emerge as adults. These surviving larvae are able to defecate and their infected-faeces contribute to the continued propagation of the disease (Bailey, 1960).

Infected larvae that escape detection by adult bees and then die, first become flaccid and turn a light yellow colour that becomes increasingly brown, and at the same time they dissolve into a semi-liquid mass. They then become dry and form a dark brown scale that can easily be removed from the cells. Severely affected brood may have a very stale or sour odour, sometimes acidic, like vinegar, but often there is no smell.

Signs of disease usually disappear spontaneously from infected colonies before the end of the active season, but are likely to return in subsequent years (Bailey & Ball, 1991; Jones, 1975).
B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Microscopy

Freshly dead larvae are best for diagnosis. Before any decomposition occurs, diseased larvae can be smeared on a microscope slide or pulled apart by pinching the cuticle about the centre of the body with two pairs of forceps, which are then pulled apart. The mid gut contents are left exposed on the slide, still within the gelatinous, transparent peritrophic membrane. This is partially or almost completely filled with bacteria, which are easily seen as opaque chalk-white clumps. The contents of the mid-guts of healthy larvae, which are less easily dissected, have a golden-brown colour. Apparently healthy larvae may contain a mixture of bacteria and pollen. The mid-gut of healthy larvae that contain much light-coloured pollen may resemble those that are filled with bacteria.

![Fig. 1. Bacteria associated with European foulbrood.](image)

(a) Melissococcus plutonius: the cause of European foulbrood occurs singly, in longitudinal chains or in clusters. Morphologically resembles Enterococcus faecalis, a common secondary invader.

(b) Paenibacillus alvei: vegetative rods 2.0–7.0 × 0.8–1.2 μm with flagella; sporulating with spores lying adjacently. Both rods and spores are larger than those of Paenibacillus larvae (see American foulbrood).

(c) Bacterium eurydice: slender, square-ended rods in vivo but can form chains of cocci in vitro in certain media.

For a bacteriological investigation, a loopful of a dilute aqueous suspension of the midgut contents is transferred to a clean microscope slide and mixed with a loopful of 5% aqueous nigrosin. This is spread over one or two square centimetres, dried gently over a flame, and examined directly by high-power microscopy. The presence of numerous lanceolate cocci, about 0.5 × 1.0 μm in size, occurring either singly or in clusters, and arranged end to end in pairs or short chains, is almost certainly diagnostic of European foulbrood. Some very slender square-ended rod-like bacteria are also usually present (Figure 1). The cocci are Gram positive and the rods are Gram negative. Similar preparations made from aqueous suspensions of whole dead or decomposing larvae are likely to present a confusing array of bacteria in which *M. plutonius* will be difficult to distinguish.

![Fig. 1. Bacteria associated with European foulbrood.](image)

(b) Culture methods

*Melissococcus plutonius* (type strain NCIMB 702443) is the most abundant bacterium during the early stages of an infection (Bailey & Collins, 1982a; 1982b). *Melissococcus plutonius* can be cultivated on a medium (expressed in g/litre or ml/litre) comprising: yeast extract or certain peptones, 10 (Bailey & Collins, 1982a); cysteine or cystine, 0.2–2.0; glucose or fructose, 10; soluble starch, 10; 1 M KH₂PO₄, 100 at pH 6.6; and agar, 2. The medium is preferably autoclaved in 100 ml lots in screw-capped bottles at 116°C for 20 minutes and poured into Petri plates immediately before use. These plates are streaked with dilute aqueous suspensions of diseased larvae, or ideally, of diseased larval mid-gut. The latter can be prepared beforehand by allowing them to dry on a slide, which may then be kept, for years if necessary, at 4°C or –20°C. All culture media should be subjected to quality control and must support the growth of *M. plutonius* from small inocula. The reference strain should also be cultured in parallel with the suspect samples to ensure that the tests are working correctly.

The preparation and storage of dried smears also eliminates most secondary organisms after a few weeks without affecting the viability of *M. plutonius*. This organism is isolated most efficiently by inoculating decimal dilutions of the aqueous suspension into agar that has been maintained molten at 45°C and which is then poured into plates. The plates must be incubated anaerobically, such as in McIntosh and Fildes jars in an atmosphere of approximately 5–10% carbon dioxide (CO₂) at 35°C. Small white opaque colonies of *M. plutonius* usually appear within 4 days. This bacterium is somewhat pleomorphic *in vitro*, often appearing in rod-like forms. The final pH of the medium may reach 5.5. Decreasingly fastidious strains become selected *in vitro*. Simplified or modified forms of the medium then support multiplication, especially of a serologically distinct *M. plutonius* group from Brazil (Allen & Ball, 1993) that will multiply on chemically
defined media (Bailey, 1984). CO₂ remains essential. Inoculated slopes should be sealed when bacterial growth is apparent and may then be kept at 4°C for up to 6 months. Alternatively, the cultures can be suspended in a medium of 10% sucrose, 5% yeast extract and 0.1 M KH₂PO₄, pH 6.6, and then lyophilised.

A number of other bacteria are often associated with and may be confused with *M. plutonius*. *Bacterium eurydice* inhabits the alimentary tract of adult bees and occurs commonly in the gut of healthy larvae in small numbers. It is more numerous in larvae infected with *M. plutonius*. The incidence of *B. eurydice* in healthy bees is very low in winter and early spring, but it increases in summer. It forms thin square-ended rods, which can grow either singly or in chains. When grown in certain media, it sometimes resembles streptococci and has been confused with *M. plutonius*. However, its cultural characteristics closely resemble those of *Corynebacterium pyogenes* (Jones, 1975), and it multiplies poorly in the form of thin rods, under the conditions necessary for the cultivation of *M. plutonius*.

*Enterococcus (= Streptococcus) faecalis* closely resembles *M. plutonius* morphologically and has often been confused with it, although they are both culturally and serologically distinct. Unlike *M. plutonius*, it does not remain viable for long when dried, or persist as mechanical contamination within bee colonies. It is probably brought into the hive by foraging adult bees, and is responsible for the sour smell sometimes encountered with European foulbrood.

*Enterococcus faecalis* grows well *in vitro* under the conditions suitable for *M. plutonius*, but it may be readily differentiated by its ability to grow aerobically. It forms small transparent colonies within 24 hours and is a facultative anaerobe. It multiplies on a variety of the more common media with or without carbohydrates or CO₂. The final pH in the presence of glucose is 4.0. *Enterococcus faecalis* rarely exceeds the number of *M. plutonius* in bee larvae, and can usually be diluted out. When it is not diluted out it produces sufficient acid to prevent the *in-vitro* multiplication of *M. plutonius*.

*Enterococcus faecalis* does not multiply in bee larvae in the absence of *M. plutonius*, so its presence in large numbers can be taken as presumptive evidence of European foulbrood.

*Paenibacillus (= Bacillus) alvei* is generally more common than *E. faecalis* in bee colonies affected with European foulbrood, but it is not invariably associated with the disease and so cannot act as a reliable indicator of it. In bee colonies, it multiplies only in the decomposing remains of larvae, and then its spores often predominate over all other bacteria, even to their apparent exclusion. *Paenibacillus alvei* forms very resistant spores and becomes well established in bee colonies with enzootic European foulbrood. It causes a characteristic stale odour. *Paenibacillus alvei* multiplies poorly under the conditions necessary for the *in-vitro* growth of *M. plutonius*. It produces a spreading growth of transparent colonies, some of which are motile and move in arcs over the surface of the agar. Cultures have the characteristic stale odour that is associated with European foulbrood when the bacillus is present. Spores are formed rapidly.

c) Immunological methods

For the identification of *M. plutonius*, antisera can be prepared in rabbits against washed cultures of *M. plutonius* either by intravenous injections (Bailey & Gibbs, 1962) or by a single intramuscular injection of 1 ml of antigen suspension mixed with an equal volume of Freund's incomplete adjuvant.

Assays are made by agglutination tests in tubes containing suspensions of bacteria equivalent to 0.25 mg dry weight/ml. End-points are noted after tubes have been incubated for 4 hours at 37°C.

A test kit for the identification of antibodies against *M. plutonius* has recently been developed and is commercially available. It provides rapid confirmatory on-site diagnosis of European foulbrood infection in honeybee larvae.

d) Polymerase chain reaction

Polymerase chain reaction (PCR) can be done on suspicious bacterial colonies transferred to and grown in liquid medium (Govan *et al.*, 1998). Genomic DNA is prepared according to standard methods (Wilson, 1990). The DNA pellet is resuspended in 50 µl of 1 × TE buffer (10 mM Tris/HCl, pH 7.5; 1 mM EDTA [ethylene diamine tetra-acetic acid]). Approximately 1–3 µg of genomic DNA is amplified in a 50 µl reaction. The PCR reaction can also be done with larvae. Each larva is incubated individually in liquid medium overnight at 30°C in an anaerobic jar containing hydrogen plus 10% CO₂. Two millilitres of each sample is then centrifuged at 1000 g for 2 minutes, and the supernatant is centrifuged at 10,000 g for 5 minutes. The resultant pellet is resuspended in 100 µl of sterile H₂O and heated at 95°C for 15 minutes. One microlitre is amplified in a 50 µl PCR mixture. Besides template DNA this mixture also contains 2 mM MgCl₂, 50 pmol of
Chapter 2.2.3. – European foulbrood of honey bees

forward (EFB-F) and reverse primer (EFB-R; primer sequences are given below) per µl, 25 mM (each) deoxynucleoside triphosphate and 1 U of Taq polymerase. Amplification of a specific DNA fragment occurs in a thermocycler under the following PCR conditions: a 95°C (1 minute) step; 30 cycles of 93°C (1 minute), 55°C (30 seconds), and 72°C (1 minute); and a final cycle of 72°C (5 minutes).

A hemi-nested PCR was first developed by Djordjevic et al. (1998) and thereafter improved for sensitive detection of M. plutonius in honey, pollen, whole larvae and adult bees (McKee et al., 2003). Here the first 50 µl reaction mixture contains 5–30 ng genomic DNA, 3 mM MgCl₂, 200 µM of each deoxyribonucleotide triphosphate, 100 ng of the primers MP1 and MP2, 5 µl of 10 × PCR buffer (100 mM Tris/HCl, pH 8.3; 15 mM MgCl₂; 500 mM KCl) and 1 U of Taq polymerase. Conditions of amplification consist of an initial denaturation cycle at 95°C for 2 minutes followed by 40 cycles of denaturation (95°C, 30 seconds), primer annealing (61°C, 15 seconds), primer extension (72°C, 1 minute) followed by an additional extension step of 5 minutes at 72°C. The third primer MP3 is used in conjunction with MP1 to amplify a DNA fragment from 1 µl of the primary PCR product obtained in the previous reaction. PCR conditions for the hemi-nested PCR are exactly as described above except that the MgCl₂ concentration is lowered to 1.5 mM and the annealing temperature to 56°C.

The molecular weights of the PCR products are determined by electrophoresis in a 1.0–1.5 % agarose gel and staining with ethidium bromide.

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<td>EFB-R</td>
<td>5’-TTA-TCT-CTA-AGG-CGT-TCA-AAG-G-3’</td>
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<td>MP2</td>
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<td>MP3</td>
<td>5’-TTA-ACC-TCG-CGG-TCT-TGC-GTC-TCT-C-3’</td>
</tr>
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2. Serological tests

No tests for detecting antibodies in bees are available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available.

ACKNOWLEDGMENT

Illustrations by Karl Weiss, extracted from Bienen-Pathologie, 1984. Reproduced with the kind permission of the author and Ehrenwirth-Verlag, Munich (Germany).

REFERENCES


Chapter 2.2.3. – European foulbrood of honey bees


* * *

NB: There are OIE Reference Laboratories for Bee diseases
(see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on diagnostic tests and reagents for bee diseases.
CHAPTER 2.2.4.
NOSEMOSIS OF HONEY BEES

SUMMARY

To date, two microsporidian parasites have been described from honey bees: Nosema apis (Zander) and N. ceranae (Fries). Nosema apis is a parasite of the European honey bee (Apis mellifera), and N. ceranae of the Asian honey bee (A. cerana) and the European honey bee. Nosema ceranae has recently been detected in several geographically separated populations of European honey bees in Europe, South and North America and Asia. The pathological consequences of N. ceranae in A. mellifera are not well known. In the following chapter, only N. apis is described. Both types are presumably very similar. Nosema apis is a parasite that invades the epithelial cells of the ventriculus of the adult honey bee. Infections are acquired by the uptake of spores during feeding or grooming. The disease occurs throughout the world, but treatment of bees can help to prevent the spread of infection to unaffected bee colonies.

The parasite invades the posterior region of the ventriculus, giving rise to large numbers of spores within a short period of time. The parasite is ubiquitous. Nosema levels generally increase when bees are confined, such as in the autumn and winter in colder climates when the amount of brood is decreasing and perhaps in the early spring when there is an increase in the brood. The disease is transmitted among bees via the ingestion of contaminated comb material and water, and by trophallaxis; honey stores and crushed infected bees may also play a role in disease transmission. Spores are expelled with the faeces where they may retain their viability for more than 1 year. Spores may also remain infective after immersion in honey and in the cadavers of infected bees; however they may lose viability after 3 days when submerged in honey at hive temperature. The relative importance of faeces, honey and cadavers as reservoirs of infective spores is not fully understood. However, it seems likely that faecal contamination of wax, especially in combs used for brood rearing, or other hive interior surfaces, provides sufficient inoculum for N. apis to be successfully transmitted to the next generation of bees. The spores are inactivated by acetic acid or by heating to 60°C for 15 minutes. To be effective, these treatments, which inactivate spores on hive surfaces and combs, can be combined with feeding colonies with the antibiotic fumagillin to suppress infections in live bees. The EU prohibits the use of antibiotic fumigation (EU 3/01/081).

Identification of the agent: In some acute cases, brown faecal marks are seen on the comb and the front of the hive, with sick or dead bees in the vicinity of the hive. However, the majority of colonies show no obvious signs of infection, even when the disease is sufficient to cause significant losses in honey production and pollination efficiency. During winter, there may be an increase in bee mortality. In affected bees, the ventriculus, which is normally brown, can be white and very fragile. Microscopic examinations (×400 magnification) of homogenates of the abdominal contents of affected bees will reveal the oval spores of N. apis, which are approximately 5–7 × 3–4 µm with a dark edge (N. ceranae is slightly smaller). Their internal contents can be distinguished after staining with Giemsa's stain. Nosema apis spores have a distinctive appearance, with a thick unstained wall and a blue-stained featureless interior. The nuclei within the spores are not visible. This method can help to distinguish N. apis from other microbes found in bees.

The appearance of N. apis spores can be confused with yeast cells, fungal spores, fat and calciferous bodies or cysts of Malpighamoeba mellifica. The latter are similar in size to Nosema spores, being 6–7 µm in diameter, but are completely spherical instead of oval.

Positive identifications can be made only by observation of typical spores in the ventriculus or faeces. Very mild infections may not be demonstrable. The extent of infection is determined by counting the spores on a microscope grid and calculating the average number of spores per area and estimating from that the number of spores per bee.
Serological tests: There are no applicable serological tests.

Requirements for vaccines and diagnostic biologicals: There are no biological products available.

A. INTRODUCTION

The microsporidium Nosema apis (Zander) is a protozoan parasite exclusive to the epithelial cells of the ventriculus of adult bees and the disease occurs throughout the world (Matheson, 1996). Infection occurs by the ingestion of spores in the feed (Bailey, 1981; Webster, 1993), via trophallaxis (Webster, 1993) or perhaps after grooming of the body hairs (Bulla, 1977; Fries, 1988; Webster, 1993). To date, two microsporidian parasites have been described from honey bees: N. apis (Zander) and N. ceranae (Fries). Nosema apis is a parasite of the European honey bee (Apis mellifera) and N. ceranae of the Asian honey bee (Apis cerana) (Fries et al., 1996) and the European honey bee. Nosema ceranae has recently been detected in several geographically separated populations of European honey bees in Europe (Higes et al., 2006), South and North America (Klee et al., 2007) and Asia (Huang et al., 2005).

The polar tube of the spore is everted and penetrates the peritrophic matrix of the intestine, particularly in the posterior region of the ventriculus. The sporoplasm passes down the tube and enters the cytoplasm of the epithelial cells, where it reproduces. Autoinfections can occur at the same time as new infections. After a short interval, spores develop in large quantities. The parasite is ubiquitous and multiplies at a specific rate throughout the year. Nosema levels generally increase when bees are confined, such as in the autumn and winter in colder climates when the amount of brood is decreasing and perhaps in the early spring when there is an increase in the brood (Webster, 1993; Weiser, 1961). In winter, spores are rarely to be found, or are only found in heavily infected bees.

Any inherent natural defence by a bee colony against a heavy infection with the parasite depends on the colony size as well as on the prevailing weather conditions during the early part of the autumn of the previous year (Steche, 1985). If these conditions are unfavourable, the overall life expectancy of the colony is reduced. This may lead to the premature death of bees during winter or early spring. In a typical case of a colony being depleted because of a Nosema infection, the queen can be observed surrounded by a few bees, confusedly attending to brood that is already sealed.

In faecal droppings, spores may retain their viability for more than 1 year (Bailey, 1962). Spores may also remain viable for up to 4 months after immersion in honey (White, 1919) and for up to 4.5 years in the cadavers of infected bees (Steche, 1985). The spores may lose viability after only 3 days when submerged in honey at hive temperature (Morgenthaler, 1939). It is likely that faecal contamination of wax, especially in combs used for brood rearing, or other hive interior surfaces, provides sufficient inoculum for N. apis to be successfully transmitted to the next generation of bees. The relative importance of faeces, honey and cadavers as reservoirs of infective spores is not fully understood and it seems that temperature may have a marked effect on the rates at which spores lose viability, regardless of their medium (Morgenthaler, 1939).

Spores may be killed by heating hive equipment or tools to a temperature of at least 60°C for 15 minutes. Combs may be sterilised by heating to 49°C for 24 hours (Cantwell & Shimanuki, 1970). Fumes from a solution of at least 60% acetic acid will inactivate any spores within a few hours, depending on the concentration; higher concentrations are even more effective and will kill spores within a few minutes (Bailey, 1957; de Ruiter & van der Steen, 1989). Such procedures come under the jurisdiction of national control authorities with protocols that vary from country to country. Disinfection can be carried out, for example, by putting acetic acid solution into bowls or on to sponges that can soak up the liquid. Following disinfection after an outbreak, all combs should be well ventilated for at least 14 days prior to use. Suppression of Nosema disease can also be achieved by feeding an antibiotic, fumagillin, in sugar syrup to the colony (Cantwell & Shimanuki). This is forbidden in many countries and in the EU.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

In acute forms of infection, especially in early spring, brown faecal marks may be noted on the comb and the front of the hive (Bailey, 1987). At the entrance to the hive, sick and dead bees may be seen, although other causes, such as pesticide poisoning and diseases of adult honey bees (such as acarapidosis should be eliminated first if this is the case. The detection of these infectious diseases requires microscopic examination. During winter, Nosema apis-infected colonies may become severely depleted of bees or die out altogether. The majority of Nosema apis-infected colonies will appear normal, with no obvious signs of disease even when the disease is sufficient to cause significant losses in honey production and pollination efficiency (Anderson & Giacon, 1992;
Fries et al., 1996). A proper diagnosis can be made only by microscopic examination of adult bee abdomen or ventriculus. To diagnose a Nosema apis infection, the posterior pair of abdominal segments is removed with a forceps to reveal the ventriculus, complete with the malpighian tubules, the small intestine and rectum. The ventriculus is normally brown but, following a Nosema infection, it can become white and fragile. However, this appearance is given by other causes of intestinal disturbance, for example feeding on indigestible food stores, such as syrup containing actively growing yeast. For a reliable diagnosis, a number of bees in a sample should be examined.

**Microscopy**

It is necessary to attempt to distinguish between a Nosema apis infection and an infection caused by Malpighamoeba mellificae (Webster, 1993). There is quite often an indication of dysentery in a Nosema apis infection. In an M. mellificae infection, there may be diarrhoea, often of a sulphur-yellow colour and with a distinct odour. Characteristics of M. mellificae cysts are described later. Secondary mixed infections may occur (Morgenthaler, 1939). A simple, nonquantitative method for detecting Nosema apis infection is as follows: sampled bees should be obtained from the hive entrance in order to avoid sampling individuals under the age of 8 days, which would lead to ‘false negatives’ because no spores from the protozoan in question would be determined. At least 60 bees should be collected in order to detect 5% of diseased bees with 95% confidence (Fries, 1988). Before sending to the laboratory, the bees should be fixed in 4% formol, 70% ethyl alcohol or frozen in a standard freezer in order to prevent them from decomposing and to improve their reception and organisation in the laboratory. The abdomens of the bees to be examined are separated and ground up in 2–3 ml of water. Three drops of the suspension are placed on a slide under a cover-slip and examined microscopically at ×400 magnification, under bright-field or phase-contrast optics. This is a slight simplification of Cantwell’s original method (Cantwell, 1970). The spores are about 5–7 µm long and 3–4 µm wide (Nosema ceranae is slightly smaller than Nosema apis). They are completely oval with a dark edge. Their contents, consisting of nucleus, sporoplasm and polar tube, cannot be seen. Dyes are usually not necessary.

Nosema spores must be differentiated from yeast cells, fungal spores, fat and calciferous bodies, and from M. mellificae cysts, which are spherical and approximately 6–7 µm in diameter.

When air-dried, ethanol-fixed smears of infected tissue are stained with Giemsa’s stain (10% in 0.02 M phosphate buffer) for 45 minutes. Nosema apis spores will have a distinctive appearance, with thick unstained walls and an indistinct blue interior, without visible nuclei. Insect cells, fungal spores and other protozoa stained in this way will generally have thinner walls, blue/purple cytoplasm and magenta-coloured nuclei.

In order to obtain accurate, reliable and meaningful quantification of levels of Nosema infections in honey bees, a standardised procedure must be used. A suitable protocol is as follows:

A sample of older worker honey bees is taken, from which the abdomens of ten individuals are macerated in 5 ml of water using a mortar and pestle. When tissue pieces have become quite fine, the suspension is filtered through two layers of muslin (thin loosely woven cotton fabric) in a funnel leading to a graduated centrifuge tube. A second 5 ml of water is used to rinse the pestle, swirl around the inside of the mortar and pour through the subsample in the funnel. Water levels are equalised in the tubes and the suspensions are centrifuged for 6 minutes at 800 g. The supernatants are decanted and the tubes are refilled to the 10 ml level. Using disposable pipettes and a rubber bulb, the pellets are resuspended by repeated uptake and forcible ejection through the pipette tips. When the solution appears to be homogenous, a sample is taken to fill the calibrated volume under the cover-slip of a haemocytometer (blood cell counting chamber). After a few minutes the spores will have settled to the bottom of the chamber. Nosema spores appear transparent but with a very distinct dark edge and are 5–7 µm long and 3–4 µm wide. They are best seen using a magnification of ×400 and bright-field or phase-contrast optics. The number of spores in each square is counted. Where a spore lies over the edge of a square, count only those spores that straddle the left and upper edges of the square, not the right and bottom edges. One Nosema apis spore, observed in the haemocytometer’s entire central square millimetre grid (25 × 16 = 400 small squares), is equal to an average of 10,000 spores per bee. If no spores are seen, the result should be designated ‘not detected’, but that does not mean that the bees are not infected. Regulatory agencies will decide on the level of infection useful for their purposes.

A laboratory method for the simultaneous detection of Nosema spores and M. mellificae cysts consists of the individual examination of the colonies using 30–60 bees per colony. A suspension of the abdomens of dead bees is prepared by grinding with 5–10 ml water; the volume of water depending on the number and condition of the bees. The suspension must be filtered to remove debris that would interfere with the examination, first through a 100 µm and then a 40 µm filter. Parts of the malpighian tubules pass through the 100 µm filter, but are collected on the 40 µm filter. They are placed on a slide or bacterial counting chamber and examined at ×400 magnification. Only a few tubules are filled with cysts after an M. mellificae infection.
The normal structure of malpighian tubules is not visible in this case. Only cysts inside the malpighian tubules can be taken as a positive result, because *M. mellificae* cysts are often confused with fungal spores and yeast cells.

b) **Culture**

There are no cultural methods for growing these organisms.

c) **Polymerase chain reaction (PCR)**

Different methods have been developed to distinguish *N. apis* from *N. ceranae*. A multiplex PCR is described below with which both pathogen types can be clearly identified at the same time (Martin-Hernandez et al., 2007).

- **Sample preparation for PCR**

  The abdomens of 10–20 adult honey bees from each sample are macerated in 10 ml distilled water (PCR grade) and the suspension is then filtered and centrifuged at 800 g for 6 minutes. For DNA extraction, spore germination is induced with 200 µl freshly prepared germination buffer (0.5 M sodium chloride, 0.5 M sodium hydrogen carbonate, pH to 6.0 with orthophosphoric acid), and the mixture is incubated at 37°C for 15 minutes. The DNA extraction can be easily carried out using routine procedures or commercial kits, such as High Pure PCR Template Preparation Kit (No. 1796828 Roche Diagnostic).

- **Multiplex PCR**

  With this technique both microsporidians (*N. apis* and *N. ceranae*) can be distinguished in just one PCR because of the use of specific primers with no interference. PCR reactions are performed in 50-µl volumes containing 5 µl of template DNA, 25 µl of High Fidelity PCR Master Mixture (catalogue no. 12140314001; Roche Diagnostic), 0.4 µM of each primer, 0.4 mM of each deoxynucleoside triphosphate, 3 mM Cl₂Mg, 0.2 mg/ml bovine serum albumin, 0.1% Triton X-100, and 5 µl of *N. apis* or *N. ceranae* DNA template. The parameters for amplification are: an initial PCR activation step of 2 minutes at 94°C, followed by 10 cycles of 15 seconds at 94°C, 30 seconds at 61.8°C, and 45 seconds at 72°C, and 20 cycles of 15 seconds at 94°C, 30 seconds at 61.8°C, and 50 seconds at 72°C plus a 5-second elongation cycle for each successive cycle and a final extension step at 72°C for 7 minutes. Negative controls (from DNA extraction) are included in all PCR experiments.

  The molecular weights of PCR products are determined by electrophoresis in a 2% agarose TAE (Tris-acetate-ethylene diamine tetra-acetic acid) gel in standard TAE buffer, stained with ethidium bromide, and visualised using UV illumination.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence⁹</th>
<th>PCR product size (bp)</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>218MITOC FOR</td>
<td>5'-CGGCCGACGATGTGATATGAAA-ATATTAA-3'</td>
<td>218–219⁹</td>
<td><em>N. ceranae</em></td>
</tr>
<tr>
<td>218MITOC REV</td>
<td>5'-CCCCTGCATTCTCAACAAAA-AACCG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>321APIS FOR</td>
<td>5'-GGGGGCATGTCTTTGACGTACTATGTA-3'</td>
<td>321</td>
<td><em>N. apis</em></td>
</tr>
<tr>
<td>321APIS REV</td>
<td>5'-GGGGGGCGTTAAAATGTGAAACAACGTATG-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

⁹CG tails added to primers are underlined.

²There is a 1-bp difference in the *N. ceranae* amplicon size depending on the sequences for *N. ceranae* available in GenBank (http://www.ncbi.nlm.nih.gov).

2. **Serological tests**

There are no serological tests available.

C. **REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS**

No biological products are available.
REFERENCES


* *

NB: There are OIE Reference Laboratories for Bee diseases
(see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list:
Please contact the OIE Reference Laboratories for any further information on diagnostic tests and reagents for bee diseases
**CHAPTER 2.2.5.**

**SMALL HIVE BEETLE INFESTATION**

*(Aethina tumida)*

**SUMMARY**

The small hive beetle *Aethina tumida* Murray 1867 (Coleoptera: Nitidulidae), is a parasite and scavenger of honey bee colonies. Adults and larvae feed on honey bee brood, honey and pollen, causing death of brood, fermenting of honey and comb destruction, often resulting in the full structural collapse of the nest and absconding of the colony. The small hive beetle can be a serious problem in honey-extracting facilities where stored comb, honey and wax cappings are all potential feeding and breeding areas. Development requires 3–12 weeks, depending on temperature and food availability. The flying adult beetles actively infest colonies.

**Identification of the agent:** An infestation by *Aethina tumida* can be recognised either indirectly via colony-wide damage or directly via eggs, larvae and adults. An early diagnosis can be made after opening the colony and finding adult beetles on the bottom board or hiding in the combs. Intra- and extra-colonial acaricides and insecticides presently are used to kill the adult beetles and larvae while intra- and extra-colonial traps can be used to find the beetles.

**Serological tests:** No serological tests are applicable.

**Requirements for vaccines and diagnostic biologicals:** No biological products are available.

**A. INTRODUCTION**

The small hive beetle, *Aethina tumida* Murray, Coleoptera: Nitidulidae (Murray, 1867), is native to sub-Saharan Africa (Hepburn & Radloff, 1998) but has been introduced to the United States of America (1996), Egypt (2000) and Australia (2002) (Neumann & Elzen; 2004). It was introduced in to Canada in 2002 but did not establish; it was reintroduced in 2006 and it has not been determined if it is established. *Aethina tumida* can be spread by active flying, migratory beekeepers, or transportation of infested hive products (Hood, 2004; Neumann & Elzen; 2004). Larvae and eggs of *A. tumida* have been identified in cages of imported queens in Portugal (2004), but all bee hives were immediately destroyed (pers. comm.). Within its native range, it is usually considered a minor pest, and reproduction appears more successful in weak and stressed colonies or in recently abandoned nests (Neumann & Elzen; 2004). However, within its new ranges it can cause considerable damage in colonies of European honey bee subspecies (Ellis, 2004; Elzen et al., 1999; Hood, 2004; Neumann & Elzen; 2004).

1. **Life cycle**

The infesting *A. tumida* females mate in the colony (more than 1000 adult beetles may occur within a colony [Elzen et al., 1999]) and oviposit several eggs in typical clutches in small cracks, in cells or within capped brood (Ellis et al., 2003c; Lundie, 1940; Neumann & Elzen; 2004). The larvae hatch after 1–6 days and feed on pollen, honey and bee brood like the adults (Lundie, 1940; Neumann & Elzen; 2004; Schmolke, 1974). Adult beetles also can be fed by worker bees via trophallaxis (Ellis et al., 2002). Larval development takes 8–29 days (depending on food availability and temperature [Ellis et al., 2002; Lundie, 1940; Neumann & Elzen; 2004; Schmolke, 1974]) following which they reach the wandering phase (Lundie, 1940) and leave for pupation in the soil, mostly in close proximity to the hive (Pettis & Shimanuki, 2000). Pupation takes 2–12 weeks depending on temperature and soil moisture (Ellis et al., 2004a). Emerging adults leave the soil and can actively fly over long distances (>10 km) to search for new host colonies, thereby completing the life cycle of *A. tumida*.

The reasons for the apparent difference in the impact of the small hive beetle with its native range compared with its new ranges are not well understood (Ellis, 2004). They may include quantitative behavioural differences between African and European honey bee subspecies, different beekeeping techniques and climatic differences (Ellis, 2004; Hood, 2004; Neumann & Elzen; 2004).
Adult beetles can survive up to 6 months and females can oviposit about 1000 eggs in their life time (Lundie, 1940). While damage due to the adults is relatively minor, it nevertheless can cause absconding of the colony (Ellis et al., 2003b). If not prevented by the bees (Ellis et al., 2003c; Neumann & Härtel, 2004), larval growth (several hundreds or thousands of individuals) is usually associated with fermentation of the honey, causes severe damage to combs and often results in the full structural collapse of the nest (Hepburn & Radloff, 1998). Economic losses can also be associated with beetle infestations in the honey-extracting facility. Environmental conditions generally associated with extracting facilities, such as high temperature and humidity, provide optimal condition for beetle development. Cryptic low-level reproduction may also occur either in the debris or underneath hive inserts without any signs of damage to the beekeeper (Schmolke, 1974).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The first sign of an infestation by *A. tumida* is the occurrence of adult beetles (~5 mm length and ~3 mm width, females slightly longer than males [Ellis et al., 2004b]), with a dark brown to black colour (lighter shortly after eclosion) in the colony (Fig. 1). During inspections, adults avoid sunlight, hide, and can be observed while running for cover into corners or in a typical fashion over the combs. Adults can be confused with other beetles from the same family, which can also be associated with colonies (e.g. *Cychramus luteus* [Neumann & Ritter, 2004]).

Fig. 1. *Aethina tumida* adult. Photo by N. Ruppert.

a) Beetle eggs, larvae and pupae

Eggs are white and bean-shaped (~2/3 of the size of a honey bee egg) and oviposited in clutches (up to 210) in cracks, on the bottom board, on the combs and underneath sealed brood (Ellis et al., 2003c). Larvae are whitish, often covered with a slimy sticky coating, up to 1.2 cm long (wandering phase) and have three pairs of legs and dorsal spikes. Larvae can be found mining in combs (Lundie, 1940) or in the debris (Spiewok & Neumann, 2006). Larval infestations are typically associated with a rotten smell (e.g. rotting orange). Wandering larvae often leave smear trails inside and outside the colony. Such wandering larvae and pupae (whitish, ~5 mm long and 3 mm wide) can be found in small pupation chambers 1–20 cm deep in the soil usually in close proximity to colonies (<180 cm, [Pettis & Shimanuki, 2000]).

b) Colony examination

When monitoring honey bee colonies for the presence of *A. tumida*, an examination of the hive may provide an early indication of infestation. Adult beetles can be observed hiding inside cells and in the debris. Colony examinations start by removing the hive roof and placing it upside down on the ground next to the hive. Remove the hive chamber, i.e. supers and upper brood chamber (in double brood chamber colonies) and place them on the upturned roof for a few minutes. Place the hive crown board on top. A few minutes later lift the boxes out of the way and scan for beetles on the inner surface of the upturned roof. Then frames are screened one by one for the presence of adults, larvae and eggs. During cool weather, adults tend to stay close to or within the bee cluster. In warmer periods beetles are found more often on the bottom board or the outer-most frames.

c) Board examination using traps

Less labour intensive diagnosis is feasible using hive inserts. Such inserts allow for the beetles to hide in the corrugations but prevent bees from entering. They can be placed on the bottom board. To detect the beetles, place a piece of corrugated cardboard (15 cm × 15 cm), with one surface peeled to expose the ridges, on the bottom board of the bee hive with the ridged side down. Cover it with wood to fit underneath the frames on the bottom board. Leave the insert in the colony for ≤ 3 days, remove it and examine for
2. Serological tests

No serological tests are available for routine laboratory diagnosis.

3. Treatment

In countries with infestations of *A. tumida*, control has focused on chemical treatments with acaricides and insecticides (Baxter *et al*., 1999a; 1999b; Elzen *et al*., 1999). Acaricides, non-toxic to bees, are used in traps intra-colonially (Elzen *et al*., 1999) to kill the adults. Similarly, insecticides are used as a ground drench to kill wandering larvae and pupae (Baxter *et al*., 1999a; 1999b). Such treatments carry the risks of both pest resistance and residues in the hive products (Neumann & Elzen, 2004). Alternative, more sustainable controls are moderately efficient so far (Ellis, 2004; Hood, 2004; Hood & Miller, 2005; Neumann & Elzen, 2004) and currently under investigation (Ellis, 2004; Ellis *et al*., 2001; 2003a; Hood, 2004; Hood & Miller, 2005; Muerrle *et al*., 2006).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available.

REFERENCES


Chapter 2.2.5. – Small hive beetle infestation (Aethina tumida)


FURTHER READING

An FAO publication, Honey bee diseases and pests: a practical guide, W. Ritter & P. Akatanakul (eds). Agricultural and Food Engineering Technical Report No. 4. FAO, Rome, Italy, 42 pp. ISSN 1814-1137 TC/D/A0849/E, is available free of charge at:

* *

NB: There are OIE Reference Laboratories for Bee diseases (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ ). Please contact the OIE Reference Laboratories for any further information on diagnostic tests and reagents for bee diseases
CHAPTER 2.2.6.

TROPILAEELAPS INFESTATION OF HONEY BEES

(Tropilaelaps spp.)

SUMMARY

The mites in the genus Tropilaelaps are parasites of honey bee brood. Feeding on bee larvae and pupae causes brood malformation, death of bees and subsequent colony decline or absconding. Development requires about 1 week, and the mites are dispersed on bees. There are at least four species in the genus Tropilaelaps. Each species is closely associated with a particular giant honey bee in Asia. Two species (T. clareae and T. mercedesae) are damaging pests of Apis mellifera. The other two species (T. koenigerum and T. thaii) appear to be harmless to A. mellifera.

Identification of the agent: Molecular and morphological methods are available for identifying each species. An infestation by Tropilaelaps can be recognised either visually on bees or by examining hive debris. Irregular brood pattern, dead or malformed immatures, bees with malformed wings that crawl at the hive’s entrance, and especially the presence of fast-running, large, red-brown, elongated mites on the combs, are diagnostic for the presence of T. clareae. An early diagnosis can be made after opening brood cells and finding immature and adult mites therein. The hive (colony) may be treated with various chemicals that cause the mites to drop off combs and bees. Sticky boards on the bottom of the colony can be used to examine hive debris and mites.

Serological tests: No serological tests are applicable.

Requirements for vaccines and diagnostic biologicals: No biological products are available.

A. INTRODUCTION

The mite species Tropilaelaps clareae, previously assumed to be ubiquitous in Asia, has been found to be two species. Tropilaelaps clareae occurs in Asia where it is a parasite of the native honey bee Apis dorsata breviligula. It is also a parasite of the introduced honey bee species A. mellifera in the Philippines and the native honey bee species A. dorsata binghami on Sulawesi Island in Indonesia. Tropilaelaps mercedesae, which until now was mistaken for T. clareae, together with T. koenigerum, parasitises the native A. dorsata dorsata in mainland Asia and Indonesia (except Sulawesi Island). Tropilaelaps mercedesae is also a parasite of the introduced A. mellifera in these and surrounding regions and, with another species, T. thaii, also parasitises A. laboriosa in mountainous Himalayan regions (Anderson & Morgan, 2007).

1. Life cycle

The colonising Tropilaelaps female (or females; as many as a dozen may occur within individual a single cells) places from one to four eggs on mature bee larvae shortly before the brood cell is capped. The drone brood is preferred by Tropilaelaps and may be almost 100% parasitised (Burgett et al., 1983). The mite progeny, usually one male and several females feed on and seriously damage the bee brood. Development of the mite requires about 1 week. The adults, including the foundress female, emerge with the adult bee and search for new hosts.

The short life-cycle, as well as a very brief stay on adult bees, explains why populations of T. clareae increase faster than those of Varroa mites. When both T. clareae and Varroa destructor infest the same colony, the former may out-compete the Varroa mite (Burgett et al., 1983; Ritter & Schneider-Ritter, 1988). It has been reported that when both mite species are in the same cell, the reproduction of both mites declines (Rath et al., 1995).
Phoretic survival on bees is quite short (only 1–2 days) because Tropilaelaps cannot pierce the integument of adult bees. The phoretic time for Tropilaelaps spp. is important in understanding the life cycle, and recent research suggests the period can be as long as 5–10 days (Wilde, 2000a; 2000b). Gravid female mites will die within 2 days unless they deposit their eggs (Woyke, 1987).

Infestation by Tropilaelaps causes the death of many bee larvae (up to 50%), resulting in an irregular brood pattern and of which the cadavers that may partially protrude from the cells. Many malformed bees occur, with distorted abdomens, stubby wings and deformed or missing legs. Some of the affected bees crawl at the hive’s entrance (Atwal & Goyal, 1971). In addition, perforated cappings are seen, the result of sanitation activities by the worker bees, which evict the infested bee pupae or young adults. Some infested colonies abscond, carrying the mites to a new location.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The first sign of an infestation by Tropilaelaps species is often the occurrence of large, red-brown, elongated mites on the combs or on adult bees (Figs 1 and 2). Tropilaelaps clareae (<1 mm in length), and T. mercedesae (< 9 mm in length) differ in body size but otherwise are alike. Tropilaelaps koenigerum is slightly smaller, only about 0.7 mm in length (Delfinado-Baker & Baker, 1982). The females also differ in the structure of their ventral anal plate and subapical tooth of the chelicerae (Anderson & Morgan, 2007). Tropilaelaps can easily be recognised and separated from the Varroa mite using a ×10 magnifying glass. The body of the Varroa mite is wider than it is long and it moves slowly, whereas the body of Tropilaelaps is elongated, with a heavily sclerotised holoventral or similar shield (Fig. 3), and it is a fast-running mite.

![Fig. 1. Tropilaelaps clarea. Photo by J. Waddell.](image1)

![Fig. 2. Tropilaelaps on Apis dorsata larvae. Photo by D. Anderson.](image2)
Chapter 2.2.6. — Tropilaelaps infestation of honey bees (Tropilaelaps spp.)

Fig. 3. Tropilaelaps offspring on Apis mellifera pupae. Photo by W. Ritter.

a) Mite collection

Methods to collect mites include an ether or sugar roll (Ritter & Schneider-Ritter, 1988). Collect approximately 100–200 bees in a wide-mouthed jar with lid. Scrape the bees into the jar or use a modified vacuum to suck them in. Knock the bees to the bottom of the jar with a sharp blow; there should be about a 1–2 inch (2.54–5.08 cm) layer of bees on the bottom. Remove the lid and spray a 2-second burst with ether starter fluid. Alternatively, use enough 70% alcohol or soapy water to cover the bees; or add around 25 g (1 oz) powdered sugar (or flour). If using ether replace the lid and agitate or roll the jar for about 10 seconds; mites should stick to walls. If using soap or alcohol, agitate and then strain out the bees with a coarse hardware cloth or mesh strainer; mites will be in the liquid. If using sugar or other powder, put screening material (such as hardware cloth) on top of the jar and shake the mites on to white paper to count; repeat every 2 minutes. For a more accurate count, finish with an alcohol or soapy water wash to collect all the mites.

b) Colony and brood examination

When monitoring honey bee colonies for the presence of Tropilaelaps (or Varroa), an examination of both drone and worker brood may provide an early indication of infestation. Mites can be observed inside capped bee brood by using a honey scratcher (with fork-like tines) to pull up capped pupae. The mites are clearly visible. The younger mite stages are whitish and may be almost motionless while feeding on their hosts’ bodies, as their mouthparts and front legs are fixed to the cuticle of the bee host (Ritter & Schneider-Ritter, 1988). The extent of parasitisation can be estimated by opening a predetermined number of brood cells; infestation rates are then calculated as per cent of capped brood containing live mites (Burgett & Kitprasert, 1990).

c) Sticky board examination

A precise diagnosis can be made using a sticky board covered with a mesh, such as fly screen, that prevents the bees from removing the dislodged mites. The mesh must be large enough for mites to pass through. Make a sticky board with poster board, cardboard or other white, stiff paper coated with Vaseline or other sticky substance (Koeniger et al., 2002; Ostiguy & Sammataro, 2000; Sammataro et al., 2000), or use a sheet of sticky shelf paper. Cut the paper to fit the bottom board of a hive. Cut a piece of hardware cloth or screen to fit on top of the sticky board. To keep the bees from cleaning off the board, fold under the outside edges of the screen to raise it off the board, and staple or tape in place. Leave the board in the colony for up to 3 days, collecting and examining the debris for mites. For faster mite diagnosis, smoke each colony adding 25 g (1 oz) pipe tobacco in the smoker. Puff the bees 6–10 times, close up the hive for 10–20 minutes. Pull out the sticky board after 10 minutes and count the mites. Acaricides are sometimes used to knock mites off bees and will appear on the sticky boards.

2. Serological tests

No serological tests are available for diagnosis.

3. Treatment

In countries with infestations of Tropilaelaps spp., fluorvalinate in slow-release formulations controls Tropilaelaps (Lubinevski et al., 1988; Pongthep, 1990), as do monthly dustings with sulphur (Atwal & Goyal, 1971) and
treatments with formic acid (Garg et al., 1984). The inability of this mite to feed on adult bees, or to survive outside sealed brood for more than a few days, such as caging the queen for a few weeks, is being used as a non-chemical control method (Woyke, 1987; 1993).

Many of the same acaricides used for Varroa will kill Tropilaelaps. Strips of plastic-impregnated fluvinate (Apistan™) will kill mites. Alternatively, tobacco smoke in the smoker will cause mites to drop off bees. Strips of filter paper, available in some countries are prepared by soaking in an aqueous solution of 15% potassium nitrate to which two drops of amitraz (usually 12.5%) are added (Lubinevski et al., 1988). After the paper dries, the strip is ignited and inserted into the hive. The smoke causes many mites to drop off. Another method is to use plates or pads soaked with 20 ml of 65% formic acid (very caustic and will burn hands and face). The pads are placed in the colonies, near the top (Hoppe et al., 1989).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available.

REFERENCES


Chapter 2.2.6. — Tropilaelaps infestation of honey bees (Tropilaelaps spp.)


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NB: There are OIE Reference Laboratories for Bee diseases (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the OIE Reference Laboratories for any further information on diagnostic tests and reagents for bee diseases.
CHAPTER 2.2.7.

VARROOSIS OF HONEY BEES

SUMMARY

The mite Varroa destructor (formerly Varroa jacobsoni) is a parasite of adult bees and their brood. It penetrates the intersegmental skin between the abdominal sclera of adult bees to ingest haemolymph. It can sometimes be found between the head and thorax. The number of parasites steadily increases with increasing brood activity and the growth of the bee population, especially late in the season when clinical signs of infestation can first be recognised. The life span of the mite depends on temperature and humidity but, in practice, it can be said to last from some days to a few months.

Identification of the agent: The clinical signs of varroosis can only be recognised at a late stage of infestation, so that diagnosis entails the examination of the hive debris. The debris produced during the summer is especially useful for diagnosis. The earliest and most precise diagnosis can be made only after the application of a medication that forces the mites to drop off the bees or kills them directly. Larger amounts of debris can be examined using a flotation procedure. Bees are washed in petroleum spirit, alcohol or detergent solution. However, this method is less accurate due to the unequal distribution of mites and the usually small sample sizes.

Serological tests: No serological tests are applicable.

Requirements for vaccines and diagnostic biologicals: No biological products are available.

A. INTRODUCTION

The Varroa mites are parasites of adult bees and their brood. Four species have been recorded: Varroa jacobsoni, V. destructor, V. underwoodi and V. rinderi. Until recently Varroa mites that affect Apis mellifera worldwide were assumed to be V. jacobsoni. However it has been shown that these mites are V. destructor (Figure 1).

Fig. 1. Varroa on pupa and adult bee. Left: pupa with four Varroa female mites. Right: worker bee with two female mites.

They are responsible for the condition of varroosis or varroatosis (Anderson, 2000; Anderson & Trueman, 2000). The mite inserts itself between the abdominal sclera in adult bees (Ritter, 1980) where it penetrates the intersegmental membranes in order to ingest haemolymph. Sometimes it can also be found between the head and thorax. For reproduction, the female enters the cells with the bee brood shortly before the cells are sealed. They prefer drone brood to worker brood. After the brood cell is sealed, the mite lays after 2 to 3 days the first egg.
(generally male). Later up to seven eggs (generally females) are laid in intervals of about 1–2 days. These hatch into nymphs, but only two to three reach the adult stage (Figs 2 and 3).

**Fig. 2.** Oviposition and development of Varroa in brood cells of worker bee (until about 9th day unsealed brood, until about 21st day sealed brood).

**Fig. 3.** Development of Varroa: E = Egg, L = Larva, P = Protonymph, D = Deutonymph, A = Adult (Sex of eggs, larvae and protonymphs can only be distinguished by examining the chromosomes).
Chapter 2.2.7. — Varroosis of honey bees

The number of mites usually increases slowly at the beginning of the season. Clinical signs may be seen at any time during the active season, although usually maximum numbers are reached late in the season (Figure 4), when the first clinical signs of infestation can be recognised. The course of this parasitism is usually lethal, except in some areas, such as tropical Latin America (De Jong, 1997; Ritter et al., 1984). The life span of mites on larval or adult bees depends on temperature and humidity. Under practical conditions, the life span may vary from some days to a few months.

![Fig. 4. Graph of populations of bees and mites over 1 year in a temperate Northern Hemisphere climate: brood numbers (solid line); mite numbers (broken line).](image)

In heavily infested bee colonies, clinical signs of varroosis can often first be seen in the latter part of the season when the brood is reduced (Ritter et al., 1984). Heavy infestations are usually reached 3–4 years after the primary invasion, but can occur within weeks if infested by bees from nearby colonies that are collapsing.

Essentially, the brood is damaged by the parasitic mites. Bees and their offspring that have been infected during the brood phase by only one parasitic mite show various ill effects, such as a shortened life span, changes in behaviour and an increased disease susceptibility (De Jong & Goncalves, 1982). The parasitism is critical if more than one mite enters the brood cell for reproduction. Only in the lethal stage immediately before the collapse of the colonies do clinical signs, such as shrunken wings and shortened abdomen, appear (Figure 5). This is due to an increased susceptibility to deformed wing and acute paralysis virus, as well as to the infection of wounds and loss of haemolymph (Bailey, 1981; Ball, 1985). If the brood dies shortly before or after sealing, clinical signs of European foulbrood appear without the presence of the specific agent *Melissococcus pluton*. If the brood survives, the emerging bees show various behavioural changes and their life span is considerably shortened (De Jong & De Jong, 1983; Ritter, 1996).

![Fig. 5. Effect of Varroa on bee morphology. Left: normal bee appearance. Right: bee heavily attacked by mites. This newly emerged bee has a deformed wing and reduced abdominal volume.](image)

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The female mite is a dark reddish/brown colour and has a flat, oval-shaped body approximately 1.1 mm × 1.5 mm. It is the only common parasite of honey bees that can be seen with the naked eye (Shimanuki & Knox, 1991).
a) Debris examination

An easy method of diagnosis of varroosis is by the examination of the debris generated by bees themselves. An insert covered with a screen mesh is placed on the floor of the hive. Unless this insert is covered with such a gauze, or smeared with grease, the bees will dispose of the mites outside the hive.

The debris produced within a few days in the late season usually contains little other than visible mites (Fries et al., 1994; Ritter, 1996). The debris collected in winter, however, must be examined in the laboratory. An insert is placed in the hive as before, but an effective medication is used to cause the mites to fall off the bees, so that after a given time, a number of mites may be observed on the floor insert. Some countries demand the diagnostic application of certain medications for proving the absence of mites.

Large amounts of debris can be examined in the laboratory using a flotation procedure (Brem, 1980).

- **Test procedure**
  i) Dry the debris for 24 hours.
  ii) Flood the debris with industrial alcohol.
  iii) Stir continuously for around 1 minute or, if debris contains wax or propolis particles, stir for 10–20 minutes.
  iv) Identify and observe the mites that float to the surface.

b) Brood examination

For the second method, drone brood is examined, if available, otherwise worker brood is examined.

When a large number of samples are examined, a rough determination of the degree of infection can be obtained.

- **Test procedure**
  i) Remove the cappings of the brood cells with a knife.
  ii) Wash the brood cells directly into a sieve system with warm water from a hand-held shower.
  iii) Collect the mites in the lower fine sieve (mesh width 1 mm) while the brood is gathered in the upper coarse sieve (mesh width 2–3 mm).
  iv) Place the contents of the sieve on a bright plate, where the mites can be easily identified and counted.

When a smaller number of samples are being studied, the individual cells are examined using an appropriate source of light. After removing the cappings and the bee brood, infected cells can be identified by the presence of small white spots – the faeces of the mite – found on the cell wall. The mites themselves should be sought for confirmation, by examining the bottom of the cell and the bee brood for attached mites.

c) Bee examination

In a third method, approximately 200–250 bees are removed from unsealed brood combs. Samples should be taken from both sides of at least three uncapped brood combs. To determine an apiary's percentage of infestation, it is necessary to collect and analyse individual samples from at least 10% of the beehives, and to determine later the average infestation rate based on these individual results.

- **Test procedure**
  i) Kill the bees in a special container by submersion in alcohol.
  ii) Stir the container for 10 minutes.
  iii) Separate the bees from the mites by means of a sieve with a mesh size of approximately 2–3 mm.

Under some circumstances, the Varroa mite may be confused with the bee louse, Braula coeca (Figure 6). The latter is round, not oval, and being an insect, has only three pairs of legs. A number of different species of mite may be associated with Varroa mites on bees, but these are easily distinguished. In addition, other parasitic mites, such as those of the Tropilaelaps spp., are known to cause similar damage to bee colonies as the Varroa mites.

2. Serological tests

No serological tests are available for routine laboratory diagnosis.
Chapter 2.2.7. — Varroosis of honey bees

Fig. 6. Diagram of Varroa destructor (formerly Varroa jacobsoni Oudemans) (female).

a) Dorsal aspect
b) Anterior aspect Note the flat shell-like back and four pairs of legs.
c) Ventral aspect
d) The bee louse (Braula coeca, female). Note the lack of shell-like back and only three pairs of legs.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products and vaccines available. Several medicaments and substances like Formic acid, oxalic acid, lactic acid and thymol can be used to control Varroa mites (http://www.apis.admin.ch/english/Themes/Varroa.htm). Some hygienic honeybee strains are less susceptible to Varroa parasites.

• Acknowledgement

Illustrations by Karl Weiss, extracted from Bienen-Pathologie, 1984. Reproduced with the kind permission of the author and Ehrenwirth-Verlag, Munich (Germany).

REFERENCES


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**NB:** There are OIE Reference Laboratories for Bee diseases
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Please contact the OIE Reference Laboratories for any further information on diagnostic tests and reagents for bee diseases.
SECTION 2.3.

AVES

CHAPTER 2.3.1.

AVIAN CHLAMYDIOSIS

SUMMARY

Avian chlamydiosis (AC) is caused by the bacterium Chlamydophila psittaci. AC occurring in humans and all birds was originally called psittacosis, but later the term ornithosis was introduced to identify the disease contracted from or occurring in domestic and wildfowl, while the name of the disease contracted from or occurring in psittacine birds remained psittacosis. These diseases are similar when contracted by humans. The genus Chlamydia was divided into two, Chlamydia and Chlamydophila. A proposal to re-combine them into the single genus Chlamydia is under consideration but has not been adopted for this chapter. The avian strains of Chlamydomphila psittaci include at least fifteen genotypes, some of which correlate with the avian species from which they are usually isolated. Chlamydiosis as it occurs naturally in mammalian species and not contracted from avian species is caused by different species of the organism.

Depending on the virulence of the chlamydial strain and the avian host defence, chlamydiae cause pericarditis, conjunctivitis, sinusitis, airsacculitis, pneumonia, lateral nasal adenitis, peritonitis, hepatitis, and splenitis. Generalised infections result in fever, anorexia, lethargy, diarrhoea, and occasionally shock and death. Special laboratory handling (biosafety level 3) is recommended because avian chlamydial strains can cause serious illness and possibly death in humans. While the disease in psittacine birds is best known, the infection in ducks and turkeys is of particular concern as transmission to humans is common during handling and slaughter of the birds. The diagnosis of AC requires the isolation and identification of the organism, the demonstration of chlamydiae in tissues, or the demonstration of a four-fold increase in specific humoral antibody, as well as typical clinical signs.

Identification of the agent: Isolation of chlamydiae requires the inoculation of embryonated eggs or cell cultures and testing for chlamydiae by cytochemical stains or immunohistochemical methods. The direct inoculation of samples into cell cultures is preferable as they are as sensitive for the isolation of most avian strains of chlamydiae as are chicken embryos. The cell cultures are then stained by immunofluorescence or by other appropriate stains at appropriate times to demonstrate the presence of inclusions.

Histochemical staining of impression smears from the liver, heart, and spleen are commonly made. The technique gives a rapid diagnosis, but requires some experience.

Enzyme-linked immunosorbent assays (ELISAs) developed for detecting Chlamydomphila trachomatis antigen in humans have been used for diagnosing chlamydiae in birds. Many of the earlier tests were developed using monoclonal or polyclonal antisera against lipopolysaccharide epitopes, some of which were shared with other Gram-negative bacteria. Their use when screening individual birds is questionable, as they lack sensitivity and specificity.

Molecular tools (conventional and real-time polymerase chain reaction, restriction length polymorphism, DNA microarray or sequencing) and immunohistochemical staining of histological sections are now widely used in diagnostic laboratories. All of them are rapid and do not require the live agent. The current PCR tests target the ompA gene or the ribosomal RNA genes (16S or 23S). Validated and standardised protocols of both species-specific and family-specific assays are
The taxonomy of the family Chlamydiaceae is currently under consideration (Kuo et al., 2011) but for the purpose of this chapter the subdivision into two genera Chlamydia and Chlamydophila has been retained (Everett et al., 1999a). The genus Chlamydia includes C. trachomatis (human), C. suis (swine), C. muridarum (mouse, hamster), C. psittaci (birds and others), C. felis (cats), C. abortus (sheep, goats, cattle), C. caviae (guinea-pigs), and C. pecorum (sheep, cattle) and C. pneumonia (human and others). While most of these organisms are highly host specific, C. pneumonia and C. psittaci have a broader host range. The latter has been reported to occur not only in birds and humans, but also in cattle, sheep, swine, horses and other animals (Sachse et al., 2009a).

The avian strains associated with AC belong to the species Chlamydophila psittaci. Until recently, nine different genotypes based on the ompA gene coding for the major outer membrane protein (MOMP) were distinguished. Seven of these "classical serovars" are thought to predominantly occur in a particular order or class of Aves and two in non-avian hosts, i.e. genotype A in psittacine birds, B in pigeons, C in ducks and geese, D in turkeys, E in pigeons, ducks and others, E/B in ducks, turkeys and pigeons, F in parakeets, WC in cattle, and M56 in rodents. Most of the avian genotypes have also been identified sporadically in isolates from cases of zoonotic transmission to humans, particularly A, B and E/B (Gaede et al., 2008; Heddema et al., 2006; Vanrompay et al., 2007). Meanwhile, subgroups for three of the more heterogeneous genotypes have been introduced, i.e. A-VS1, A-6BC, A-8455, EB-E30, EB-B89, EB-KKCP, D-NJ1, D-9N, and six new provisional genotypes to cover the strains that were previously non-typable have been suggested, i.e. 1V, 6N, Mat116, R54, YP84, and CPX0308, thus bringing the total number of genotypes to 15 (Sachse et al., 2008).

**Serological tests:** The standard serological test for chlamydial antibodies is the complement fixation (CF) test. The modified direct CF test can be used with most sera. The antigen is a group-reactive lipopolysaccharide antigen present in all strains. The occurrence of high CF titres in the majority of individuals in a flock with clinical signs is presumptive evidence of active infection. The demonstration of a four-fold increase in titre in an individual bird is considered to be diagnostic of a current infection.

Other serological tests, such as the ELISA, latex agglutination, elementary body agglutination, micro-immunofluorescence, and the agar gel immunodiffusion tests can be used. These tests are of value in specific cases and may replace the CF test; however, comparisons of reliability and reproducibility are not yet available.

**Requirements for vaccines:** There are no commercial vaccines available for chlamydiosis control in poultry. Antibiotics are the only current means of control. Chlamydophila psittaci is susceptible to a number of antibiotics. The drug of choice varies from country to country.

### A. INTRODUCTION

Avian chlamydiosis (AC) is caused by the bacterium Chlamydia psittaci. The disease in birds was originally called psittacosis, but the term ornithosis was introduced later to differentiate the disease in domestic and wild fowl from the disease in psittacine birds. The two syndromes are currently considered to be the same (Andersen & Vanrompay, 2003). Their earlier separation was based on the assumption that in humans, ornithosis was a milder disease than psittacosis. However, it should be noted that the disease in humans contracted from turkeys and ducks is often as severe as that contracted from psittacine birds.

Infection of birds with Chlamydia psittaci is common all over the world and has been found in about 465 avian species (Kaleta & Taday, 2003). Outbreaks of AC in psittacine birds and domestic poultry farms cause considerable economic damage (European Commission, 2002). The infection can lead to systemic and occasionally fatal disease in birds. The clinical signs are generally nonspecific and vary greatly in severity, depending on the species and age of the bird and the strain of chlamydia. AC can produce lethargy, hyperthermia, abnormal excretions, nasal and eye discharges, and reduced egg production. Mortality rates will vary greatly. In pet birds the most frequent clinical signs are conjunctivitis, anorexia and weight loss, diarrhoea, yellowish droppings, sinusitis, biliverdinuria, nasal discharge, sneezing, lachrymation and respiratory distress (Mohan, 1984). Many birds, especially older psittacine birds, may show no clinical signs; nevertheless, they may often shed the agent for extended periods. Necropsy of affected birds will often reveal multifocal hepatic necrosis, spleen and liver enlargement, fibrinous airsacculitis, pericarditis and peritonitis (Andersen & Vanrompay, 2003; Vanrompay et al., 1995). Histological lesions are suggestive of infection but are non-pathognomonic unless there are identifiable chlamydiae present.

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Recent evidence suggests that *C. psittaci* is not the only chlamydial agent occurring in birds (Gaede et al., 2008; Laroucau et al., 2009). The taxonomic classification of these new agents within or outside the family *Chlamydiaceae* has yet to be defined and their epidemiological importance is still unclear. They seem to be quite widespread in ducks, chickens and pigeons, and some strains appear to act as facultative pathogens. It is important to use diagnostic methods that are capable of differentiating between these organisms and *C. psittaci*.

Antibiotics are the only current means of control. *Chlamydia psittaci* is susceptible to a number of antibiotics: the drug of choice varies from country to country. Chlortetracycline, doxycycline, and other tetracyclines are the most commonly used. Treatment needs to be maintained for extended periods of time. For pet birds, 45 days is often recommended (Smith et al., 2005; Vanrompay et al., 1995).

- **Zoonotic risk and biosafety requirements**

The strains of avian chlamydiae can infect humans and should be handled carefully, at biosafety level 3 (Smith et al., 2005) as outlined in Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities*. Most infections occur through inhalation of infectious aerosols. While the disease from psittacine birds is best known, the infection in ducks and turkeys is of particular concern as transmission to humans is common during handling and slaughter of the birds (Dickx et al., 2010). Post-mortem examinations of infected birds and handling of cultures should be done in laminar flow hoods or with proper protective equipment. Appropriate zoonotic agent decontamination procedures should be followed because human infection can result from transient exposures. The incubation period is usually 5–14 days; however, longer incubation periods are known. Human infections vary from inapparent to severe systemic disease with interstitial pneumonia and encephalitis. The disease is rarely fatal in properly treated patients; therefore, awareness of the danger and early diagnosis are important. Infected humans typically develop headache, chills, malaise and myalgia, with or without signs of respiratory involvement. Pulmonary involvement is common; auscultatory findings, however, may appear to be normal or to underestimate the extent of involvement. Diagnosis can be difficult and is usually established through testing paired sera for antibodies to chlamydia by the complement fixation (CF) test. In humans, tetracycline, doxycycline, or azithromycin are usually the drugs of choice unless contraindicated. The length of treatment will vary with the drug, but should be continued for at least 14 days for tetracycline.

### B. DIAGNOSTIC TECHNIQUES

#### 1. Identification of the agent

The preferred method for the identification of AC is the isolation and identification of the organism. Because of the time involved, the need for high-quality samples, and the hazard to laboratory personnel, other techniques are often used. These include histochemical staining of smears of exudate and faeces, and impression smears of tissues, immunohistochemical staining of cytological and histological preparations, antigen-capture enzyme-linked immunosorbent assays (ELISA), conventional and real-time polymerase chain reaction (PCR), PCR-RFLP (restriction fragment length polymorphism), DNA microarray-based detection and DNA sequencing.

- **a) Collection and treatment of samples for isolation**

The samples to be collected will depend on the disease signs in evidence. They must be taken aseptically. Contaminant bacteria may interfere with the isolation of the chlamydiae. Specimens from acute cases should include inflammatory or fibrinous exudate in or around organs that display lesions, ocular and nasal exudates, impression smears of liver, whole blood and tissue samples from kidney, lung, pericardium, spleen, and liver. In cases with diarrhoea, colon contents or excrement should be cultured. In live birds, the preferred samples are pharyngeal and nasal swabs (Andersen, 1996). Intestinal excrement, cloacal swabs, conjunctival scrapings, and peritoneal exudate can also be taken.

Proper handling of clinical samples is necessary to prevent loss of infectivity of chlamydiae during shipping and storage. A special medium consisting of sucrose/phosphate/glutamate (SPG) was developed for rickettsiae and has proven to be satisfactory for transport of chlamydial field samples. The medium as recommended for chlamydiae (Spencer & Johnson, 1983) consists of SPG buffer: sucrose (74.6 g/litre); KH₂PO₄ (0.512 g/litre); K₂HPO₄ (1.237 g/litre); and L-glutamic acid (0.721 g/litre), which can be sterilised by autoclaving or filtering. Added to this are fetal calf serum (10%), vancomycin, kanamycin, and streptomycin (200–500 µg/ml), amphotericin B and gentamicin (50 µg/ml). The addition of antibiotics reduces the effect of contamination, even when samples are shipped at ambient temperatures. In the absence of refrigerative storage, the organism remains viable for up to 30 days and at 4°C for up to 34 days (Spencer & Johnson, 1983). This medium can also be used as a laboratory diluent and for freezing of chlamydiae.

Contaminated samples must be pre-treated before being used to inoculate animals or cell cultures. There are three basic methods: treatment with antibiotics (Bevan et al., 1978), treatment with antibiotics together
Chapter 2.3.1. – Avian chlamydiosis

with low-speed centrifugation (Andersen & Vanrompay, 2003; 2005), and treatment with antibiotics with filtration (Andersen & Vanrompay, 2005; Bevan et al., 1978). A number of antibiotics that do not inhibit chlamydia can be used. Samples are homogenised in phosphate buffered saline (PBS), pH 7.2, containing a maximum of the following: streptomycin (1 mg/ml), vancomycin (1 mg/ml), and kanamycin (1 mg/ml). Gentamicin (200 µg/ml) can be used. Amphotericin B (50 µg/ml) can be added to control yeast and fungal growth. Penicillin, tetracycline and chloramphenicol should be avoided as these inhibit the growth of chlamydiae.

When contamination is light, samples should be homogenised in an antibiotic solution prior to inoculation into chicken embryos or tissue cultures. Samples are often left to stand in the antibiotic solution for 24 hours at 5°C before inoculation. Heavily contaminated samples, such as faecal samples, should be homogenised in antibiotics and then centrifuged at 500 g for 20 minutes. The surface layer and the bottom layer are discarded. The supernatant fluid is collected and recentrifuged. The final supernatant fluid is used for inoculation. Samples should be passed through a filter of 450–800 µm average pore size if contamination persists.

b) Isolation in cell culture

Cell cultures are the most convenient method for the isolation of C. psittaci. The most common cell lines are buffalo green monkey (BGM), McCoy, HeLa, African green monkey kidney (Vero), and L cells (Vanrompay et al., 1992). The cells are grown as monolayers using standard tissue culture media containing 5–10% fetal calf serum and antibiotics that are not inhibitory to chlamydia (as described previously).

When selecting cell culture equipment, it is important to remember that:

i) Chlamydiae can be identified by direct or indirect immunofluorescence or some other appropriate staining technique;
ii) The inoculum is usually centrifuged on to the monolayer to enhance its infectivity;
iii) The sample may need to be blind passaged at 4–5 days to increase sensitivity of isolation;
iv) The sample will need to be examined from two to three times during any one passage; and
v) Chlamydiae can be infectious to humans.

Small flat-bottomed vials, such as 1-dram (3.7 ml, 15 × 45 mm) shell vials or bottles containing cover-slips that are 12 mm in diameter, will meet these requirements (Bevan et al., 1978). A number of vials, often four to six, are inoculated with each sample to permit fixing and staining at various intervals, and to permit repassing of apparently negative samples 6 days after inoculation. When testing multiple samples, 96-well multiwell dishes can also be used as they have a labour-saving advantage. However, it should be noted that cross-contamination between samples can be a problem.

Chlamydiae can be isolated from cells that are replicating normally, but the use of non-replicating cells is preferable as these may provide increased nutrients for the growth of chlamydiae. Suppressed cells can also be observed for longer periods. Host cell division can be suppressed either by irradiation or, more commonly, by cytotoxic chemicals. The latter include 5-iodo-2-deoxyuridine, cyto-cholasin B, cycloheximide, and emetine hydrochloride. Cycloheximide is the most commonly used and can be added to the medium at the rate of 0.5–2.0 µg/ml at the time of inoculation of the monolayer (Andersen & Vanrompay, 2003; 2005; Bevan et al., 1978). Emetine is removed after treatment and replaced by medium. The monolayer is first treated for 5 minutes with emetine (0.5 µg/ml), after which the emetine is removed and replaced with culture medium; the monolayer is then ready for use. The growth of most chlamydial strains will be enhanced by the treatment of the monolayer by one of these drugs.

Attachment of chlamydia to cells is increased by centrifuging the inoculum on to the monolayer at 500–1500 g for 30–90 minutes at 37°C. The inoculum is removed and replaced with tissue culture medium containing a cell-division inhibitor, and then incubated at 37–39°C. Cultures must be examined for chlamydiae at regular intervals using an appropriate staining method. This is usually done on day 2 or 3, as well as on day 4 or 5. Cultures that appear to be negative at the fifth day are harvested and repassaged. When repassing chlamydiae, cells and culture media should be passaged without using freeze–thawing to disrupt cells, as this will destroy the chlamydiae.

Before staining the cultures, the medium is first removed, the cultures are washed with PBS and fixed with acetone for 2–10 minutes. The fixation time will depend on the tissue culture vessel used. As acetone will soften most plastics, the use of a mixture of 50% acetone and 50% methyl alcohol may be preferable. A number of staining methods can be employed to demonstrate chlamydial inclusions. The preferred method is direct immunofluorescence (Andersen & Vanrompay, 2005; Bevan et al., 1978; Moore & Petrak, 1985). A chlamydial fluorescein-conjugated antiserum is applied to the infected cells and incubated in a humid chamber for 30 minutes at 37°C. The cover-slips are then washed three times with PBS, air-dried, mounted, and examined. Chlamydial inclusions fluoresce a green colour. Commercial conjugate preparations using
monoclonal antibodies (MAbs) are available and are highly specific. Conjugates may also be prepared from polyclonal sera, but it is important to obtain specific, high-titred antisera. Polyclonal antisera can be prepared in rabbits, guinea-pigs, sheep or goats. Sheep and goats are excellent sources because of the volume and high titres that are readily obtained following infection. Conjugates are then prepared using standard techniques (Andersen & Vanrompay, 2003; 2005).

Chlamydial inclusions can also be demonstrated by indirect fluorescent antibody and immunoperoxidase techniques (Andersen & Vanrompay, 2005; Page, 1974). Direct staining can be done with Gimenez, Giemsa, Ziehl–Neelsen, or Macchiavello’s stains. Except for immunofluorescence, all these stains have the advantage that standard light microscopes can be used.

c) Isolation in eggs

Chicken embryos are still used for the primary isolation of chlamydiae. The standard procedure is to inject up to 0.5 ml of inoculum into the yolk sac of a specific pathogen free 6–7-day-old embryo (Andersen & Vanrompay, 2003; 2005). The eggs are then incubated in a humid atmosphere at 39°C, rather than at 37°C, as multiplication of chlamydiae is greatly increased at the higher temperature. Replication of the organism usually causes the death of the embryo within 3–10 days. If no deaths occur, two additional blind passages are usually made before designating any sample as negative. Chlamydial infections will give rise to a typical vascular congestion of the yolk sac membranes. These are harvested and homogenised as a 20% (w/v) suspension in SPG buffer, and can be frozen to preserve the strain, or inoculated into eggs or on to cell cultures.

The organism can be identified by preparing an antigen from an infected yolk sac and testing it by direct staining of smears using appropriate stains or by using the antigen in a serological test. Cell culture monolayers can be inoculated with the yolk sac suspension and examined by direct immunofluorescence 48–72 hours later for the presence of chlamydial inclusions. Typical inclusions are intracytoplasmic round or hat-shaped bodies. With some virulent strains, the inclusions rapidly break up and the chlamydial antigen is dispersed throughout the cytoplasm.

d) Differentiating among species/strains

Chlamydothila psittaci can be identified using PCR-RFLP (Everett & Andersen, 1999) or species-specific conventional PCR (Messmer et al., 1997; Sachse & Hotzel, 2003; Van Loock et al., 2005), or real-time PCR (Everett et al., 1999b; Geens et al., 2005; Pantchev et al., 2009; reviewed in Sachse et al., 2009b). A DNA microarray assay was shown to differentiate among all nine species of the family Chlamydiaceae (Sachse et al., 2005).

Serotyping in its classical form (Andersen, 1991; 1997) is only rarely conducted because the serovar-specific monoclonal antibodies are not available from a commercial supplier. Instead, genotyping is a practical alternative because i) the classical genotypes A-F are equivalent to the corresponding serotypes, and ii) nine of the more recently defined genotypes cannot be characterised by serotyping because of the absence of specific antibodies. Genotypes A-F can be identified using PCR-RFLP (Vanrompay et al., 1997). A DNA microarray procedure, which was shown to differentiate among all currently known genotypes (Sachse et al., 2008; 2009a), can be used for genotyping of C. psittaci strains from cell culture and tissue samples. In addition, all genotypes can be identified by complete sequencing of the ompA gene.

As mentioned above, C. psittaci is not the only chlamydial agent encountered in birds. The recently described new chlamydial agents (Gaede et al., 2008; Laroucau et al., 2009) have to be taken into consideration, when a given avian sample is positive in a general chlamydial test, e.g. Chlamydiaceae-specific PCR or immunohistochemistry, but negative in a species-specific test for C. psittaci. In such a case, partial or complete sequencing of the ompA gene and the 16–23S rRNA operon will reveal the identity of the strain. The occurrence of C. abortus in birds is a rare event, but should also be considered as a possible differential diagnosis.

e) Histochemical staining

Giemsa, Gimenez, Ziehl–Neelsen and Macchiavello’s stains are commonly used to detect chlamydiae in impression smears of liver and spleen. The following modified Gimenez technique is used by several laboratories (Andersen & Vanrompay, 2005):

- **Modified Gimenez technique or (Pierce-van der Kamp) stain**
- **Reagents:**

  Solution 1: Distilled H₂O (450.0 ml) and phenol (5.0 ml) added to basic fuchsin (2.5 g) and 95% ethanol (50.0 ml). Incubate at 37°C for 48 hours. Filter and store in the dark at room temperature.

  Solution 2: Na₂HPO₄ (11.65 g); Na₂HPO₄.H₂O (2.47 g); distilled H₂O, pH 7.5 (to 1.0 litre).
Solution 3: Solution 1 (20.0 ml); and solution 2 (25.0 ml). Let stand for 10 minutes, filter and use.
Solution 4: 0.5% citric acid.
Solution 5: Fast green (0.2 g); distilled H₂O (100.0 ml); and glacial acetic acid (0.2 ml).
Solution 6: Solution 5 (20.0 ml); and distilled H₂O (50.0 ml).

- Procedure for smears is as follows:
  i) Fix in methanol for 5 minutes.
  ii) Stain in Solution 3 for 10 minutes and rinse in tap water.
  iii) Counterstain in Solution 6 for 2 minutes.
  iv) Rinse in tap water and air-dry.

- Procedure for paraffin sections is as follows:
  i) Deparaffinise and hydrate with distilled H₂O.
  ii) Stain in Solution 3 for 10 minutes and rinse in tap water.
  iii) Dip in Solution 4 until no more red runs out of the section. Rinse in tap water.
  iv) Counterstain in Solution 6 for 20 dips.
  v) Dip in two changes of 95% alcohol, for five dips each. Dehydrate, clear, and mount.

Chlamydiae will appear red against a green background.

f) Immunohistochemical staining

Immunohistochemical staining can be used to detect chlamydiae in cytological and histological preparations. The technique is more sensitive than histochemical staining, but some experience is necessary as cross-reactions with some bacteria and fungi require that morphology must be considered.

Most widely used immunohistochemical staining procedures can be adapted to give satisfactory results. The selection of the primary antibody is very important. Both polyclonal and monoclonal antibodies have been used. Because formalin affects chlamydial antigens, it is recommended that polyclonal antibodies be made to purified formalin-inactivated chlamydiae. The chlamydial strain used is not important, as the antibodies will be mainly to the group-reactive antigens. MAbs should also be selected for reactions to formalin-fixed chlamydiae. A pool of group-reactive MAbs can be used.

g) Enzyme-linked immunosorbent assays

The ELISA has been extensively promoted in kit format for use in the diagnosis of human chlamydiosis. These test kits detect the lipopolysaccharide (LPS) antigen (group reactive) and will detect all species of Chlamydiaceae. A number of these kits have been tested for use in detecting chlamydiae in birds (Vanrompay et al., 1994), but none of the kits has been licensed for detection of C. psittaci. One problem with some of these tests is that the chlamydial LPS shares some epitopes with other Gram-negative bacteria, and these epitopes can cross-react, resulting in a high number of false-positive results. This problem has been reduced or eliminated in more recently developed kits by careful selection of the MAbs used. These kits, however, still lack sensitivity because a few hundred organisms are still needed to give a positive reaction. Most diagnosticians believe that a diagnosis of AC can be made when a strong positive ELISA reaction is obtained from birds with signs of psittacosis. Because of the number of false-positive results, a positive in an individual bird without signs of disease is not considered to be significant, but indicates the need for further testing using different methods.

h) DNA detection systems

- Polymerase chain reaction

PCR techniques have been replacing isolation for the detection of chlamydiae from animal tissue. Infection risks to laboratory staff are avoided by inactivation of the sample prior to testing. The sensitivity and specificity will usually exceed that of isolation. Current PCR tests for detection of C. psittaci target the ompA gene or the 16S–23S rRNA gene (Everett et al., 1999b; Geens et al., 2005; Messmer et al., 1997; Pantchev et al., 2009; Sachse & Hotzel, 2003; Van Loock et al., 2005; and reviewed in Sachse et al., 2009b). The sensitivity and specificity varies on sample preparation and the PCR test. Reagents designed to stabilise the DNA should be considered when a delay in processing the sample is anticipated (DeGraves et al., 2003).
DNA samples can be prepared using inexpensive reagents or using commercially available kits (Andersen &
Vanrompay, 2005). Sensitivity is increased by targeting a relatively short DNA segment, using a nested procedure or using real-time PCR techniques. The nested PCR can equal isolation in sensitivity and specificity (Messmer et al., 1997; Sachse & Hotzel, 2003; Van Loock et al., 2005). However, the risk of contamination is increased if extreme care is not taken when manipulating the reactions (see Chapter 1.1.5 Principles and methods of validation of diagnostic assays for infectious diseases). In the last few years, real-time PCR has become the preferred method in diagnostic laboratories for its rapidity, high throughput and ease of standardisation (Sachse et al., 2009b). This technology requires a fluorescent-labelled probe and special equipment, which increases costs. Its sensitivity can be equivalent to that of the nested system, but contamination problems and labour are reduced as it is based on one reaction in a closed system (Everett et al., 1999b; Geens et al., 2005; Pantchev et al., 2009).

- **Real-time PCR procedure (Pantchev et al., 2009)**

This assay is conducted as a duplex amplification that includes an internal amplification control (IAC). A detection limit of 2 inclusion-forming units per reaction mix was determined for this assay.


ii) The amplification is conducted in 96-well microtitre plates on an Mx3000P thermocycler or comparable equipment. Each 25-µl reaction contains 12.5 µl of 2 × TaqMan Universal PCR Master Mix supplemented with ROX 2 or a comparable product. The final concentration is 0.9 µM for each *C. psittaci* primer, 0.3 µM for each IAC primer, and 0.2 µM for each probe.

iii) IAC template DNA (0.5 µl containing 10^4 copies) is added to each reaction before the final volume is made up with water.

iv) The following cycling parameters are used: initial heating cycle at 95°C for 10 minutes (single denaturation step), 45 cycles of 95°C for 15 seconds and 60°C for 1 minute (annealing and extension).

v) The cycle threshold value (Ct) automatically calculated by the software should be used. Ct values higher than 36 should be treated with caution as they may represent cross-reaction with related microorganisms. In such cases, the respective samples should be re-examined by an alternative test.

- **DNA microarray**

DNA microarray technology was recently shown to be a powerful tool in the diagnosis of chlamydial infections (Sachse et al., 2005). The assay for detection and identification of *Chlamydiaceae* spp. includes identification of *C. psittaci*. It has been validated and proved suitable for routine diagnosis (Borel et al., 2008). Its sensitivity is comparable to that of real-time PCR and specificity is even higher because sample DNA is hybridised to 36 specific oligonucleotide probes. This methodological approach enables detection of mixed chlamydial infections and identification of unexpected chlamydial species directly from clinical samples.

2. **Serological tests**

a) **Modified direct complement fixation test for *Chlamydophila***

The following is a widely used modified direct CF test for the detection of antibody. The reagents are relatively easy to prepare and standardise. There are other CF tests; each has advantages. The modified direct CF test is performed in 96-well round-bottom multiwell dishes. Incubation steps are usually done by floating the plates in a 37°C water bath. The chlamydial antigen can be prepared from either infected yolk sacs or cell culture preparations. The modified direct CF test differs from the direct CF test in that normal, unheated chicken serum from chickens without chlamydial antibody is added to the complement dilution. The normal serum increases the sensitivity of the CF procedure so that it can be used to test sera from avian species whose antibodies do not normally fix guinea-pig complement.

- **Test procedure**

i) **Dilution of sera**

Figure 1 gives a suggested pattern for performing the test in round-bottom, 96-well multiwell dishes. All sera must be heat-inactivated at 60°C for 30 minutes prior to use. The sera are diluted in Veronal

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1 Available from Intype IC-DNA, Labordiagnostik Leipzig, Germany.
2 Available from Applied Biosystems.
ii) Addition of antigen

To each well in columns 1, 4, 7, and 10, add 25 µl of positive chlamydial antigen. In columns 2, 5, 8, and 11, add 25 µl of VBS (anticomplementary control wells), and in columns 3, 6, 9, and 12, add 25 µl of negative antigen (normal yolk sac or cell culture prepared the same as the chlamydial antigen). The chlamydial antigens are stored undiluted at 4°C and diluted to proper concentration in VBS prior to use.

iii) Addition of complement

Complement (C’) is stored at –70°C and should be thawed and diluted in VBS prior to the addition of the antigen. Fresh chicken serum is added before diluting the C’ to give a 5% concentration in the complement. Dilutions of C’ are made as in previous tests or from titrations. C’ should be allowed to stand in an ice bath to stabilise for 15 minutes. The diluted C’ should be stored at 4°C following stabilisation and should be used within 2 hours; 50 µl of the C’ is added to each well immediately following the addition of the antigens. The plates are incubated uncovered in a 37°C water bath for 2 hours.

iv) Addition of sheep red blood cells

Mix 4% standardised sheep red blood cells (SRBCs) with an equal volume of VBS. To this add an equal volume of haemolysin diluted in VBS. The final dilution is incubated in a 37°C water bath for 15 minutes to sensitise the SRBCs. To each well add 50 µl of sensitised SRBCs. The plates are then incubated for 1 hour in a 37°C water bath. The plates can be centrifuged at 400 g for 5 minutes before reading or they can be refrigerated at 4°C overnight prior to reading.

v) Interpretation of the results

The wells are often scored 1+, 2+, 3+, or 4+ corresponding to reduction of haemolysis of 25, 50, 75, or 100%. A positive reaction is 2+ or higher, which is equivalent to 50% or less lysis of the SRBCs. This indicates that the C’ was fixed by antibody prior to the addition of the SRBCs. Negative wells are indicated by the complete lysis of the cells; the C’ remains unbound and reacts with the SRBCs and the haemolysin to produce lysis of the SRBCs.

Invalid tests occur when the serum is anticomplementary and a positive reaction occurs in the dilution with VBS as the antigen. Nonspecific serum reactions give positive reactions in both the positive and negative wells.

- Reagents

i) Antigen preparation

The simplest methods start with the growth of chlamydiae in cell culture. The two methods described below produce antigens that can be used in the micro-CF test. The procedures are quite similar: both include the growth of chlamydiae in cell culture, the inactivation of the chlamydiae, partial purification of
the antigen, mechanical disruption, and dilution into the appropriate buffer. The method selected will depend on the equipment available.

The first procedure (Grimes, 1985; Grimes et al., 1970) starts with the chlamydiae and cell culture debris harvested when cytopathic effects are noted. The culture is inactivated by the addition of phenol to a final concentration of 1.0%, incubated for 24 hours at 37°C, and concentrated by centrifugation at 10,000 \( g \) for 1 hour. The sediment is reconstituted to 10% of the original volume using VBS, pH 7.2, containing 1.0% phenol and 1.0% glycerol.

The sediment is then homogenised in an omnimixer at top speed for three 1-minute periods while cooled in ice water. The homogenate is centrifuged for 15 minutes at 100 \( g \) to remove debris. Some procedures suggest heating the antigen for 30 minutes in a boiling water bath at this time. The supernatant is saved and diluted to the desired concentration.

In the second procedure for the production of antigen for the CF test (Bracewell & Bevan, 1986), antigen is prepared from L cells infected with a psittacine strain. The cell culture medium is discarded, and the cells are heated for 40 minutes at 56°C. The cells are lysed in distilled water, the chlamydiae are disrupted by ultrasonication, and then made isotonic in VBS. The antigen is tested against a standard sheep convalescent serum and used at 2 units in the micro-CF test.

There are a number of procedures for preparing the antigen from infected yolk sacs, some of which are quite elaborate. However, with the following procedure it is relatively easy to prepare a crude infected yolk sac antigen that works well in the modified direct CF test. An egg-adapted strain of \textit{Chlamydia} is used to inoculate 6–7-day-old embryonated chicken eggs via the yolk sac. The yolk sacs are harvested from embryos that die between 3 and 7 days post-inoculation. The yolk-sac harvest is diluted 1/3 in PBS, Tris buffer, or cell culture medium, and then autoclaved for 20 minutes. The suspension is cooled and then homogenised thoroughly. The use of a high-speed tissue homogeniser for 3–5 minutes is recommended. After homogenisation, phenol is added to make a final concentration of 0.5% phenol (prepare a 5% phenol stock solution and add 1 ml for every 9 ml of antigen). The antigen preparation is prepared, held for 3 days, and then the supernatant is used after centrifugation for 20 minutes at 1000 \( g \). The antigen can be stored for long periods of time at 4°C.

### ii) Preparation of sensitised SRBCs

Defibrinated SRBCs are preserved by mixing in an equal volume of Alsever’s solution. These can be stored at 4°C for up to 4 weeks. Wash 25 ml of the stock SRBCs with 25 ml of VBS. Centrifuge at 400 \( g \) for 10 minutes. Aspirate off the VBS and resuspend in 50 ml of VBS. Repeat the wash a total of three times. Following the final wash, dilute the SRBCs at a ratio of 2.2 ml of packed SRBCs to 98 ml of VBS. The SRBCs can then be standardised by optical density: mix 1 ml of the diluted, washed SRBCs with 9 ml distilled H\(_2\)O, determine the absorbance using a spectrophotometer, and standardise to 0.25 at a wavelength of 550 mm. The reading obtained can be used in the following formula to determine the dilution needed:

\[
\text{Final volume of SRBCs} = \frac{(\text{absorbance reading} \times \text{current volume})}{0.25 \text{ desired absorbance}}
\]

The SRBCs are sensitised by rapidly adding an equal volume of VBS containing the appropriate dilution of haemolysin (dilution determined by titration). Incubate at 37°C for 15 minutes prior to use.

### iii) Veronal buffer saline

VBS is prepared as a 5 × stock solution and diluted 1/5 with distilled H\(_2\)O prior to use. The following formula makes 4 litres. To distilled water add sodium barbital (7.5 g); barbital H\(_2\)O (dissolve in boiling H\(_2\)O) (11.5 g); MgSO\(_4\).7 H\(_2\)O (4.056 g); NaCl (170.0 g); and CaCl\(_2\) (0.078 g). Add distilled H\(_2\)O to make to 4 litres.

### iv) Complement titration

Complement (C’) is unstable and will deteriorate if improperly handled. Normally it should be kept frozen at −70°C in aliquots that are used at one time to eliminate refreezing. To obtain the desired working concentration (2 units per test well) first add 5% normal chicken serum for the modification to enhance sensitivity as described earlier. Then estimate a starting point based on previous lots. A good starting point is a dilution of 1/30 after the chicken serum has been added. Set up a series of tubes with various amounts of complement in VBS. The VBS should contain the antigen to be used in the reaction and take into account any anticomplementary properties of the antigen. A common method is to dilute 0.10 ml C’ + 0.90 ml VBS; 0.12 ml complement + 0.88 ml VBS, etc. through 0.25 ml C’ + 0.75 ml VBS. Incubate the tubes for 2 hours in a 37°C water bath. Add 0.5 ml of sensitised SRBCs to each tube. Incubate for 1 additional hour in the 37°C water bath. The highest dilution giving complete haemolysis
equals 1 unit. Twice that amount equals 2 units. The following formula can be used to obtain 2 units/0.05 ml:

\[
x = \frac{(d_i)(v)}{2d_h}
\]

where:
- \(x\) = reciprocal of C’ dilution desired to yield 2 units C'/well
- \(d_i\) = reciprocal of C’ initial dilution used in titration (1/30)
- \(v\) = volume of diluted C’ to be added
- \(d_h\) = twice the volume of C’ giving complete haemolysis in titration

\[v)\]

v) **Titration of haemolysin**

Haemolysin can be obtained from commercial sources. It must be standardised by titration. The following procedure is recommended:

Prepare a 1/100 dilution of the stock haemolysin in VBS. From this, prepare 1/300, 1/400, and 1/500 dilutions in tubes. From each of these dilutions, make 0.5 ml of twofold dilutions in VBS for a block titration.

To determine haemolysin concentration, add the following to 0.5 ml of each dilution: 0.5 ml of C’ at 1/30 dilution, 0.5 ml of unsensitised SRBCs at 0.25 optical density, and 1.5 ml of VBS. Incubate for 1 hour at 37°C, and then centrifuge at 400 g for 5 minutes. One unit of the haemolysin is the dilution that gives complete lysis of the SRBCs. The haemolysin solution is prepared in VBS at the dilution containing 2 units of haemolysin. This is then added to an equal volume of SRBCs at the proper concentration.

vi) **Titration of antigen and positive control serum**

In order to standardise the CF test, it is also necessary to have titres of both the antigen and the positive control serum. If the titre is known for either the positive serum or antigen, the titre of the other component can be determined by performing the CF test using dilutions of the component being titred. If titres of both the positive serum and antigen are unknown, a block titration (chequerboard) can be used to determine the limiting dilutions of both the antigen and the antibody where haemolysis starts. It is very critical to obtain these titres accurately.

For both the antigen and the positive control serum, 4 units are used. A unit is the highest dilution that will give a positive test. That is, if a dilution of 1/160 gives a positive test, then a 1/40 dilution has 4 units and is used for the test.

Complement-fixing antibodies usually appear within 7–10 days of infection. For a positive diagnosis, a four-fold rise in CF antibody titre is required. A presumptive diagnosis by serological tests on a flock can only be made if typical clinical signs are present and a majority of the birds have antibody titres of >1/64.

b) **Other tests**

Other serological tests have been developed, but their specificity has not yet been sufficiently evaluated. The ELISA for group-specific chlamydial antibodies is more rapid and sensitive than the CF test; it can be automated. Evaluations of ELISAs for the detection of antibodies to both *C. trachomatis* and *C. psittaci* indicate that the tests are highly sensitive but lack specificity. New tests are being developed that use peptides or recombinant antigens which may correct the specificity problem (Sachse *et al.*, 2009b).

Other tests include the agar gel immunodiffusion test (Page, 1974), the latex agglutination (LA) test, the elementary body agglutination (EBA) test (Grimes & Arizmendi, 1996; Grimes *et al.*, 1994) and the micro-immunofluorescence test (MIFT). Immunodiffusion is less sensitive than the CF test. The LA test will detect antibodies to *C. psittaci*, and is easy and rapid to perform (Grimes *et al.*, 1993). Latex beads are coated with purified chlamydial antigen, mixed thoroughly with the test serum on a glass plate, and rotated for 2 minutes to enhance agglutination. The test is read against a dark background. Sera giving positive reactions should be retested with uncoated beads to eliminate possible nonspecific agglutination. The LA and direct CF tests correlate in 72.5% of tests with paired sera. The LA test has a sensitivity of 39.1% and a specificity of 98.8% relative to the direct CF test (Grimes *et al.*, 1993). The test detects both IgM and IgG, but it is best at detecting IgM. It has been suggested for use in detecting recent or active infections. The EBA test detects only IgM, and it is indicative of a current infection. The MIFT is rapid and easy to perform; however, fluorescence-conjugated anti-species sera are not always available.
C. REQUIREMENTS FOR VACCINES

There are no commercial vaccines available for chlamydiosis in poultry. Attempts to produce a vaccine have met with limited success, and most have been based on bacterins produced by formalin inactivation of concentrated suspensions of chlamydiae. There is evidence that immunity involves cell-mediated immune responses (Beeckman, & Vanrompay, 2010; Smith et al., 2005), but vaccine manufacture has not been directed towards reactions of this type.

REFERENCES


Chapter 2.3.1. — Avian chlamydiosis


Chapter 2.3.1. — Avian chlamydiosis


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**NB:** There is an OIE Reference Laboratory for avian chlamydiosis (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the OIE Reference Laboratories for any further information on diagnostic tests and reagents for avian chlamydiosis.
CHAPTER 2.3.2.

AVIAN INFECTIOUS BRONCHITIS

SUMMARY

Avian infectious bronchitis (IB) is caused by coronavirus infectious bronchitis virus (IBV). The virus causes infections mainly in chickens and is a significant pathogen of commercial meat and egg type birds. IB is an acute, contagious disease characterised primarily by respiratory signs in growing chickens. In hens, decreased egg production and quality are often observed. Some strains of the virus are nephropathogenic and produce interstitial nephritis and mortality. The severity of IBV-induced respiratory disease is enhanced by the presence of bacterial pathogens leading to chronic complicated airsacculitis. Diagnosis of IB requires virus isolation or demonstration of viral nucleic acid from diseased flocks. Demonstration of an ascending serum antibody response may also be useful. The widespread use of live and inactivated vaccines may complicate both the interpretation of virus isolation and serology findings. The occurrence of antigenic variant strains may overcome immunity induced by vaccination.

Diagnosis requires laboratory testing. Virus isolation and identification is preferred. Reverse-transcription polymerase chain reaction (RT-PCR) techniques are commonly used to identify the IBV genotype. Haemagglutination inhibition (HI) tests to determine serotype and enzyme-linked immunosorbent assays (ELISA) for general monitoring are often used for sero-diagnosis. Supplementary tests include electron microscopy, the use of monoclonal antibodies, virus neutralisation (VN), immunohistochemical or immunofluorescence tests, and immunisation-challenge trials in chickens.

Identification of the agent: For the common respiratory form, IBV is most successfully isolated from tracheal mucosa and lung several days to one week following infection. For other forms of IB, kidney, oviduct, the caecal tonsils of the intestinal tract or proventriculus tissues are better sources of virus depending on the pathogenesis of the disease.

Specific pathogen free chicken embryos or tracheal organ cultures (TOCs) from embryos may be used for virus isolation. Following inoculation of the allantoic cavity, IBV produces embryo stunting, curling, clubbing of the down, or urate deposits in the mesonephros of the kidney, usually within three serial passages. Isolation in TOCs has the advantage that IBV produces stasis of the tracheal cilia on initial inoculation. RT-PCR is increasingly being used to identify the spike (S) glycoprotein genotype of IBV field strains. Genotyping using primers specific for the S1 subunit of the S gene or sequencing of the same gene generally provides similar but not always identical findings to HI or VN serotyping. Alternatively, VN or HI tests using specific antiserum may be used to identify the serotype.

Serological tests: Commercial ELISA kits may be used for monitoring serum antibody responses. The antigens used in the kits are broadly cross-reactive among serotypes and allow for general serological monitoring of vaccinal responses and field challenges. The HI test is used for identifying serotype-specific responses to vaccination and field challenges especially in young growing chickens. Because of multiple infections and vaccinations, the sera of breeders and layers contain cross-reactive antibodies and the results of HI testing cannot be used with a high degree of confidence.

Requirements for vaccines and diagnostic biologicals: Both live attenuated and oil emulsion inactivated vaccines are available. Live vaccines, attenuated by serial passage in chicken embryos or by thermal heat treatment, confer better local immunity of the respiratory tract than inactivated vaccines. The use of live vaccines carries a risk of residual pathogenicity associated with vaccine
back-passage in flocks. However, proper mass application will generally result in safe application of live vaccines.

Inactivated vaccines are injected and a single inoculation does not confer protection unless preceded by one or more live IBV priming vaccinations. Both types of vaccines are available in combination with Newcastle disease vaccine; in some countries inactivated multivalent vaccines are available that include Newcastle disease, infectious bursal disease, reovirus and egg-drop syndrome 76 viral antigens.

A. INTRODUCTION

Avian infectious bronchitis (IB) was first described in the United States of America (USA) in the 1930s as an acute respiratory disease mainly of young chickens. A viral aetiology was established, and the agent was termed avian infectious bronchitis virus (IBV). The virus is a member of the genus Coronavirus, family Coronaviridae, in the order Nidovirales. IBV and other avian coronaviruses of turkeys and pheasants are classified as group 3 coronaviruses, with mammalian coronaviruses comprising groups 1, 2 and 4 (group 4 being the more recently identified severe acute respiratory syndrome (SARS) coronavirus) (Cavanagh, 2003). Coronaviruses have a nonsegmented, positive-sense, single-stranded RNA genome.

IB affects chickens of all ages, which, apart from pheasants (Cavanagh et al., 2002) are the only species reported to be naturally affected. The disease is transmitted by the air-borne route, direct chicken-to-chicken contact and indirectly through mechanical spread (contaminated poultry equipment or egg-packing materials, manure used as fertiliser, farm visits, etc.). IB occurs world-wide and assumes a variety of clinical forms, the principal one being respiratory disease that develops after infection of the respiratory tract tissues following inhalation or ingestion. Infection of the oviduct can lead to permanent damage in immature birds and, in hens, can lead to cessation of egg-laying or production of thin-walled and misshapen shells with loss of shell pigmentation. IB can be nephropathogenic causing acute nephritis, urolithiasis and mortality (Cavanagh & Naqi, 2003). After apparent recovery, chronic nephritis can produce death at a later time. IBV has also been reported to produce disease of the proventriculus (Yu et al., 2001). Vaccine and field strains of IBV may persist in the caecal tonsils of the intestinal tract and be excreted in faeces for weeks or longer in clinically normal chickens (Alexander et al., 1978).

There have been no reports of human infection with IBV.

B. DIAGNOSTIC TECHNIQUES

Confirmation of diagnosis is based on virus isolation, often assisted by serology. Extensive use is made of live and inactivated vaccinations, which may complicate diagnosis by serological methods as antibodies to vaccination and field infections can not always be distinguished. Persistence of live vaccines may also confuse attempts at recovering the causative field strain.

1. Identification of the agent

a) Sampling

Samples appropriate to the form of IB observed must be obtained as soon as signs of clinical disease are evident. Samples must be placed in cold transport media and be frozen as soon as possible. The cold chain from bird to laboratory should be maintained. For acute respiratory disease, swabs from the upper respiratory tract of live birds or tracheal and lung tissues from diseased birds should be harvested, placed in transport medium containing penicillin (10,000 International Units [IU]/ml) and streptomycin (10 mg/ml) and kept on ice and then frozen. For birds with nephritis or egg-production problems, samples from the kidneys or oviduct, respectively, should be collected in addition to respiratory specimens. In some cases, IBV identification by reverse-transcription polymerase chain reaction (RT-PCR) may be desirable without virus isolation. In this case, swabbings from the respiratory tract or cloaca may also be submitted alone, without being placed in liquid transport media (Cavanagh et al., 1999). In situations where IB-induced nephritis is suspected, kidney samples should also be selected from fresh carcasses for histopathological examination as well as virus isolation. Blood samples from acutely affected birds as well as convalescent chickens should be submitted for serological testing. A high rate of virus recovery has been reported from the caecal tonsil or faeces (Alexander et al., 1978). However, isolates from the intestinal tract may have no relevance to the latest infection or clinical disease. IBV isolation may be facilitated using sentinel specific pathogen free (SPF) chickens placed at one or more times in contact with commercial poultry (Gelb et al., 1989).
b) Culture

Suspensions of tissues (10–20% w/v) are prepared in sterile phosphate buffered saline (PBS) or nutrient broth for egg inoculation, or in tissue culture medium for tracheal organ culture (TOC) inoculation (Cook et al., 1976). The suspensions are clarified by low-speed centrifugation and filtration through bacteriological filters (0.2 µ) before inoculation of eggs or TOCs.

Embryonated chicken eggs and TOCs are used for primary isolation of IBV. Cell cultures are not recommended for primary isolation as it is often necessary to adapt IBV isolates to growth in chicken embryos before cytopathic effect (CPE) is produced in chick embryo kidney cells.

Embryonated eggs used for virus isolation should originate preferably from SPF chickens or from breeder sources that have been neither infected nor vaccinated with IBV. Most commonly, 0.1–0.2 ml of sample supernatant is inoculated into the allantoic cavity of 9–11-day-old embryos. Eggs are candled daily for 7 days with mortality within the first 24 hours being considered nonspecific. The initial inoculation usually has limited macroscopic effects on the embryo unless the strain is derived from a vaccine and is already egg adapted. Normally, the allantoic fluids of all eggs are pooled after harvesting 3 days after infection; this pool is diluted 1/5 or 1/10 in antibiotic broth and further passaged into another set of eggs for up to a total of three to four passages. Typically, a field strain will induce observable embryonic changes consisting of stunted and curled embryos with feather dystrophy (clubbing) and urate deposits in the mesonephros on the second to fourth passage. Embryo mortality in later passages may occur as the strain becomes more egg adapted. Other viruses, notably adenoviruses that are common to the respiratory tract, also produce embryo lesions indistinguishable from IBV. The IBV-laden allantoic fluid should not agglutinate red blood cells and isolation of IBV must be confirmed by serotyping or genotyping. Infective allantoic fluids are kept at −20°C or below for short-term storage, −60°C for long-term storage or at 4°C after lyophilisation.

TOCs prepared from 20-day-old embryos can be used to isolate IBV directly from field material (Cook et al., 1976). An automatic tissue-chopper is desirable for the large-scale production of suitable transverse sections or rings of the trachea for this technique (Darbyshire et al., 1978). The rings are about 0.5–1.0 mm thick, and are maintained in a medium consisting of Eagle’s N-2-hydroxyethylpiperazine N’-2-ethanesulphonic acid (HEPES) in roller drums (15 rev/hour) at 37°C. Infection of tracheal organ cultures usually produces ciliostasis within 24–48 hours. Ciliostasis may be produced by other viruses and suspect IBV sources that have been neither infected nor vaccinated with IBV. Most commonly, 0.1–0.2 ml of sample supernatant is inoculated into the allantoic cavity of 9–11-day-old embryos. Eggs are candled daily for 7 days with mortality within the first 24 hours being considered nonspecific. The initial inoculation usually has limited macroscopic effects on the embryo unless the strain is derived from a vaccine and is already egg adapted. Normally, the allantoic fluids of all eggs are pooled after harvesting 3 days after infection; this pool is diluted 1/5 or 1/10 in antibiotic broth and further passaged into another set of eggs for up to a total of three to four passages. Typically, a field strain will induce observable embryonic changes consisting of stunted and curled embryos with feather dystrophy (clubbing) and urate deposits in the mesonephros on the second to fourth passage. Embryo mortality in later passages may occur as the strain becomes more egg adapted. Other viruses, notably adenoviruses that are common to the respiratory tract, also produce embryo lesions indistinguishable from IBV. The IBV-laden allantoic fluid should not agglutinate red blood cells and isolation of IBV must be confirmed by serotyping or genotyping. Infective allantoic fluids are kept at −20°C or below for short-term storage, −60°C for long-term storage or at 4°C after lyophilisation.

The initial tests performed on IBV isolates are directed at eliminating other viruses from diagnostic consideration. Chorioallantoic membranes from infected eggs are collected, homogenised, and tested for avian adenovirus group 1 by immunodiffusion or PCR. Group 1 avian adenovirus infections of commercial chickens are common, and the virus often produces stunted embryos indistinguishable from IBV-infected embryos. Furthermore, harvested allantoic fluids do not hemagglutinate (HA) chick red blood cells. Genetic based tests (RT-PCR or RT-PCR-RFLP [restriction fragment length polymorphism]) are used commonly to identify an isolate as IBV. Other techniques may be used, for example cells present in the allantoic fluid of infected eggs may be tested for IBV antigen using fluorescent antibody tests (Clarke et al., 1972) and direct negative-contrast electron microscopy will reveal particles with typical coronavirus morphology in allantoic fluid or TOC fluid concentrates. The presence of IBV in infective allantoic fluid may be detected by RT-PCR amplification and use of a DNA probe in a dot-hybridisation assay (Jackwood et al., 1992). Direct immunofluorescence staining of infected TOCs for the rapid detection of the presence of IBV has been described (Bhattacharjee et al., 1994). Immuno-histochemistry, with a group-specific monoclonal antibody (MAb), can be used to identify IBV in infected chorioallantoic membranes (Naqi, 1990).

c) Methods for identification

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d) Serotype identification

Antigenic variation among IBV strains is common (Cavanagh & Naqi, 2003; Cook, 1984; Dawson & Gough, 1971; Hofstad, 1958; Ignjatovic & Sapats, 2000), but at present there is no agreed definitive classification system. Nevertheless, antigenic relationships and differences among strains are important, as vaccines based on one particular serotype may show little or no protection against viruses of a different antigenic group. As a result of the regular emergence of antigenic variants, the viruses, and hence the disease situation and vaccines used, may be quite different in different geographical locations. Ongoing assessment of the viruses present in the field is necessary to produce vaccines that will be efficacious in the face of antigenic variants that arise. Serotyping of IBV isolates and strains has been done using haemagglutination inhibition (HI) (Alexander et al., 1983; King & Hopkins, 1984) and virus neutralisation (VN) tests in chick embryos (Dawson & Gough, 1971), TOCs (Darbyshire et al., 1979) and cell cultures (Hopkins, 1974). Neutralisation of fluorescent foci has also been applied to strain differentiation (Csermelyi et al., 1988).

MAbs, usually employed in enzyme-linked immunosorbent assays (ELISA), have proven useful in grouping and differentiating strains of IBV (Ignjatovic et al., 1991; Koch et al., 1986). The limitations of MAb analysis for IB serotype definition are the lack of availability of MAbs or hybridomas and the need to produce new
MAbs with appropriate specificity to keep pace with the ever-growing number of emerging IB-variant serotypes (Karaca et al., 1992).

e) Genotype identification

RT-PCR genotyping methods have largely replaced HI and VN serotyping for determining the identity of a field strain. The molecular basis of antigenic variation has been investigated, usually by nucleotide sequencing of the gene coding for the spike (S) protein or, more specifically, nucleotide sequencing of the gene coding for the S1 subunit of the S protein (Cavanagh, 1991; Kusters et al., 1989) where most of the epitopes to which neutralising antibodies bind are found (Koch et al., 1992). An exact correlation with HI or VN results has not been seen, in that while different serotypes generally have large differences (20–50%) in the deduced amino acid sequences of the S1 subunit (Kusters et al., 1989), other viruses that are clearly distinguishable in neutralisation tests show only 2–3% differences in amino acid sequences (Cavanagh, 1991). However, there is, in general, good agreement between data represented by the S1 sequence and the VN serotype, and it may eventually be possible to select vaccine strains on the basis of sequence data.

The primary advantages of genotyping methods are a rapid turnaround time, and the ability to detect a variety of genotypes, depending on the tests used. RFLP RT-PCR differentiates IBV serotypes based on unique electrophoresis banding patterns of restriction enzyme-digested fragments of S1 following amplification of the gene by RT-PCR (Jackwood et al., 1997; Kwon et al., 1993). The RFLP RT-PCR procedure may be used in conjunction with a biotin-labelled DNA probe to first detect IBV in egg fluids harvested following the inoculation of eggs with clinical samples (Jackwood et al., 1992). The RFLP RT-PCR test can identify all known serotypes of IBV as well as variant viruses.

S1 genotype-specific RT PCR may be used to identify specific IBV serotypes (Keeler et al., 1998). S1 gene primers specific for serotypes Massachusetts (Mass), Connecticut, Arkansas, and JMK are used in conjunction with a universal primer set that amplifies all IBV serotypes. Primers for the DE/072/92 and California serotypes have also been developed. Other variant serotypes may be determined to be IBV using the general primers, but the specific serotype cannot be identified. Infections caused by multiple IBV serotypes may be identified.

Nucleotide sequencing of a diagnostically relevant fragment of the S1 gene is the most useful technique for the differentiation of IBV strains and has become the genotyping method of choice in many laboratories. Nucleotide sequencing has also produced evidence that recombination between IB strains occurs often (Cavanagh et al., 1992; Zwaagstra et al., 1992). RT-PCR product cycle sequencing of the hypervariable amino terminus region of S1 may be used diagnostically to identify previously recognised field isolates and variants (Kingham et al., 2000). Comparison and analysis of sequences of unknown field isolates and variants with reference strains for establishing potential relatedness are significant advantages of sequencing.

Recently, it has been shown that coronaviruses isolated from turkeys and pheasants are genetically similar to IBV, having approximately 90% nucleotide identity in the highly conserved region II of the 3' untranslated region (UTR) of the IBV genome (Cavanagh et al., 2001; 2002). The potential role of these coronaviruses in IBV infections has not been determined.

The major uses of RT-PCR tests are virus identification and its application in the understanding of epidemiological investigations during IBV outbreaks. The RT-PCR tests, as they now exist however, do not provide information on viral pathogenicity.

- RT-PCR test procedure
  i) Extraction of viral RNA

  Any RNA extraction method can be used. There are many protocols available in journals, books and on the web. However, for extracting high quality RNA from allantoic fluid, the Qiagen Viral RNA Mini Kit (www.qiagen.com) is recommended. The Qiagen RNeasy Mini Kit is recommended for extracted IBV RNA from tissue or swabbings. All extracted RNA should be stored between –20°C and –80°C until tested. It is advised that for long-term storage, RNA be kept at –80°C.

  ii) Custom oligos

  Custom oligos can be purchased through any commercial supplier. Operon (www.operon.com) has been making quality custom oligos for years. The target gene for IBV characterisation is the S1 subunit of the spike glycoprotein gene. A commonly used primer pair for amplification of genotypically diverse IBV strains is oligo S15' mod (forward): 5'-TGA-AAA-CTG-AAC-AAA-AGA-3' and CK2 (reverse): 5'-CNG-TRT-TRT-AYT-GRC-A-3' (Gelb et al., 2005). The oligo S15' mod/CK2 amplicon is approximately 700 bp in length beginning from the start of the S1 gene spanning two hypervariable regions used for genotyping.
iii) **Reverse-transcription polymerase chain reaction**

Many one and two-step RT-PCR kits are commercially available from manufacturers claiming superior enzyme sensitivity and fidelity. The recommended RT-PCR kit is the basic, two-step, RNA PCR kit from Applied Biosystems (http://www.appliedbiosystems.com). Reverse transcription is performed according to the manufacturer’s instructions. RT priming is accomplished with the use of random hexamers (supplied with the kit) or with the reverse PCR primer, in this case CK2 (Keeler et al., 1998). One cycle of RT is performed with the following parameters: 25°C for 10 minutes, 42°C for 25 minutes, 95°C for 5 minutes, hold at 4°C. The full RT reaction volume is added to the PCR sample master mix. PCR is performed using the following parameters: 95°C for 2 minutes, 45 cycles of 95°C for 30 seconds, 52°C for 30 seconds, 68°C for 30 seconds, final extension of 68°C for 12 minutes, hold at 4°C. Samples are concentrated in a desiccator overnight or by the use of a vacuum centrifuge. Dried samples are resuspended in 12 µl of DEPC-treated water and 6 µl of loading buffer prior to electrophoresis on a 1.8% agarose gel containing ethidium bromide. Gels are visualised with a UV light box. Bands are compared to a commercially available 100 bp ladder and an IBV positive control.

iv) **S1 gene sequencing**

Bands visualised in the agarose gel that are of similar size to the positive control are excised from the gel. The PCR product is separated from the agarose gel using the Qiagen Gel Extraction kit (www.qiagen.com) or any other commercial gel extraction kit. Purified PCR products are run on a second 1.8% agarose-ethidium bromide gel to determine the quantity of product present. Approximately 20 µl (10 ng/µl) of PCR product is required for sequencing. Sequencing can be performed at the University of Delaware Sequencing & Genotyping Center, Newark, DE (http://www.udel.edu/dnasequence) or another university or commercial sequencing facility. Sequence chromatograms are edited using the DNAStar analysis software or on-line freeware 4peaks (http://www.mekentosj.com/4peaks/), or chromas lite(http://www.technelysium.com.au/chromas_lite.html). Edited sequences of IBV isolates are characterised using BLASTn for nucleotide or BLASTp for protein analysis (http://www.ncbi.nlm.nih.gov/BLAST?).

### 2. Serological tests

A number of tests have been described. Those considered here include VN (Dawson & Gough, 1971), agar gel immunodiffusion (AGID) (Witter, 1962), HI (Alexander et al., 1983) and ELISA (Mockett & Darbyshire, 1981). Each test has advantages and disadvantages in terms of practicality, specificity, sensitivity and cost. In general, for routine serological testing, the VN tests are too expensive and impractical, and AGID tests lack sensitivity. ELISA and HI tests are most suitable for routine serology. ELISAs are useful for general monitoring of IBV exposure and can detect antibody responses to all serotypes. HI when used on sera from young growing chickens such as pullets and broilers can give information on the serotype-specific antibody status of a flock. Regular monitoring of sera from flocks for IBV antibody titres may help to indicate the level of vaccine or field exposure and can detect antibody responses to all serotypes. HI when used on sera from young growing chickens such as pullets and broilers can give information on the serotype-specific antibody status of a flock. Routine monitoring of sera from flocks for IBV antibody titres may help to indicate the level of vaccine or field challenge responses. Because chicken sera from older birds contain antibodies that are highly cross-reactive against antigenically unrelated strains, serodiagnosis of suspected disease outbreaks of IB cannot be used with a high degree of confidence.

#### a) Virus neutralisation

In VN tests, all sera should first be heated to 56°C for 30 minutes. Virus is mixed with serum and incubated for 30–60 minutes at 37°C or room temperature. Chicken embryos are most often employed, but antibodies can be measured using TOC or cell culture systems. Two methods have been used to estimate neutralising antibodies. One employs a constant serum concentration reacted with varying dilutions of virus (the alpha method) and the other employs a constant amount of virus and varying dilutions of serum (the beta method).

In the alpha method, tenfold dilutions of egg-adapted virus are reacted with a fixed dilution (usually 1/5) of antiserum, and the mixtures are inoculated into groups of from five to ten eggs. The virus alone is titrated in parallel. End-points are calculated by the Kärber or the Reed and Muench methods. The results are expressed as a neutralisation index (NI) that represents the log10 difference in the titres of the virus alone and that of the virus/antiserum mixtures. The NI values may reach 4.5–7.0 in the case of homologous virus/serum mixtures; values of <1.5 are not specific, but a heterologous virus will give a value as low as 1.5.

The beta method is the more widely used neutralisation test for antibody assay with chicken embryos. Two- or four-fold dilutions of antiserum are reacted in equal volumes with a dilution of virus, usually fixed at 100 or 200 EID50 (median embryo-infective doses) per 0.05 ml and 0.1 ml of each mixture inoculated into the allantoic cavity of each of from five to ten embryonated eggs. A control titration of the virus is performed simultaneously to confirm that the fixed virus dilution in the virus/serum mixtures was between 10^1.5 and 10^2.5 EID50. End-points of the serum titres are determined by the Kärber or Reed and Muench method as before, but here are expressed as reciprocals of log2 dilutions. This fixed-virus/varying-serum method is also employed for neutralisation tests in tracheal organ cultures using five tubes per serum dilution, as is...
conventional with other viruses (Darbyshire et al., 1979). The results are calculated according to Reed and Muench, and the virus titres are expressed as median ciliostatic doses per unit volume (log$_{10}$ CD$_{50}$). Serum titres are again expressed as log$_{2}$ dilution reciprocals. This test is more sensitive than others, but technical logistics hamper its more widespread adoption.

b) Haemagglutination inhibition

A standard protocol for a HI test for IBV has been described (Alexander et al., 1983), and the test procedure detailed below is based on that standard. Strains and isolates of IBV will agglutinate chicken red blood cells (RBCs) after neuraminidase treatment (Ruano et al., 2000; Schultze et al., 1992). The strain selected to produce antigen may be varied, depending on the requirements of diagnosis. The antigen for the HI test is prepared from IBV-laden allantoic fluids.

For HA and HI tests, procedures are carried out at 4°C.

- **Haemagglutination test**
  i) Dispense 0.025 ml of PBS, pH 7.0–7.4, into each well of a plastic U or V-bottom microtitre plate.
  ii) Place 0.025 ml of virus antigen in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/3, 1/4, 1/5, 1/6, etc.
  iii) Make twofold dilutions of 0.025 ml volumes of the virus antigen across the plate.
  iv) Dispense a further 0.025 ml of PBS into each well.
  v) Dispense 0.025 ml of 1% (v/v) chicken RBCs to each well.
  vi) Mix by tapping the plate gently and allow the RBCs to settle for about 40 minutes at 4°C, when control RBCs should be settled to a distinct button.
  vii) HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA in which there is no streaming; this is 100% HA and represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

- **Haemagglutination-inhibition test**

The HI test is used in the diagnosis and routine flock monitoring of vaccine responses.

i) Dispense 0.025 ml of PBS into each well of a plastic U or V-bottom microtitre plate.

ii) Place 0.025 ml of serum into the first well of the plate.

iii) Make twofold dilutions of 0.025 ml volumes of the serum across the plate.

iv) Add 4 HAU of virus antigen in 0.025 ml to each well and leave for 30 minutes.

v) Add 0.025 ml of 1% (v/v) chicken RBCs to each well and, after gentle mixing, allow the RBCs to settle for about 40 minutes when control RBCs should be settled to a distinct button.

vi) The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed more exactly by tilting the plates. Only those wells in which the RBCs ‘stream’ at the same rate as the control wells (containing 0.025 ml RBC and 0.05 ml PBS only) should be considered to show inhibition.

vii) The validity of results should be assessed against a negative control serum, which should not give a titre >24, and a positive control serum, for which the titre should be within one dilution of the known titre.

viii) Sera are usually regarded as positive if they have a titre of 24 or more. However, it should be noted that even in SPF flocks, a very small proportion of birds may show a nonspecific titre of 24, but usually in birds over 1 year of age.

c) Enzyme-linked immunosorbent assay

The ELISA technique is a sensitive serological method and gives earlier reactions and higher antibody titres than other tests (Mockett & Darbyshire, 1981). It lacks type or strain specificity, but is valuable for monitoring vaccination responses under field conditions. Commercial kits for ELISAs are available – these are based on several different strategies for the detection of IBV antibodies. Usually, such tests have been evaluated and validated by the manufacturer, and it is therefore important that the instructions specified for their use be followed carefully. The ELISA is widely used to identify IBV-infected flocks (broilers) based on high antibody titres. If IB reoccurs in the next flock on the farm, virus isolation attempts are performed and the virus is genotyped by RFLP or S1 sequencing.
d) Agar gel immunodiffusion

AGID can be used in diagnosis (Witter, 1962). The antigen is prepared from a homogenate of the chorioallantoic membranes of infected chicken embryos. The Beaudette embryo-lethal strain is often employed to produce antigen. The test lacks sensitivity and is liable to yield inconsistent results as the presence and duration of precipitating antibodies may vary with individual birds.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

All live and inactivated commercial vaccines must be licensed. Strains used in live virus vaccines generally require attenuation. At present, many countries only permit live vaccines of the Massachusetts type, such as the H 120. Some countries may also have licensed vaccines to other live strains such as Connecticut, Arkansas, or Delaware 072 (USA) or the 4/91 (United Kingdom). Live vaccines may be given as aerosols, in the drinking water, or by the intraocular route (eyedrop).

The efficacy of inactivated vaccines depends heavily on proper priming with a live vaccine(s). Inactivated vaccines must be administered to birds individually, by intramuscular or subcutaneous injection. Variant strains may be used to prepare inactivated autogenous vaccines for controlling IB in layers and breeders.

Live vaccines confer better local immunity in the respiratory tract and also may protect against a wider antigenic spectrum of field strains (Cook et al., 1999). However, vaccination with live vaccines may not protect layer flocks against variant serotype challenge especially common on farms with flocks of multiple ages where production drops as early as 40 weeks of age are not uncommon (Gelb et al., 1991). Live vaccines carry a risk of residual pathogenicity associated with vaccine back-passage in flocks. However, proper mass application techniques (e.g. spray or drinking water) can achieve uniform distribution of the vaccine in the flock and avoid backpassage. Furthermore, the use of vaccines at manufacturer’s recommended dosages will also help avoid backpassage reversion that may be caused by fractional dose application.

There are prospects for genetically engineered vaccines (Casais et al., 2003), and in-ovo vaccination (Wakenell et al., 1995).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.6 are intended to be general in nature. National and international standards that apply in the country in which IB vaccines are manufactured must be complied with. The licensing authority should provide information and guidance on requirements. These are now often presented in general terms, as applying to all vaccines – avian and mammalian, live and inactivated, or viral and bacterial vaccines. There may also be specific requirements applying to IB vaccines, live and inactivated. As examples, references are given to the European and USA regulations (Commission of the European Communities, 1999; European Pharmacopoeia [Council of Europe, 2010a; 2010b]; USDA Code of Federal Regulations 113.327).

The list of extraneous agents that must be shown to be absent continues to grow. Manufacturers must be familiar with those that currently apply in their country. Recent additions are avian nephritis virus and avian pneumovirus.

For IB vaccines, important differences among countries may arise regarding the challenge virus to be used for potency tests, and its validation. Traditionally, the virulent M-41 (Mass 41) strain of the Massachusetts type has been used for challenge tests of both live and inactivated vaccines. Although this type is still common, it is often not the only or the dominant type in many countries and it may be advisable to prepare vaccines from other types. It is logical for challenges to be made by the same type as present in the vaccine. Establishing criteria for validating the challenge virus may be more difficult for non-Massachusetts types, because of their lower virulence in general. Inactivated vaccines are usually expected to protect against drops in egg production. The traditional M-41 challenge, as described in this chapter, should cause a drop of at least 67% in the unvaccinated controls, but when using other types much lower drops may be regarded as satisfactory, depending on published evidence of the effects of these strains in the field. There is also a tendency to relax the criteria for Massachusetts type challenges, and the European Pharmacopoeia now defines a satisfactory drop for Massachusetts types to be at least 35%, and for non-Massachusetts types to be at least 15%, provided that the drop is ‘commensurate with the documented evidence’ (European Pharmacopoeia).

1. Seed management

a) Characteristics of the seed

The seed-lot (master seed) system should be employed for whatever type of vaccine is produced. Each virus must be designated as to strain and origin and must be free from contamination with other strains of IBV and extraneous agents. Separate storage facilities should be provided between the strains of virus intended for vaccines or for challenge.
For live virus vaccines, many countries permit only strains of the Massachusetts type. Some countries allow other strains, usually on the basis that those strains are already present in their national flocks. The antigenic type incorporated in both live and inactivated vaccines requires justification if there is doubt as to its existence in a country.

b) Method of culture

All seed viruses are grown in the allantoic sac of developing chicken embryos or in suitable cell cultures. The eggs should be from an SPF flock.

c) Validation as a vaccine

- **Purity**

Every seed lot must be free from bacterial, fungal, mycoplasmal and viral contamination.

For the detection of extraneous viruses, the seed is first treated with a high-titred monospecific antiserum prepared against the strain under examination or against one of identical type. This mixture is cultured in a variety of ways, designed to confirm the absence of any viruses considered from past experience to be potential contaminants. The antiserum must not contain antibodies to adenovirus, avian encephalomyelitis virus, avian rotavirus, chicken anaemia virus, fowlpox virus, infectious laryngotracheitis virus, influenza A virus, Newcastle disease virus, infectious bursal disease virus, leukosis virus, reovirus, Marek’s disease virus, turkey herpesvirus, adeno-associated virus, egg-drop syndrome 76 (EDS76) virus, avian nephritis virus, avian pneumovirus or reticulo-endotheliosis virus. The inoculum given to each unit of the culture system used should contain a quantity of the neutralised IBV component under test that had an initial infectivity of at least ten times the minimum field dose. These systems include:

1. SPF chicken embryos, incubated for 9–11 days, inoculated via both allantoic sac and chorioallantoic membrane (two passages);
2. Chicken embryo fibroblast cultures, for leukosis virus subgroups A and B. The COFAL test (test for avian leukosis using complement fixation) or double-antibody sandwich ELISA for group-specific leukosis antigen is performed on cell extracts harvested at 14 days. An immunofluorescence test for reticulo-endotheliosis virus is done on cover-slip cultures after two passages.
3. SPF chicken kidney cultures that are examined for CPEs, cell inclusions and haemadsorbing agents passaged at intervals of no fewer than 5 days for up to 20 days’ total incubation.
4. SPF chickens of minimum vaccination age inoculated intramuscularly with 100 field doses, and on to the conjunctiva with ten field doses; this is repeated 3 weeks later when the chickens are also inoculated both into the foot pad and intranasally with ten field doses. Observations are made for 6 weeks overall, and serum is collected for tests for avian encephalomyelitis, infectious bursal disease, Marek’s disease, Newcastle disease and *Salmonella pullorum* infection.

- **Potency**

Vaccines intended to protect against loss of egg production should be tested for duration of antibody response. Mean HI titres should be >6 log₂ up to at least 60 weeks of age. Serological tests should be done at intervals frequent enough to show that titres have not been boosted by extraneous IBV infection.

Vaccines intended for protection of broiler chickens or rearing chickens against the respiratory form of the disease should be similarly tested for duration of antibody responses; in the case of broilers this would be up to the normal age for slaughtering, and in the case of pullets up to the age when a booster vaccination would be administered (often at 16–18 weeks of age).

- **Safety**

Tests on seed virus should include a test for any potential ability to revert to virulence. Live and inactivated vaccine seed must be tested for safety as in Section C.4.b.

- **Efficacy**

To demonstrate efficacy, a trial vaccine must be made from the master seed and the working seed at five passages from the master seed and subjected to tests that demonstrate their protective effect.

For live vaccines, a minimum of ten SPF chickens aged 3–4 weeks are vaccinated intranasally or by eyedrop with the recommended dose. Ten unvaccinated control birds from the same age and source are retained separately. All birds of both groups are challenge inoculated intranasally or by eyedrop 3–4 weeks later, each with $10^{3.0–3.5} \text{EID}_{50}$ of the virulent Massachusetts M-41 strain. A swab of the trachea is taken.
from each bird 4–5 days after challenge and placed in 3 ml of antibiotic broth. Each fluid is tested for IBV by the inoculation (0.2 ml) of five embryonated eggs after 9–11 days of incubation. An alternative test to that of taking swabs is to kill birds at 4–6 days after challenge and examine microscopically the tracheal rings for ciliary activity (Darbyshire, 1985). Failure to resist challenge is indicated by an extensive loss of ciliary motility. The live vaccine is suitable for use if at least 90% of the challenge vaccinated birds show no evidence of IBV in their trachea, while 90% or more of the control birds should have evidence of the presence of the virus.

To assess an inactivated vaccine intended to protect laying birds, 30 or more SPF chickens are vaccinated as recommended at the earliest permitted age. If a primary vaccination with live vaccine is first undertaken, an additional group of birds is given only the primary vaccination. In both cases, these primary vaccinations should be done at no later than 3 weeks of age. The inactivated vaccine is given 4–6 weeks after the live priming vaccination. A further group of 30 control birds are left unvaccinated. All groups are housed separately until 4 weeks before peak egg production, and then are housed together. Individual egg production is monitored and once it is regular, all birds are challenged, egg production being recorded for a further 4 weeks. The challenge should be sufficient to ensure loss of production during the 3 weeks after challenge. The loss in the control group should be at least 67%; the group that received primary live virus vaccine followed by inactivated vaccine should remain at the previous level, and the group given only a primary vaccination should show an intermediate drop in production. Sera are collected from all birds at vaccination, 4 weeks later, and at challenge; there should be no response in the control birds.

To assess an inactivated vaccine intended to protect birds against respiratory disease, 20 SPF chickens aged 4 weeks are vaccinated as recommended. An additional 20 control birds of the same age and origin are housed with this first group. Antibody responses are determined 4 weeks later; there should be no response in the control birds. All birds are then challenged with 10^3 CID_{50} (50% chick infective dose) of virulent virus, killed 4–7 days later, and tracheal sections are examined for ciliary motility. At least 80% of the unvaccinated controls should display complete ciliostasis, whereas the tracheal cilia of a similar percentage of the vaccinated birds should remain unaffected.

Both live and inactivated vaccines containing Newcastle disease, infectious bursal disease, reovirus and EDS76 viruses are available in some countries. The efficacy of the different components of these vaccines must each be established independently and then as a combination in case interference between different antigens exists.

2. Method of manufacture

All virus strains destined for live vaccines are cultured in the allantoic sac of SPF chicken embryos or in suitable cell cultures. For inactivated vaccines, hens’ eggs from healthy non-SPF flocks may be used. The pooled fluid is clarified and then titrated for infectivity. For live vaccines this fluid is lyophilised in vials, and for inactivated vaccines it is blended with high-grade mineral oil to form an emulsion to which a preservative is added.

3. In-process control

The required antigen content is based on initial test batches of vaccine of proven efficacy in laboratory and field trials. Infectivity titrations are done in chicken embryos.

Live vaccine should contain not less than 10^{3.5} EID_{50} per dose per bird until the expiry date indicated, and not less than 10^{2.5} EID_{50} per dose per bird after incubation at 37°C for 7 days at the time of issue. For inactivated vaccine, the inactivating agent and inactivation procedure must be shown under manufacture to be effective on both IBV and potential contaminants. With the use of beta-propiolactone or formalin, any live leukosis viruses and Salmonella species must be eliminated; and with other inactivating agents, the complete range of potential contaminants must be rendered ineffective. Before inactivation procedures, it is important to ensure homogeneity of suspensions, and a test of inactivation should be conducted on each batch of both bulk harvest after inactivation and the final product.

Tests of inactivation should be appropriate to the vaccine concerned and should consist of two passages in cell cultures, embryos or chickens, using inoculations of 0.2 ml and ten replicates per passage.

4. Batch control

a) Sterility

Every batch of live vaccine should be tested for the absence of extraneous agents as for the seed virus (see Chapter 1.1.7 Tests for sterility and freedom from contamination of biological materials).
b) Safety

- For live vaccines

Use no fewer than ten chickens from an SPF flock that are of the minimum age stated on the label for vaccination. Administer by eyedrop to each chicken ten doses of the vaccine reconstituted so as to obtain a concentration suitable for the test. Observe the chickens for 21 days. For vaccines intended for chickens that are 2 weeks old or more, use the chickens inoculated in the ‘test for extraneous agents using chickens’ (see Section C.1.c.4). If during the period of observation, more than two chickens die from causes not attributable to the vaccine, repeat the test. The vaccine complies with the test if no chicken shows serious clinical signs, in particular respiratory signs, and no chicken dies from causes attributable to the vaccine.

- For inactivated vaccines

Inject a double dose of vaccine by the recommended route into each of ten 14–28-day-old chickens from an SPF flock. Observe the chickens for 21 days. Ascertain that no abnormal local or systemic reaction occurs.

c) Potency

The potency test is developed from the results of efficacy tests on the master seed virus. Live vaccines are tested for potency by titration of infectivity, and inactivated vaccines by measuring antibody production. The potency test for a batch of inactivated vaccine consists of vaccinating 20 SPF chickens, 4 weeks of age, and showing that their mean HI titre 4 weeks later is not less than 6 log₂.

d) Duration of immunity

Vaccine must be shown to have the required potency to achieve the claimed duration of immunity at the end of the claimed shelf life.

e) Stability

At least three batches should be tested for stability and must give satisfactory results for 3 months beyond the claimed shelf life.

The stability of a live vaccine must be measured by maintenance of an adequate infectivity titre.

The stability of an inactivated vaccine is measured at intervals by batch potency tests. The concentration of preservative and persistence through the shelf life should be assessed. There should be no physical change in the vaccine and it should regain its former emulsion state after one quick shake.

f) Preservatives

There are maximum level requirements for the use of antibiotics, preservatives and residual inactivating agents.

g) Precautions (hazards)

IBV itself is not known to present any danger to staff employed in vaccine manufacture or testing. Extraneous agents may be harmful, however, and the initial stages of handling a new seed virus should be carried out in a safety cabinet. It is a wise precaution with all vaccine production to take steps to minimise exposure of staff to aerosols of foreign proteins. Persons allergic to egg materials must never be employed in this work.

5. Tests on the final product

a) Safety

A safety test must be carried out on each batch of final product, as in Section C.4.b.

b) Potency

A potency test must be carried out on each batch of final product, as in Section C.4.c, at manufacture and at the end of the stated shelf life.

REFERENCES

Chapter 2.3.2. — Avian infectious bronchitis

technique for haemagglutination inhibition tests for antibodies to avian infectious bronchitis virus. Vet. Rec., 113, 64.


Chapter 2.3.2. – Avian infectious bronchitis


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CHAPTER 2.3.3.

AVIAN INFECTIOUS LARYNGOTRACHEITIS

SUMMARY

Avian infectious laryngotracheitis (ILT) is a respiratory disease caused by Herpesviridae alphaherpesvirinae gallid herpesvirus 1. It is principally a disease of chickens, although it can also affect pheasants, partridges and peafowl. The clinical signs and pathological reactions may vary from extremely severe, with some birds dying due to asphyxiation, to very mild, indistinguishable from other mild respiratory diseases of chickens. The principal lesion is tracheitis. In infected birds the virus can become latent and re-excreted at a later date without clinical signs.

Laboratory diagnosis depends on isolation of the virus, demonstration of the presence of the virus or viral antigens, and detection of specific antibodies in the serum. Histopathological examination of the trachea for characteristic intranuclear inclusions may be of value.

Identification of the agent: Virus isolation may be done by inoculation of suspected material on to the dropped chorioallantoic membrane of embryonated eggs, or into avian embryonic cell cultures. These methods are time-consuming but sensitive. Rapid methods include direct electron microscopy on tracheal exudate, immunofluorescence on tracheal exudate or frozen sections, agar gel immunodiffusion (AGID) to detect viral antigens in tracheal samples or infected egg material, and an enzyme-linked immunosorbent assay (ELISA) to demonstrate viral antigen in mucosal scrapings. Polymerase chain reaction (PCR) methodology has been shown to be more sensitive than virus isolation for the examination of clinical material and is now widely used. Virus characterisation and differentiation of vaccine and wild-type viruses are possible using PCR followed by restriction fragment length polymorphism.

Serological tests: Antibodies to ILT virus (ILTV) can be detected by virus neutralisation (VN) tests conducted in eggs or in cell cultures, or by AGID reactions, indirect immunofluorescence, or ELISA. The latter is preferred for screening flocks.

Requirements for vaccines and diagnostic biologicals: Vaccines against ILT are usually prepared from attenuated live virus. Those available at present afford some degree of protection, but are not completely satisfactory. There have been recent promising studies on the efficacy of a genetically engineered vaccine.

A. INTRODUCTION

Avian infectious laryngotracheitis (ILT) is a respiratory disease of chickens caused by an alphaherpesvirus. It can also affect pheasants, partridges and peafowl. In the virulent form, the history, clinical signs and very severe tracheal lesions are highly characteristic of the disease, but the mild form may be indistinguishable from other mild respiratory diseases. Laboratory diagnosis depends on the demonstration of the presence of the virus or viral antigens or products (Guy et al., 2003; Scholz et al., 1994; Williams et al., 1994) or specific antibodies in the serum (Adair et al., 1985; Meulemans & Halen, 1978).

Clinically, the disease may appear in three forms, namely peracute, subacute, and chronic or mild. In the peracute form, onset of disease is sudden with a rapid spread. The morbidity is high and mortality may exceed 50%. Some birds may die in good body condition before the appearance of signs, which are characteristic and comprise difficulty in breathing with extension of the neck and gasping in an attempt to inhale. There is also gurgling, rattling and coughing when birds try to expel obstructions in the trachea. Clots of blood may be coughed up and can be found on the floor and walls of the house. Post-mortem changes are confined to the upper respiratory tract and are also characteristic, consisting of haemorrhagic tracheitis with blood clots, mucoid rhinitis, and blood-stained mucus along the length of the trachea.
In the subacute form, the onset of illness is slower and respiratory signs may extend over some days before deaths are seen. The morbidity is high but the mortality is lower than in the peracute form, between 10% and 30%. Post-mortem findings are less severe and consist of mucoid exudate with or without blood in the trachea. Yellow caseous diphtheritic membranes may be found adherent to the larynx and upper tracheal mucosa.

Chronic or mild ILT may be seen among survivors of either of the above forms of the disease, although some outbreaks themselves may be entirely mild. Incidence of chronic ILT within a flock may be only 1–2%, with most affected birds dying of suffocation. Signs include spasms of coughing and gasping, with nasal and oral discharge and reduced egg production. Infection is acquired via the upper respiratory tract and transmission occurs most readily from acutely infected birds, but clinically inapparent infection can persist for long periods with intermittent re-excretion of the virus, and these recovered carrier birds are also a potential means of transmission of the disease (Hughes et al., 1987). On post-mortem examination, diphtheric and caseous necrotic plaques and plugs are found in the trachea, larynx and mouth. Outbreaks of mild ILT may affect large numbers of birds simultaneously, in which case gross lesions may consist only of conjunctivitis, sinusitis and mucoid tracheitis. Given that transmission of ILT takes place by close contact, transmission is slower in cage houses than where birds are loose-housed, and the path of infection through a cage house may be apparent. Recent work has confirmed considerable variation among ILTV strains in their tropism for trachea or conjunctiva and those with affinity for the latter site can severely affect weight gain (Kirkpatrick et al., 2006).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The virus may be isolated in chick embryo liver (McNulty et al., 1985), chicken embryo kidney (Chang et al., 1960) or in chicken kidney (Van Kammen & Spadbrow, 1976) cell cultures. Of these, monolayers of chicken embryo liver cells have been found to be the most sensitive (Hughes & Jones, 1988). The virus can also be grown on the dropped chorioallantoic membrane (CAM) of 10–12-day-old specific pathogen free embryonated chicken eggs (Jordan, 1964).

The causative herpesvirus may be demonstrated directly in tracheal exudate by electron microscopy (Van Kammen & Spadbrow, 1976). Viral antigens may be detected by immunofluorescence (Braune & Gentry, 1985; Wilks & Kogan, 1979), agar gel immunodiffusion (AGID) (Jordan & Chubb, 1962), or enzyme-linked immunosorbent assay (ELISA), using tracheal mucosal scrapings (York & Fahey, 1988). Histopathological examination of the trachea for typical herpesvirus intranuclear inclusions may also be helpful (Armstrong, 1959; Pirozok et al., 1957). Methods of detecting ILT virus (ILTIV) using polymerase chain reaction (PCR) have been described and PCR has been reported to be generally more sensitive than virus isolation (Alexander & Nagy, 1997; Keam et al., 1991; McNulty et al., 1985; Williams et al., 1994).

a) Virus isolation

When samples are taken from live birds for virus isolation, tracheal swabs are superior to oropharyngeal or conjunctival swabs. These are placed in transport medium containing antibiotics. When selecting material for virus isolation from chronic outbreaks, it is more productive to cull a bird in the early stages of the infection, rather than to attempt to isolate virus from a bird that has died of asphyxiation after a long illness. The quality of sample is further improved if the bird is killed by barbiturate or other injection rather than by cervical dislocation. The whole head and neck from dead birds may be submitted, or only the trachea and larynx after their removal with minimal contamination. Tracheas should be transported in antibiotic broth for virus isolation, but wrapped in moist tissue paper if destined for electron microscopy. Any prolonged storage of infected tissues should be at −70°C or below to minimise loss of virus titre. Repeated freezing and thawing must be avoided as this reduces virus infectivity.

Exudate and epithelial cells are scraped from the tracheas, diluted approximately 1/5 in nutrient broth containing penicillin and streptomycin, and agitated vigorously. The resulting suspension is centrifuged at low speed to remove debris, and 0.1 ml of the supernatant fluid is inoculated on to the dropped CAM of at least three embryonated chicken eggs of 10–12 days' incubation. The eggs are sealed with paraffin wax and incubated at 37°C for up to 7 days. They are candled daily and the CAMs of dead embryos or of those surviving for 7 days are examined for typical pocks. Alternatively, at least two confluent chick embryo liver or chicken embryo kidney cell monolayers, with their medium removed, are inoculated and allowed to adsorb for 1–2 hours. Cultures are overlaid with fresh medium, incubated for up to 7 days and examined daily under the microscope for evidence of a typical syncytial cell cytopathic effect (CPE).

In each instance, up to three passages of material may be necessary before a specimen is considered to be negative. A virus isolate can be confirmed as ILTV by a neutralisation test in eggs or cell culture using hyperimmune antiserum to ILTV. Alternatively, virus particles may be identified rapidly in cell culture fluid or
in pocks on CAMs by electron microscopy, viral antigens by immunofluorescence in acetone-fixed ILT-virus-infected cell cultures or in frozen sections of CAM and viral nucleic acid by PCR.

b) Electron microscopy

To demonstrate the presence of virus by electron microscopy, tracheal exudate or epithelial scrapings from the trachea are smeared on to a microscope slide and mixed with a few drops of distilled water. One drop of suspension is placed on a carbon and formvar-coated grid and left for 2 minutes, after which excess moisture is removed using filter paper. One drop of 4% phosphotungstic acid, pH 6.4, is added and the excess removed after a further 3 minutes. The grid is allowed to dry thoroughly and examined using the electron microscope at a magnification of \( \times30-45,000 \) for typical herpesvirus particles, measuring 100 nm diameter with icosahedral symmetry.

c) Immunofluorescence

In immunofluorescence tests for viral antigens, epithelial cell scrapings from the trachea are smeared on to a glass slide. Alternatively, 5 µm thick cryostat sections of trachea, snap-frozen in liquid nitrogen may be used. The preparations are fixed in acetone at room temperature for 10 minutes. These can be stained directly by applying chicken anti-ILTv immunoglobulin labelled with fluorescein isothiocyanate (FITC) for 1 hour, followed by rinsing for 15 minutes in a bath of phosphate buffered saline (PBS), pH 7.2, agitated with a magnetic stirrer. Otherwise, they can be stained indirectly by applying an appropriate dilution of chicken anti-ILTv serum for 1 hour. The slide is rinsed thoroughly with PBS for 15 minutes as above, and an FITC-labelled anti-chicken immunoglobulin is applied for 30 minutes. After a final rinse, cover-slips are applied over non-fade mountant. The preparations are examined for specific intranuclear fluorescence in the epithelial cells using a microscope with epifluorescent ultraviolet illumination. Suitable controls include the use of known uninfected specimens and, for the indirect method, the application of nonimmune chicken serum. Particular care should be taken in the reading of indirect immunofluorescence preparations, as endogenous chicken IgG in the trachea may cause unwanted attachment of FITC-labelled anti-chicken IgG.

d) Agar gel immunodiffusion

ILT viral antigens may be demonstrated by AGID tests on tracheal exudate, infected CAMs and infected cell culture material using hyperimmune ILTV antiserum. The gel is made with Noble agar (1.5%) containing sodium chloride (8%) and sodium azide (0.02%) – as preservative – in distilled water. The ingredients are autoclaved at 15 lb/sq. inch (2.4 bar) for 15 minutes; 5 ml of the molten agar is poured into a 5 cm diameter Petri dish. When the agar has set, a pattern of wells is punched in the agar, consisting of a central well and six surrounding wells. The wells are usually 8 mm in diameter and 4 mm apart. The hyperimmune serum is pipetted into the central well, while the surrounding wells are filled with suspect virus samples under test, but with at least one well containing positive viral antigen. Dishes are incubated in a humid atmosphere at room temperature or at 37°C, and examined 24–48 hours later by oblique illumination for lines of precipitation (reactions of identity). Tests should include uninfect ed material as negative antigen and known negative antiserum as controls. For economy of materials, the test can be done on a microscale – the agar being poured in a thin layer on to a microscope slide and holes punched of 4 mm diameter and 2 mm apart.

e) Enzyme-linked immunosorbent assay

When the monoclonal antibody (MAb) ELISA is used for detecting viral antigens (McNulty et al., 1985), tracheal exudate is mixed with an equal volume of PBS containing 1% (v/v) of a detergent, such as Nonidet P40 (BDH Chemicals, Poole, United Kingdom), then vortexed for 30 seconds and centrifuged at 10 \( g \) for 1 minute. The supernatant fluid is dropped in 50 µl volumes in wells of microtitre plates previously coated with rabbit IgG against ILTV, diluted 1/200 in 0.05 M carbonate/bicarbonate buffer, pH 9.0, and incubated for 1 hour. Next, 50 µl of MAb against major glycoproteins of ILTV, diluted 1/50 in PBS, is added to each well, followed by 50 µl of a 1/1000 dilution of affinity-purified goat anti-mouse IgG conjugated to horseradish peroxidase. The substrate, 5-aminosalicylic acid (6.5 mM), is added to the wells in 100 µl volumes. After 30 minutes, the plates are read at 450 nm on a spectrophotometer and the absorbance reading for each well is corrected by subtracting the reading obtained for wells containing diluting buffer instead of tracheal exudate. The positive/negative cut-off point is taken as the mean absorbance value for several negative (i.e. tracheal material without ILTV) samples plus 3 standard deviations.

f) Histopathology

Tracheas for histopathological examination should be placed in formol saline immediately after removal from the birds and embedded in paraffin wax. Eyelids and lung are sometimes examined. Intranuclear inclusions may be seen in the epithelial cells of the trachea in longitudinal sections after staining by haematoxylin and eosin. They are the classical Cowdry type A inclusions of herpesviruses, but they may be present for only 3–5 days after infection. In severe cases where most infected cells have detached from the tracheal lining, inclusions may be seen in intact cells among the cellular debris in the lumen of the trachea. Longitudinal rather than transverse sections of trachea permit examination of the whole length of the organ.
g) Molecular methods

Several molecular methods for identifying ILTV DNA in clinical samples have been reported, but the PCR has proved the most useful. Dot-blot hybridisation assays and cloned virus DNA fragments were shown to be highly sensitive for detecting virus when isolation and ELISA were negative (Keam et al., 1991; Key et al., 1994). Humberd et al. (2002), using a nested PCR, showed that ILTV DNA could be detected in formalin-fixed, paraffin-embedded tissues independently of the presence of syncytial cells, intranuclear inclusions or both.

PCR has been found to be more sensitive than virus isolation for clinical samples, especially when other contaminant viruses such as adenoviruses are present (Williams et al., 1994). Alexander & Nagy (1997) found that during the middle to the end of the infection phase, PCR and virus isolation were similar in sensitivity, but PCR was superior in the recovery phase.

A problem with the PCR for ILTV was that initially it was not able to differentiate between field and vaccine strains. However, the combination of PCR with restriction fragment length polymorphism (RFLP) analysis of single and multiple viral genes and genome regions has enabled the characterisation of different strains within a country or region (Chang et al., 1997). Several reports have shown that while some field strains are closely related to and likely to be derived from vaccines viruses, others are true ‘wild types’ (Ojkic et al., 2006). Genes commonly examined by different international authors include ICP4, TK (thymidine kinase), glycoprotein G (gG), glycoprotein E (gE) and UL47. Oldoni & Garcia (2007) used 36 restriction enzymes, while others have used as few as four.

i) PCR protocol

In a typical PCR protocol for ILTV, viral DNA is extracted from clinical samples (swabs, tissues pieces), chorioallantoic membrane plaques, cell culture supernatants or vaccines using DNA extraction kits. Primers used can be obtained from previously published work or designed using ILTV sequences on the Genbank international database. Amplifications are made using Taq DNA polymerase. Typical amplification reactions use an initial denaturing step of 94°C for 1 minute followed by 35 amplification cycles of 94°C for 1 minute with annealing temperatures ranging from 54–60°C for 30 seconds. Extension may be performed at 68°C, with extension times varying according to the size of the target region amplified and a final extension at 68°C for 7 minutes. The PCR products are separated by electrophoresis in 1% agarose gels, stained with ethidium bromide and exposed to UV light for visualisation.

ii) Real-time PCR

Recently a real-time PCR assay had been described for ILTV (Creelan et al., 2006). This has the advantage that, including amplification and melt-curve analysis, it can be conducted in less than 2 hours. It therefore provides a very rapid method of ILT diagnosis in comparison with traditional virus isolation, or even the standard PCR followed by gel electrophoresis.

iii) Restriction fragment length polymorphism (RFLP)

A range of restriction endonucleases (RE) have been described for RFLP analysis of ILTV PCR products and several genes have been targeted for digestion. They include ICP4, TK (thymidine kinase), UL 15, UL47 glycoprotein G and ORF-BTK genes. Amplification products are digested separately with 10U RE for 3 hours. Digestion fragments are separated in 15% polyacrylamide gels. Fragments are observed after DNA silver staining and analysed under a light box. Pattern differences are recorded for each enzyme and results can be developed into dendrograms. The combination of PCR and RFLP has enabled field strains of ILTV to be distinguished from vaccine strains (Creelan et al., 2006; Han & Sim, 2001; Ojkic et al., 2006; Van Kammen & Spadbrow, 1976).

2. Serological tests

Antibodies to ILTV in chicken serum can be detected by virus neutralisation (VN), AGID, indirect immunofluorescence tests and ELISA (Adair et al., 1985).

a) Virus neutralisation

VN tests may be conducted on the dropped CAMs of embryonating chicken eggs that have been incubated for 9–11 days, where antibody specifically neutralises pock formation due to ILTV. Alternatively, the tests can be performed in cell cultures, where antibody specifically neutralises the ILTV thus preventing CPE. Doubling dilutions of serum are added to equal volumes of a constant concentration of virus. This concentration may either be 100 median egg infectious doses (EID₅₀) for egg inoculations, or 100 median tissue culture infectious doses (TCID₅₀) for the inoculation of cultures. The mixtures are incubated at 37°C for 1 hour to allow any neutralisation to occur.
When the test is performed in eggs, the virus/serum mixtures are inoculated on to the dropped CAMs, using at least five eggs per dilution. Eggs are sealed and incubated at 37°C for 6–7 days. The end-point is recorded as the highest dilution of the serum where no pocks are present on the CAMs. When the tests are done in cell cultures, serum dilutions are prepared in 96-well microculture plates and virus is then added. After the period allowed for neutralisation, freshly prepared chicken embryo liver or kidney cells are added to each well. The plates are incubated at 37°C in an atmosphere of 5% CO₂ and examined daily for CPE; 50% end-points are read after approximately 4 days when the virus control titre indicates that 30–300 TCID₅₀ of virus have been used in the test. For the cell culture method of testing, virus neutralisation at 1/8 (initial dilution) or greater is considered positive.

b) Agar gel immunodiffusion

For AGID tests, antigen is prepared from virus-infected CAMs or infected cell cultures. For the former, at least 10⁴ TCID₅₀ of ILTV is inoculated into the allantoic cavity of a batch of 10-day-old embryonating specific pathogen-free (SPF) chicken eggs. The CAMs are harvested after 4 days’ incubation, and those with large pocks are homogenised and sonicated in a small amount of PBS, pH 7.1. Alternatively, heavily infected monolayers of chicken embryo liver or kidney, or chicken kidney cells are incubated at 37°C until the CPE is maximal. Any remaining attached cells are scraped from the culture vessel into the medium. Total culture harvests may be concentrated up to 100-fold by dialysis against polyethylene glycol (PEG 20,000 or PEG 30,000). For the test, the agar is prepared as described previously for antigen detection, but this time the CAM or cell culture antigen is placed in the central well with test sera in the surrounding wells. Known positive and negative antisera are incorporated in the test, which is read after 24–48 hours’ incubation at room temperature or at 37°C. AGID tests are simple, economical to perform, and useful for flock screening, although they are less sensitive than the other methods.

c) Indirect fluorescent antibody test

For indirect fluorescent antibody tests, the antigen consists of ILT-virus-infected cell monolayers grown on teflon-coated multispot slides. When CPE is beginning to develop, the cultures are fixed in acetone for 10 minutes. Dilutions of test sera prepared in PBS are applied to each spot culture and the slides are incubated at 37°C for 1 hour. The slides are washed in PBS as described previously, drained and treated with an appropriate dilution of a commercial FITC-labelled rabbit anti-chicken IgG. After incubation at 37°C for 1 hour, the slides are re-washed and cover-slips are applied over a non-fade mountant. They are examined by epifluorescence with ultraviolet illumination, and end-point titres are read as the highest serum dilutions giving specific fluorescent staining. This test is more sensitive than AGID, but the interpretation of results may be subjective.

d) Enzyme-linked immunosorbent assay

The antigen for ELISA is obtained by sonication of heavily infected cell cultures at the time of maximum CPE, which is then absorbed on to the wells of microtitre plates. A negative antigen is provided by uninfected cell culture material treated in the same way. The test consists essentially of the addition of 0.1 ml of 1/10 dilutions of test sera to duplicate wells coated with positive or negative antigen. After incubation at 37°C for 2 hours, the plates are washed four times and a 1/4000 dilution of a rabbit anti-chicken IgG conjugated with peroxidase is added. After incubation at 37°C for 1 hour, the plates are washed again four times. Finally a substrate consisting of 5-aminosalicylic acid is added to each well followed by hydrogen peroxide to a final concentration of 0.005%, and the absorbance of the fluid in each well is read at 450 nm on a spectrophotometer. The result for each serum is expressed as the difference between the mean absorbance produced with the positive and negative antigens. The positive/negative cut-off point is taken as the mean absorbance value for numerous negative sera plus 3 standard deviations. The test is very sensitive and possibly the best available for surveillance purposes. Antibody responses as measured by ELISA are detectable 7–10 days after infection and peak at about 2 weeks. The response to ILT vaccines may be variable and testing is not worthwhile before 14 days post-vaccination. Several laryngotracheitis antibody ELISA kits are available commercially.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

ILT is usually controlled with live vaccines, although inactivated vaccines have also been used for safety reasons. There has been some recent work with genetically engineered vaccines and the results of the initial studies look promising (Davison et al., 2006; Veits et al., 2003). The live virus seed is a suitably attenuated or naturally avirulent strain of ILTV. Vaccines may be administered by eye-drop, spray or in the drinking water. If administered by spray and a small droplet size is produced and inhaled, clinical disease may be precipitated. Young chickens may require vaccinating in endemic areas, but show more severe reactions to the vaccine. Repeated doses may be required to afford good protection. The level of virulence of the vaccine virus is critical. Strains of low virulence may not be effective, and those of higher virulence may cause severe disease. The spray route of administration requires care over droplet size and uniformity of application. It can be more effective with low virulence strains, but may be more dangerous with high virulence strains. At present, the available vaccines attempt to make a compromise between lack of efficacy and poor safety. Because of persistence of virulent vaccine virus on a site, it
may be difficult to discontinue vaccination once it has been started. Subclinical mixed infections of vaccine and field virus, in vaccinated birds, can cause severe disease in unvaccinated in-contacts.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements (e.g. see Code of Federal Regulations [of the USA], 2000).

1. Seed management

a) Characteristics of the seed

The master seed virus (MSV) is selected and can be propagated in SPF chicken embryos or tissue cultures derived from such embryos. The MSV is tested in chicken embryos or chickens for the following: 1) purity, 2) *Mycoplasma* spp., 3) *Salmonella* spp., 4) avian leukosis virus, 5) haemagglutinating viruses, 6) virus identity, and 7) extraneous pathogens. Additionally, initial tests are performed to demonstrate the safety and efficacy of the chosen master seed. The safety test on the MSV must include tests to show lack of reversion to virulence on serial passage and also safety in birds. Evidence of shed and spread is also required. The MSV is stored in aliquots at –70°C. The MSV should not cause mortality or a severe respiratory reaction in chickens following ocular instillation, although pheasants are more susceptible. Administration by spray is convenient but may cause quite severe respiratory disease in some flocks.

b) Method of culture

In large-scale vaccine production, the virus is propagated in SPF chicken embryos or tissue culture derived from such embryos, up to the fifth passage from the MSV. The acceptable passage level is supported experimentally by the passage level used to prepare the experimental product used in the efficacy study.

c) Validation as a vaccine

A test must be carried out to establish the efficacy of the vaccine in birds of the minimum age for which the product is destined and also for each avian species. This is repeated in further batches of chickens for each of the recommended routes of administration and/or age of bird. Three weeks later (or 10–14 days in the USA), the birds, together with ten controls of the same age and source, are challenged intratracheally or in the orbital sinus with a strain of ILTV of known high virulence. To be satisfactory, only 5% of the vaccinated birds should die or show severe signs of ILT. No more than four should show mild signs of ILT. At least eight of the controls should die or show severe signs of ILT.

2. Method of manufacture

The vaccine is made by inoculation of the production seed virus into 9–11-day-old chicken embryos or tissue culture prepared from chicken embryos derived from SPF flocks. Eggs are inoculated through a hole in the shell, on to the dropped CAM. They are sealed and incubated at 37°C for 4–6 days. All eggs are candled before harvest and only those with living embryos are used. To harvest the virus, the eggs are chilled, then cleansed and opened aseptically. The CAMs and fluids are pooled in sterile, cooled containers. The CAMs should show the thick grey plaques typical of ILTV growth. Tissue culture-derived product would be prepared from virus-bearing cell culture fluids, which would also be subsequently pooled and tested.

3. In-process control

The infected tissue or tissue culture homogenate may be tested for purity, potency, and virus content, mixed with a stabiliser (usually beef peptone and sucrose) and then lyophilised and stored at 4°C.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

b) Safety

Using the recommended route of administration, each batch of vaccine is tested in ten SPF chickens, or ten birds of other target species, using ten doses per bird. The birds are observed for at least 21 days for adverse effects attributable to the vaccine.
Chapter 2.3.3. – Avian infectious laryngotracheitis

c) Potency

Once the *in-vivo* efficacy of the vaccine has been established, the batch potency may be determined by measuring the virus content. Serial dilutions of the vaccine are inoculated on to the dropped CAM of 9–11-day-old SPF chicken embryos, using at least seven eggs per dilution, in a volume of 100 µl. The eggs are incubated for 5 days and the virus titre is calculated by observing characteristic lesions on the CAMs. The virus content should be at or above a release value and above and expiration titre during dating of the product. Both the release and expiration titres are based on the minimum protective dose described above.

d) Duration of immunity

The results of vaccination will depend on many factors, including dose schedule and route of administration. Some degree of protection should be given, over a period of several months.

e) Stability

Stability is tested by taking samples of correctly stored vaccine at intervals and measuring virus content. Tests should be carried out on at least six batches of the vaccine or until a statistically valid number of serials have been evaluated and be continued for 3 months after the claimed shelf-life.

f) Preservatives

Preservatives may not be required, but some antibiotics may be added to the tissue harvest or at serial assembly during manufacture. For products licensed in the USA, any antibiotics added are listed on the label.

g) Precautions (hazards)

Care should be taken over diluting and administering the vaccine, and over the proper disposal of unused vaccine.

5. Tests on the final product

a) Safety

In the USA, 25 susceptible chickens are injected intratracheally and observed for 14 days. Deaths are counted as failures. Four or fewer failures are allowed for satisfactory serials. In the European Union, tests of virus content are carried out. The virus titre shall normally be no higher than one-tenth of the dose at which the vaccine has been shown to be safe.

b) Potency

The test of virus content (see above) may also be used as a measure of potency. It must be no lower than the agreed minimum release titre. Each serial or subserial shall have a virus titre of $10^7$ greater than the minimum protective dose, but not less than $10^{25}$ EID$_{50}$ (or TCID$_{50}$ for tissue culture prepared product)/dose.

c) Tests of final product

The lack of chicken pathogens should be confirmed in embryos or chickens. It should also be confirmed by testing for *Mycoplasma* spp., *Salmonella* spp., avian leukosis virus, and haemagglutinating viruses.

REFERENCES


Chapter 2.3.3. — Avian infectious laryngotracheitis


* *
CHAPTER 2.3.4.

AVIAN INFLUENZA

SUMMARY

Avian influenza (AI) is caused by specified viruses that are members of the family Orthomyxoviridae and placed in the genus influenzavirus A. There are three influenza genera – A, B and C; only influenza A viruses are known to infect birds. Diagnosis is by isolation of the virus or by detection and characterisation of fragments of its genome. This is because infections in birds can give rise to a wide variety of clinical signs that may vary according to the host, strain of virus, the host's immune status, presence of any secondary exacerbating organisms and environmental conditions.

Identification of the agent: Suspensions in antibiotic solution of oropharyngeal and cloacal swabs (or faeces) taken from live birds, or of faeces and pooled samples of organs from dead birds, are inoculated into the allantoic cavity of 9- to 11-day-old embryonated chicken eggs. The eggs are incubated at 37°C (range 35–39°C) for 2–7 days. The allantoic fluid of any eggs containing dead or dying embryos during the incubation and all eggs at the end of the incubation period are tested for the presence of haemagglutinating activity. The presence of influenza A virus can be confirmed by an immunodiffusion test between concentrated virus and an antiserum to the nucleocapsid and/or matrix antigens, both of which are common to all influenza A viruses. Isolation in embryos has recently been replaced, under certain circumstances, by detection of one or more segments of the influenza A genome using real-time reverse-transcription polymerase chain reaction (rRT-PCR) or other validated molecular techniques.

For subtyping the virus, a reference laboratory should conduct haemagglutination and neuraminidase inhibition tests against a battery of polyclonal or monospecific antisera to each of the 16 haemagglutinin (H1–16) and 9 neuraminidase (N1–9) subtypes of influenza A virus, or identify the genome of specific H and N subtypes using RNA detection technologies with subtype specific primers and probes (e.g. rRT-PCR) or sequencing and phylogenetic analysis.

As the term highly pathogenic avian influenza and the historical term ‘fowl plague’ refer to infection with virulent strains of influenza A virus, it is necessary to assess the virulence of an isolate for domestic poultry. Any highly pathogenic avian influenza isolate is classified as notifiable avian influenza (NAI) virus. Although all naturally occurring virulent strains isolated to date have been either of the H5 or H7 subtype, most H5 or H7 isolates have been of low virulence. Due to the risk of a low virulent H5 or H7 becoming virulent by mutation in poultry hosts, all H5 and H7 viruses have also been classified as NAI viruses. The methods used for the determination of strain virulence for birds have evolved over recent years with a greater understanding of the molecular basis of pathogenicity, but still primarily involve the intravenous inoculation of a minimum of eight susceptible 4- to 8-week-old chickens with infectious virus; strains are considered to be highly pathogenic if they cause more than 75% mortality within 10 days or inoculation of 10 susceptible 4- to 8-week-old chickens resulting in an intravenous pathogenicity index (IVPI) of greater than 1.2. Characterisation of suspected virulent strains of the virus should be conducted in a virus-secure biocontainment laboratory. All virulent AI isolates are designated as highly pathogenic notifiable avian influenza (HPNAI) viruses. Regardless of their virulence for chickens, H5 or H7 viruses with a HA0 cleavage site amino acid sequence similar to any of those that have been observed in virulent viruses are considered HPNAI viruses. H5 and H7 isolates that are not pathogenic for chickens and do not have an HA0 cleavage site amino acid sequence similar to any of those that have been observed in HPNAI viruses are designated as low pathogenicity notifiable avian influenza (LPNAI) viruses and non-H5 or non-H7 AI isolates that are not highly pathogenic for chickens are designated as low pathogenicity avian influenza (LPAI) viruses.

Serological tests: As all influenza A viruses have antigenically similar nucleocapsid and matrix antigens, agar gel immunodiffusion tests are used to detect antibodies to these antigens.
Concentrated virus preparations containing either or both type of antigens are used in such tests. Not all species of birds develop demonstrable precipitating antibodies. Haemagglutination inhibition tests have also been employed in routine diagnostic serology, but it is possible that this technique may miss some particular infections because the haemagglutinin is subtype specific. Enzyme-linked immunosorbent assays have been used to detect antibodies to influenza A type-specific antigens in either species-dependent (indirect) or -independent (competitive) test formats.

**Requirements for vaccines and diagnostic biologicals:** Historically, in most countries, vaccines specifically designed to contain or prevent HPNAI were banned or discouraged by government agencies because they may interfere with stamping-out control policies. The first use of vaccination in an avian influenza eradication programme was against LPAI and LPNAI. The programmes used inactivated oil-emulsion vaccines with the same haemagglutinin and neuraminidase subtypes, and infected flocks were identified by detection of virus or antibodies against the virus in non-vaccinated sentinel birds. During the 1990s the prophylactic use of inactivated oil-emulsion vaccines was employed in Mexico and Pakistan to control widespread outbreaks of NAI, and a recombinant fowl poxvirus vaccine expressing the homologous HA gene was also used in Mexico, El Salvador and Guatemala. During the 1999–2001 outbreak of LPNAI in Italy, an inactivated vaccine was used with the same haemagglutinin type as the field virus, but with a different neuraminidase. This allowed the differentiation of non-infected vaccinated birds from vaccinated birds infected with the field virus and ultimately resulted in eradication of the field virus. Prophylactic use of H5 and H7 vaccines has been practised in parts of Italy, aimed at preventing LPNAI infections, and several countries in Asia, Africa and the Middle East as an aid in controlling HPNAI H5N1 virus infections. HPNAI viruses should not be used as the seed virus for production of vaccine.

If HPNAI is used in challenge studies, the facility should meet the OIE requirements for Containment Group 4 pathogens.

**A. INTRODUCTION**

Notifiable avian influenza (NAI) is caused by infection with viruses of the family Orthomyxoviridae placed in the genus *influenzavirus A*. Influenza A viruses are the only orthomyxoviruses known to naturally affect birds. Many species of birds have been shown to be susceptible to infection with influenza A viruses; aquatic birds form a major reservoir of these viruses, and the overwhelming majority of isolates have been of low pathogenicity (low virulence) for chickens and turkeys. Influenza A viruses have antigenically related nucleocapsid and matrix proteins, but are classified into subtypes on the basis of their haemagglutinin (H) and neuraminidase (N) antigens (World Health Organization Expert Committee, 1980). At present, 16 H subtypes (H1–H16) and 9 N subtypes (N1–N9) are recognised (Swayne & Halvorson, 2008). To date, naturally occurring highly virulent influenza A viruses that produce acute clinical disease in chickens, turkeys and other birds of economic importance have been associated only with the H5 and H7 subtypes. Most viruses of the H5 and H7 subtype isolated from birds have been of low virulence for poultry. As there is the risk of a H5 or H7 virus of low virulence becoming virulent by mutation, all H5 and H7 viruses have been designated as NAI viruses.

Depending on the species, age and type of bird, specific characteristics of the viral strain involved, and on environmental factors, the highly pathogenic disease, in fully susceptible birds, may vary from one of sudden death with little or no overt clinical signs to a more characteristic disease with variable clinical presentations including respiratory signs, such as ocular and nasal discharges, coughing, snicking and dyspnoea, swelling of the sinuses and/or head, apathy, reduced vocalisation, marked reduction in feed and water intake, cyanosis of the unfeathered skin, wattles and comb, incoordination and nervous signs and diarrhoea. In laying birds, additional clinical features include a marked drop in egg production, usually accompanied by an increase in numbers of poor quality eggs. Typically, high morbidity is accompanied by high and rapidly escalating unexplained mortality. However, none of these signs can be considered pathognomonic. In certain host species such as Pekin ducks some HPAI viruses do not necessarily provoke significant clinical disease. In addition, low pathogenicity avian influenza (LPAI) viruses, which normally cause only a mild or no clinical disease, may in certain circumstances produce a spectrum of clinical signs, the severity of which may approach that of highly pathogenic avian influenza (HPAI), particularly if exacerbating infections and/or adverse environmental conditions are present. Confirmatory diagnosis of the disease, therefore, depends on the isolation or detection of the causal virus and the demonstration that it fulfils one of the defined criteria described in section B.2. Testing sera from suspect birds using antibody detection methods may supplement diagnosis, but these methods are not suitable for a detailed identification. Diagnosis for official control purposes is established on the basis of agreed official criteria for pathogenicity according to in-vivo tests or to molecular determinants (i.e. the presence of a cleavage site of the haemagglutinin precursor protein HA0 consistent with HPNAI virus) and haemagglutinin subtyping. These definitions evolve as scientific knowledge of the disease increases.
NAI are subject to official control. The viruses that cause NAI have the potential to spread from the laboratory if adequate levels of biosecurity and biosafety are not in place. Consequently, a risk assessment should be carried out to determine the level of biosecurity needed for laboratory diagnosis and chicken inoculation; characterisation of the HPAI virus should be conducted at biocontainment level 3 and LPNAI at biocontainment level 2 (at least). The facility should meet the requirements for the appropriate Containment Group as determined by the risk assessment and as outlined in Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities. Countries lacking access to such a specialised national or regional laboratory should send specimens to an OIE Reference Laboratory.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent (the prescribed test for international trade)

Samples taken from dead birds should include intestinal contents (faeces) or cloacal swabs and oropharyngeal swabs. Samples from trachea, lungs, air sacs, intestine, spleen, kidney, brain, liver and heart should also be collected and processed either separately or as a pool.

Samples from live birds should include both oropharyngeal and cloacal swabs. To avoid harming them, swabbing of small delicate birds should be done with the use of especially small swabs that are usually commercially available and intended for use in human paediatrics. Where these are not available, the collection of fresh faeces may serve as an alternative.

The samples should be placed in isotonic phosphate-buffered saline (PBS), pH 7.0–7.4 with antibiotics or a solution containing protein and antibiotics. The antibiotics can be varied according to local conditions, but could be, for example, penicillin (2000 units/ml), streptomycin (2 mg/ml), gentamycin (50 µg/ml) and mycostatin (1000 units/ml) for tissues and oropharyngeal swabs, but at five-fold higher concentrations for faeces and cloacal swabs. It is important to readjust the pH of the solution to pH 7.0–7.4 following the addition of the antibiotics. It is recommended that a solution for transport of the swabs should contain protein to stabilise the virus (e.g. brain–heart infusion, up to 5% [v/v] cattle serum, 0.5% [w/v] bovine albumen or similar commercially available transport media). Faeces and finely minced tissues should be prepared as 10–20% (v/v) suspensions in the antibiotic solution. Suspensions should be processed as soon as possible after incubation for 1–2 hours at room temperature. When immediate processing is impracticable, samples may be stored at 4°C for up to 4 days. For prolonged storage, diagnostic samples and isolates should be kept at –80°C. Repeated freezing and thawing should be avoided.

The preferred method of growing avian influenza A viruses is by the inoculation of specific pathogen free (SPF) embryonated chicken eggs, or specific antibody negative (SAN) eggs. The supernatant fluids of faeces or tissue suspensions obtained through clarification by centrifugation at 1000 g are inoculated into the allantoic sac of three to five embryonated SPF or SAN chicken eggs of 9–11 days’ incubation. The eggs are incubated at 37°C (range 35–39°C) for 2–7 days. Eggs containing dead or dying embryos as they arise, and all eggs remaining at the end of the incubation period, should first be chilled to 4°C for 4 hours or overnight, and the allantoic fluids should then be recovered and tested with a screening test (such as haemagglutination [HA] test), influenza A type-specific test (such as agar gel immunodiffusion test [AGID] or solid-phase antigen-capture enzyme-linked immunosorbent assays [ELISA]) or influenza A subtype-specific test (such as haemagglutinin inhibition [HI] and neuraminidase inhibition [NI] tests) or a molecular test to detect influenza A specific nucleic acid signatures (such as real-time reverse transcriptase polymerase chain reaction [RT-PCR] test) as described later (see Section B.3.b). Detection of HA activity, in bacteria-free amnio-allantoic fluids verified by microbiological assay, indicates a high probability of the presence of an influenza A virus or of an avian paramyxovirus. Fluids that give a negative reaction should be passaged into at least one further batch of eggs.

The presence of influenza A virus can be confirmed in AGID tests by demonstrating the presence of the nucleocapsid or matrix antigens, both of which are common to all influenza A viruses (see Section B.3.a). The antigens may be prepared by concentrating the virus from infective allantoic fluid or extracting the infected chorioallantoic membranes; these are tested against known positive antisera. Virus may be concentrated from infective allantoic fluid by ultracentrifugation, or by precipitation under acid conditions. The latter method consists of the addition of 1.0 M HCl to infective allantoic fluid until it is approximately pH 4.0. The mixture is placed in an ice bath for 1 hour and then clarified by centrifugation at 1000 g at 4°C. The supernatant fluid is discarded. The virus concentrates are re-suspended in glycine/sarcosyl buffer: this consists of 1% (w/v) sodium lauroyl sarcosinate buffered to pH 9.0 with 0.5 M glycine. These concentrates contain both nucleocapsid and matrix polypeptides.

Preparations of nucleocapsid-rich antigen can also be obtained from chorioallantoic membranes for use in the AGID test (Beard, 1970). This method involves removal of the chorioallantoic membranes from infected eggs that have allantoic fluids with HA activity. The membranes are then homogenised or ground to a paste. This is subjected to three freeze–thaw cycles, followed by centrifugation at 1000 g for 10 minutes. The pellet is discarded and the supernatant is used as an antigen following treatment with 0.1% formalin.
Use of the AGID test to demonstrate nucleocapsid or matrix antigens is a satisfactory way to indicate the presence of avian influenza virus (AIV) in amniotic fluid, but various experimental and commercial rapid, solid-phase antigen-capture ELISAs (AC-ELISAs) are an effective alternative (Swayne & Halvorson, 2008). Most AC-ELISAs have been licensed and marketed to detect human influenza A virus in clinical specimens. Some have demonstrated effectiveness for detection of AIV, but many of these commercial tests have had low sensitivity (Woolcock & Cardona, 2005). Those validated for veterinary use are preferred.

Any HA activity of sterile fluids harvested from the inoculated eggs is most likely to be caused by an influenza A virus or an avian paramyxovirus, but a few strains of avian reovirus, as well as nonsterile fluid containing HA of bacterial origin can cause the agglutination of RBCs. There are currently 10 recognised serotypes of avian paramyxoviruses (Miller et al., 2010). Most laboratories will have antisera specific to Newcastle disease virus (avian paramyxovirus type 1), and in view of its widespread occurrence and almost universal use as a live vaccine in poultry, it is best to evaluate its presence by haemagglutination inhibition (HI) tests (see Chapter 2.3.14 Newcastle disease).

Alternatively, the presence of influenza virus can be confirmed by the use of RT-PCR or rRT-PCR using nucleoprotein-specific or matrix-specific conserved primers (Altmuller et al., 1991; Spackman et al., 2002). Also, the presence of subtype H5 or H7 influenza virus can be confirmed by using H5- or H7-specific primers (Monne et al., 2008; Slomka et al., 2007; Spackman et al., 2002).

Antigenic subtyping can be accomplished by monospecific antisera prepared against purified or recombinant H and N subtype-specific proteins, used in HI and NI tests, or polyclonal antisera raised against a battery of intact influenza viruses and used in HI and NI tests. Genotyping can be accomplished using H and N subtype specific primers in RT-PCR and rRT-PCR tests; or 4) using sequence analysis of H and N genes. Subtype identification by these techniques is beyond the scope of most diagnostic laboratories not specialising in influenza viruses. Assistance is available from the OIE Reference Laboratories (see Table given in Part 4 of this Terrestrial Manual).

2. Assessment of pathogenicity

The term HPAI relates to the assessment of virulence in chickens and implies the involvement of virulent strains of virus. It is used to describe a disease of fully susceptible chickens with clinical signs such as ocular and nasal discharges, coughing, snicking and dyspnoea, swelling of the sinuses and/or head, listlessness, reduced vocalisation, marked reduction in feed and water intake, cyanosis of the unfeathered skin, wattles and comb, incoordination, nervous signs and diarrhoea. In laying birds, additional clinical features include a marked drop in egg production usually accompanied by an increase in numbers of poor quality eggs. Typically, high morbidity is accompanied by high and rapidly escalating unexplained mortality. However, none of these signs can be considered pathognomonic and high mortality may occur in their absence. In addition, LPAI viruses that normally cause only mild or no clinical disease, may cause a much more severe disease if exacerbating infections or adverse environmental factors are present and, in certain circumstances, the spectrum of clinical signs may mimic HPAI.

The historical term ‘fowl plague’ has been abandoned in favour of the more accurate term HPAI. Because all naturally occurring HPAI viruses to date have been H5 and H7 subtypes and genomic studies have determined HPAI viruses arise by mutation of H5 and H7 LPAI viruses, all H5 and H7 LPAI have been recognised as potentially pathologic. Pathogenicity shifts have been associated with changes to the proteolytic cleavage site of the haemagglutinin including: 1) substitutions of non-basic with basic amino acids (arginine or lysine); 2) insertions of multiple basic amino acids from codons duplicated from the haemagglutinin cleavage site; 3) short inserts of basic and non-basic amino acids from unknown source; 4) recombination with inserts from other gene segments that lengthen the proteolytic cleavage site; and 5) loss of the shielding glycosylation site at residue 13 in combination with multiple basic amino acids at the cleavage site. Amino acid sequencing of the cleavage sites of H5 and H7 subtype influenza isolates of low virulence for birds should identify viruses that have the capacity, following simple mutation, to become highly pathogenic for poultry.

The following criteria have been adopted by the OIE for classifying an AIV as HPAI:

a) One of the following two methods to determine pathogenicity in chickens is used. A HPAI virus is:

i) any influenza virus that is lethal\(^1\) for six, seven or eight of eight 4- to 8-week-old susceptible chickens within 10 days following intravenous inoculation with 0.2 ml of a 1/10 dilution of a bacteria-free, infective amniotic fluid

or

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\(^1\) When birds are too sick to eat or drink, they should be killed humanely.
a) For all H5 and H7 viruses of low pathogenicity in chickens, the amino acid sequence of the connecting peptide of the haemagglutinin must be determined. If the sequence is similar to that observed for other highly pathogenic AI isolates, the isolate being tested will be considered to be highly pathogenic (see the table that lists all the reported haemagglutinin proteolytic cleavage site of HA0 protein for H5 and H7 LPAI and HPAI viruses based on deduced amino acid sequence, which can be found on the OFFLU site at: http://www.offlu.net/index.php?id=123).

b) All AI isolates that meet the above criteria are designated as HPNAI.

c) Non-H5 or non-H7 AI isolates that are not virulent for chickens are designated as LPAI.

A variety of strategies and techniques have been used successfully to sequence the nucleotides at that portion of the HA gene coding for the cleavage site region of the haemagglutinin of H5 and H7 subtypes of avian influenza, enabling the amino acids there to be deduced. This can be done by extraction the specimen and direct sequencing of the hemagglutinin proteolytic cleavage site, or first, cloning the hemagglutinin and then sequencing the cDNA. Various stages in the procedure can be facilitated using commercially available kits and automatic sequencers.

determination of the cleavage site by sequencing or other methods has become the method of choice for initial assessment of the virulence of these viruses and has been incorporated into agreed definitions. This has reduced the number of in-vivo tests, although at present the inoculation of birds is still required to confirm a negative result as the possibility of virus populations containing mixtures of viruses of high and low virulence cannot be ruled out.

Although all the truly HPAI viruses isolated to date have been of H5 or H7 subtypes, at least two isolates, both of H10 subtype (H10N4 and H10N5), have been reported that would have fulfilled both the OIE and EU in vivo definitions for highly pathogenic AI viruses (Wood et al., 1996) as they killed 7/10 and 8/10 chickens with IVPI values >1.2 when the birds were inoculated intravenously. However, these viruses did not induce death or signs of disease when inoculated intranasally and did not have a haemagglutinin cleavage site sequence compatible with HPNAI virus. Similarly, other intravenously inoculated LPAI viruses are nephrotropic and birds that die have high titres of virus in their kidneys indicating a renal pathogenic mechanism (Slemons & Swayne, 1990), but such laboratory-induced pathobiology does not equal to the multi-organ infection and systemic disease caused by HPNAI viruses. Conversely, four viruses have been described that have HA0 cleavage sites containing multiple basic amino acids, but which show low virulence (IVPI <1.2) when inoculated intravenously into 6-week-old chickens (Londt et al., 2007). Other anomalies are the Chile 2002 (Suarez et al., 2004) and the Canada 2004 (Pasick et al., 2005) H7N3 HPAI viruses, which show distinct and unusual cleavage site amino acid sequences of PEKPKTCPLSLRCRERTR*GLF and PENPKQAYRKRMRTR*GLF, respectively. These viruses appear to have arisen as a result of a recombination between the HA, nucleoprotein and matrix genes, respectively, resulting in

2 When birds are too sick to eat or drink, they should be killed humanely and scored as dead at the next observation.
an insertion at the HA0 cleavage site of 11 amino acids for the Chile virus and 7 amino acids for the Canadian virus. Both are extremely virulent when inoculated into 6-week-old chickens intravenously.

A table is available on the OFFLU web site that lists all the reported haemagglutinin proteolytic cleavage site of HA0 protein for H5 and H7 LPAI and HPAI viruses based on deduced amino acid sequence. This table will be updated as new virus are characterised; it can be found on the OFFLU site at:

http://www.offlu.net/index.php?id=123

3. Serological tests

a) Agar gel immunodiffusion (an alternative test for international trade)

All influenza A viruses have antigenically similar nucleocapsid and antigenically similar matrix antigens. Owing to this fact AGID tests are able to detect the presence or absence of antibodies to any influenza A virus. Concentrated virus preparations, as described above, contain both matrix and nucleocapsid antigens; the matrix antigen diffuses more rapidly than the nucleocapsid antigen. AGID tests have been widely and routinely used to detect specific antibodies in chicken and turkey flocks as an indication of infection, but AGID tests are less reliable at detecting antibodies following infection with influenza A viruses in other avian species. These have generally employed nucleocapsid-enriched preparations made from the chorioallantoic membranes of embryonated chicken eggs (Beard, 1970) that have been infected at 10 days of age, homogenised, freeze–thawed three times, and centrifuged at 1000 g. The supernatant fluids are inactivated by the addition of 0.1% formalin or 1% betapropiolactone, recentrifuged and used as antigen. Not all avian species may produce precipitating antibodies following infection with influenza viruses.

Tests are usually carried out using gels of 1% (w/v) agarose or purified agar and 8% (w/v) NaCl in 0.1 M phosphate buffer, pH 7.2, poured to a thickness of 2–3 mm in Petri dishes or on microscope slides. Using a template and cutter, wells of approximately 5 mm in diameter are cut into the agar. A pattern of wells must place each suspect serum adjacent to a known positive serum and antigen. This will make a continuous line of identity between the known positive, the suspect serum and the nucleocapsid antigen. Approximately 50 µl of each reagent should be added to each well.

Precipitin lines can be detected after approximately 24–48 hours, but this may be dependent on the concentrations of the antibody and the antigen. The precipitin lines are best observed against a dark background that is illuminated from behind. A specific, positive result is recorded when the precipitin line between the known positive control wells is continuous with the line between the antigen and the test well. Crossed lines are interpreted to be caused by the test serum lacking identity with the antibodies in the positive control well.

b) Haemagglutination and haemagglutination inhibition tests

Variations in the procedures for HA and HI tests are practised in different laboratories. The following recommended examples apply to the use of V-bottomed microwell plastic plates in which the final volume for both types of test is 0.075 ml. The reagents required for these tests are isotonic PBS (0.01 M), pH 7.0–7.2, and red blood cells (RBCs) taken from a minimum of three SPF or SAN chickens and pooled into an equal volume of Alsever’s solution. Cells should be washed three times in PBS before use as a 1% (packed cell v/v) suspension. Positive and negative control antigens and antisera should be run with each test, as appropriate.

- Haemagglutination test
  i) Dispense 0.025 ml of PBS into each well of a plastic V-bottomed microtitre plate.
  ii) Place 0.025 ml of virus suspension (i.e. infective allantoic fluid) in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/3, 1/4, 1/5, 1/6, etc.
  iii) Make twofold dilutions of 0.025 ml volumes of the virus suspension across the plate.
  iv) Dispense a further 0.025 ml of PBS to each well.
  v) Dispense 0.025 ml of 1% (v/v) chicken RBCs to each well.
  vi) Mix by tapping the plate gently and then allow the RBCs to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C, if ambient temperatures are high, by which time control RBCs should have formed a distinct button.
vii) HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA (no streaming); this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

- **Haemagglutination inhibition test (an alternative test for international trade)**
  i) Dispense 0.025 ml of PBS into each well of a plastic V-bottomed microtitre plate.
  ii) Place 0.025 ml of serum into the first well of the plate.
  iii) Make twofold dilutions of 0.025 ml volumes of the serum across the plate.
  iv) Add 4 HAU of virus/antigen in 0.025 ml to each well and leave for a minimum of 30 minutes at room temperature (i.e. about 20°C) or 60 minutes at 4°C.
  v) Add 0.025 ml of 1% (v/v) chicken RBCs to each well and mix gently, allow the RBCs to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C if ambient temperatures are high, by which time control RBCs should have formed a distinct button.
  vi) The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (containing 0.025 ml RBCs and 0.05 ml PBS only) should be considered to show inhibition.
  vii) The validity of results should be assessed against a negative control serum, which should not give a titre >1/4 (>22 or >log2 2 when expressed as the reciprocal), and a positive control serum for which the titre should be within one dilution of the known titre.

HI titres may be regarded as being positive if there is inhibition at a serum dilution of 1/16 (2^4 or log2 4 when expressed as the reciprocal) or more against 4 HAU of antigen. Some laboratories prefer to use 8 HAU in HI tests. While this is permissible, it affects the interpretation of results so that a positive titre is 1/8 (2^3 or log2 3) or more. The meaning of a minimum positive titre should not be misinterpreted; for example, that immunised birds with that titre will be protected against challenge or that birds with lower titres will be susceptible to challenge. Appropriate virus/antigen control, positive control serum and RBC control well should be included with each batch of HI tests.

Chicken sera rarely give nonspecific positive agglutination reactions in this test and any pretreatment of the sera is unnecessary. Sera from species other than chickens may sometimes cause agglutination of chicken RBCs resulting in nonspecific agglutination. Therefore, each serum should first be tested for this idiosyncrasy and, if present, it should be inhibited by adsorption of the serum with chicken RBCs. This is done by adding 0.025 ml of packed chicken RBCs to each 0.5 ml of antisera, mixing gently and leaving for at least 30 minutes; the RBCs are then pelleted by centrifugation at 800 g for 2–5 minutes and the adsorbed sera are decanted. Alternatively, RBCs of the avian species under investigation could be used. Nonspecific inhibition of agglutination can be caused by steric inhibition when the H antigen and serum in the HI test have the same N subtype. The steric inhibition reaction can result in RBC buttoning in the bottom of the plate or streaming at the same rate as the control. To prevent steric nonspecific inhibition the H antigen used to test unknown serum must be of a different N subtype than the unknown sera, or the H antigen use can be recombinant or purified H protein that lacks N protein. The HI test is based on antigenic binding between the H antigen and antisera and thus other factors may cause nonspecific binding of the H antigen and sera leading to a nonspecific inhibition reaction. At this time there are no documented cross reactions or nonspecific inhibition reactions between the different haemagglutinin subtypes of avian influenza.

The neuraminidase-inhibition test has been used to identify the AI neuraminidase type of isolates as well as to characterise the antibody in infected birds. The procedure requires specialised expertise and reagents; consequently this testing is usually done in an OIE Reference Laboratory. The DIVA (differentiating infected from vaccinated animals) strategy used in Italy also relies on a serological test to detect specific anti-N antibodies; the test procedure has been described (Capua et al., 2003).

- **Enzyme-linked immunoassay (ELISA) (an alternative test for international trade)**

Commercial ELISA kits that detect antibodies against the nucleocapsid protein are available. Kits with an indirect and competitive format have been developed and are now being used to detect AIV-specific antibodies. The kits should be validated for the specific species of interest and for the specific purpose(s) for which they are to be used. Several different test and antigen preparation methods are used. Such tests have usually been evaluated and validated by the manufacturer, and it is therefore important that the instructions specified for their use be followed carefully. Please see the OIE Register for kits certified by the OIE (http://www.oie.int/vcda/eng/en_vcda_registre.htm).
4. Antigen capture and molecular techniques

At present, the conventional virus isolation and characterisation techniques for the diagnosis of AI remain the methods of choice, for at least the initial diagnosis of AI infections. However, conventional methods tend to be costly, labour intensive and slow. There have been enormous developments and improvements in molecular and other diagnostic techniques, many of which have been applied to the diagnosis of AI infections.

a) Antigen detection

There are several commercially available AC-ELISA kits that can detect the presence of influenza A viruses in poultry (Swayne & Halvorson, 2008; Woolcock & Cardona, 2005). Most of the kits are enzyme immunoassays and use a monoclonal antibody against the nucleoprotein; they should be able to detect any influenza A virus. The main advantage of these tests is that they can demonstrate the presence of AI within 15 minutes. The disadvantages are that they may lack sensitivity, they may not have been validated for different species of birds, subtype identification is not achieved and the kits are expensive. The tests should only be interpreted on a flock basis and not as an individual bird test. Oropharyngeal or tracheal samples from clinically affected or dead birds provide the best sensitivity. Nevertheless, the lack of sensitivity is a major drawback to the use of available antigen detection tests. Chua et al. (Chua et al., 2007) evaluated five detection tests and showed overall sensitivities from 36.3% to 51.4%; these authors pointed out that in terms of sensitivity using cloacal and tracheal swabs, the tests performed less well with samples from waterfowl or wild birds than they did with samples from chickens. Woolcock & Cardonna (Woolcock & Cardona, 2005) examined five commercial tests licensed for human clinical use and found a wide variation in the ability to detect AIV in poultry specimens with minimal detection limits of $10^{4.7}$ EID<sub>50</sub> (50% egg-infective dose) of virus per ml with the best test, and a minimum of $10^{5.7}$ EID<sub>50</sub> per ml for the remaining tests.

b) Direct RNA detection

As demonstrated by the current definitions of HPNAI, molecular techniques have been used in the diagnosis of AI for some time now. Furthermore, there have recently been developments towards their application to the detection and characterisation of AI virus directly from clinical specimens of infected birds. It is imperative that when using highly sensitive molecular detection methods that allow rapid direct detection of viral RNA for confirmatory laboratory diagnosis of avian influenza infections, stringent protocols are in place to prevent the risk of cross-contamination between clinical samples. In addition, RNA detection test methodologies should be validated to the OIE standard (see Chapter 1.1.5 Principles and methods of validation of diagnostic assays for infectious diseases) using clinical material to demonstrate the tests as being ‘fit for purpose’ for application in a field diagnostic setting, which may include the use of internal test standards. The control reactions enable greater confidence in the integrity of the molecular reactions, clinical samples and results.

RT-PCR techniques on clinical specimens can, with the correctly defined primers, result in rapid detection and subtype identification (at least of H5 and H7), including a cDNA product that can be used for nucleotide sequencing (Starick et al., 2000; Suarez, 2007). This technique was used with success during the 2003 HPAI outbreaks in The Netherlands.

However, the preferred molecular detection tests for AI virus is the rRT-PCR, a modification to the RT-PCR that reduces the time for both identification of virus subtype and sequencing. For example, Spackman et al. (2002) used a single-step rRT-PCR primer/fluorogenic hydrolysis probe system to allow detection of AI viruses and determination of subtype H5 or H7. The test performed well relative to virus isolation and offered a cheaper and much more rapid alternative, with diagnosis on clinical samples in less than 3 hours. In additional studies, the rRT-PCR was shown to have sensitivity and specificity equivalent to virus isolation based on field validation in the live poultry market control programme of New York and New Jersey during the winter of 2002, and the H7N2 LPAI outbreak and eradication programme in Virginia during 2002 (Elvinger et al., 2007; Spackman et al., 2003). The test provides high sensitivity and specificity similar to virus isolation from tracheal and oropharyngeal swabs of chickens and turkeys, but may lack sensitivity for detection of influenza A virus in faecal swabs, faeces and tissues in some bird species, because of the presence of PCR inhibitors resulting in false-negative results (Das et al., 2006). Incorporation of a positive internal control into the test will verify a proper test run. In addition, improved RNA extraction methods have been developed to eliminate most PCR inhibitors from test samples.

rRT-PCR, usually based around the hydrolysis probe or ‘TaqMan’ method for generation of the target-specific fluorescence signal, has become the method of choice in many laboratories for at least partial diagnosis directly from clinical specimens. The method offers rapid results, with sensitivity and specificity comparable to virus isolation. These are ideal qualities for AI outbreak management, where the period of time in which an unequivocal diagnosis can be obtained is crucial for decision making by the relevant
Veterinary Authority. In addition, rRT-PCR systems can be designed to operate in a 96-well format and combined with high-throughput robotic RNA extraction from specimens (Agüero et al., 2007).

The approach to diagnosis using rRT-PCR adopted in most laboratories has been based on initial generic detection of AIV in clinical specimens, primarily by initially targeting the matrix (M) gene, which is highly conserved for all type A influenza viruses, followed by specific rRT-PCR testing for H5 and H7 subtype viruses. For subtype identification, primers used in TaqMan rRT-PCRs are targeted at the HA2 region, as this is relatively well conserved within the haemagglutinin genes of the H5 and H7 subtypes (Spackman et al., 2008; Spackman & Suarez, 2008). It has therefore served as the target region for these subtypes. Spackman et al. (2002) demonstrated specific detection of these subtypes, but cautioned that their H5 and H7 primer/probe sequences had been designed for the detection of North American H5 and H7 isolates and might not be suitable for all H5 and H7 isolates. This proved to be the case. Slomka et al. (Slomka et al., 2007) described modification of the H5 oligonucleotide sequences used by Spackman et al. (2002) to enable the detection of the Asian lineage HPAI H5N1 Al virus and other Eurasian H5 Al viruses that have been isolated within the past decade in both poultry and wild birds. Validated rRT-PCR protocols for the simultaneous detection and typing of H5, H7 and H9 RNA have been developed (Monne et al., 2008). These validated Eurasian rRT-PCR have proven valuable in the investigation of many H5N1 HPAI clinical specimens and other subtypes submitted to International Reference Laboratories from Europe, Africa and Asia since autumn 2005 (Monne et al., 2008; Slomka et al., 2007). Each set of primers and probes needs to be validated against a diverse set of viruses to make the test applicable to a diverse avian species, and in viruses from broad geographic areas and time periods.

One of the problems with rapidly emerging new tests is that methods and protocols may be developed and reported without the test being properly validated. This has been addressed for some of the rRT-PCR protocols (Slomka et al., 2007b; Suarez et al., 2007). In the European Union, National Reference Laboratories have collaborated to define and validate protocols that can be recommended for use within the European Union (Monne et al., 2008; Slomka et al. 2007b).

rRT-PCR protocols have been described that amplify regions across the cleavage site of the HA0 gene. This may result in useful tests for specific viruses. For example, Hoffman et al. (2007) have described an rRT-PCR test specific to the Asian HPAI H5N1 Qinghai-like clade 2.2 viruses that represents a rapid means of determining the pathotype for this subgroup of H5N1 HPAI viruses without sequencing. Fereidouni et al. (2008) have developed a restriction fragment polymorphism-based assay that enables the pathotyping of NAI of subtype H5 independent of sequencing or animal experiments after RT-PCR and restriction enzyme digest of the amplificate.

Modifications to the straightforward RT-PCR method of detection of viral RNA have been designed to reduce the effect of inhibitory substances in the sample taken, the possibility of contaminating nucleic acids and the time taken to produce a result. For example, nucleic acid sequence-based amplification (NASBA) with electrochemiluminescent detection (NASBA/ECL) is a continuous isothermal reaction in which specialised thermocycling equipment is not required. NASBA assays have been developed for the detection of AIV subtypes H7 and H5 in clinical samples within 6 hours (Ko et al., 2004). The loop-mediated isothermal amplification (LAMP) system for H5 detection appeared to show high sensitivity and reliable specificity (Imai et al., 2006), but may have limited application because of susceptibility to viral mutations affecting the target regions, reducing virus detection (Postel et al., 2010).

It seems highly likely that within a very short time molecular-based and improved antigen-based technologies will have developed sufficiently to allow rapid ‘flock-side’ tests for the detection of the presence of AIV specific subtypes and virulence markers. The extent to which such tests are employed will depend very much on the agreement on and adoption of definitions of what constitutes statutory infections for control and trade purposes.

C. REQUIREMENTS FOR VACCINES

1. Background

It is important that vaccination alone is not considered the solution to the control of NAI or LPAI subtypes if eradication is the desired result. Without the application of monitoring systems, strict biosecurity and depopulation in the face of infection, there is the possibility that NAI viruses could become endemic in vaccinated poultry populations. Long-term circulation of the virus in a vaccinated population may result in both antigenic and genetic changes in the virus and this has been reported to have occurred in Mexico, China (People’s Rep. of), Egypt, Indonesia and other countries (Grund et al., 2011; Lee et al., 2004; Smith et al., 2006; Swayne & Kapczynski, 2008b). Currently used vaccines and the use of vaccination have been reviewed (Capua & Alexander, 2008; Swayne, 2003, 2004; Swayne & Kapczynski, 2008a, 2008b).
In this chapter, conventional vaccines are limited to inactivated avian influenza virus vaccines. These vaccines have been used against NAI or LPAI, having been prepared from infective allantoic fluid inactivated by beta-propiolactone or formalin and emulsified with mineral oil. Live conventional influenza vaccines against any subtype are not recommended.

The existence of a large number of virus subtypes, together with the known variation of different strains within a subtype, poses serious problems when selecting strains to produce inactivated avian influenza vaccines, especially for LPAI. In addition, some isolates do not grow to a sufficiently high titre to produce adequately potent vaccines without costly pre-concentration. While some vaccination strategies use autogenous vaccines, i.e. vaccines for LPAI, some isolates do not grow to a sufficiently high titre to produce adequately potent vaccines possessing the same haemagglutinin subtype and capable of yielding high concentrations of antigen.

Since the 1970s in the USA, inactivated AI vaccines have been used primarily in turkeys against LPAI and LPNAI viruses. These viruses may cause severe clinical signs, especially in exacerbating circumstances. Significant quantities of this vaccine have been used (Swayne & Halvorson 2008). In recent years in the USA, most of the inactivated Al vaccine has been used in breeder turkeys to protect against H1 and H3 swine influenza viruses. Vaccination against H9N2 LPAI virus has been used extensively in Asia and the Middle East (Swayne & Kapczyński, 2008a). Vaccination against HPNAI of H5N2 subtype was used in Mexico following outbreaks in 1994–1995 (Villareal, 2007), and against H7N3 subtype in Pakistan (Naeem, 1998) following outbreaks in 1995. In Mexico, the HPNAI virus appears to have been eradicated, but LPNAI virus of H5N2 has continued to circulate, while in Pakistan, HPNAI viruses genetically close to the original HPNAI virus were still being isolated in 2004. Following the outbreaks of HPNAI caused by H5N1 virus in Hong Kong in 2002 (Sims, 2003), a vaccination policy was adopted using H5N2 vaccine. Beginning in 2004, the widespread outbreaks of HPAI H5N1 in several countries of South-East Asia and Africa resulted in emergency and prophylactic vaccination being applied in China (the People’s Rep. of), Indonesia, Vietnam and Egypt. Inactivated H7N7 AI vaccine was used in Korea (Dem. Rep. of) during 2005 to control a HPAI outbreak. Similarly, preventive vaccination against H5N1 HPAI has been permitted for outdoor poultry and zoo birds in several European Union countries in recent years. Italy has extensively used the tool of serological (heterologous neuraminidase) DIVA with vaccination to control recurrent epidemics of LPNAI caused by viruses of the H7 subtype. A bivalent H5/H7 prophylactic vaccination programme was also developed as a result of an evolving epidemiological situation (Capua & Marangon, 2008).

Live recombinant virus vectored vaccines with avian influenza virus haemagglutinin gene inserts have been licensed and used in a few countries since 1997, mostly in chickens, and include recombinant fowl poxvirus, recombinant Newcastle disease virus and recombinant herpesvirus turkey vaccines.

a) Rationale and intended use of the product

Experimental work has shown, for both NAI and LPAI, that properly administered AI vaccines protect against clinical signs and mortality, reduces virus shedding and increases resistance to infection, protects from diverse field viruses within the same haemagglutinin subtype, protects from low and high challenge exposure, and reduces excretion and thus contact transmission of challenge virus (Capua et al., 2004; Swayne, 2003; Swayne & Suarez, 2000). However, the virus is still able to infect and replicate in clinically healthy vaccinated SPF birds when given in high challenge doses. Most of the work evaluating vaccines has been done in chickens and turkeys and some care must be taken in extrapolating the results obtained to other species. For example, in an experimental system using HPAI H7N7 as a challenge virus it was shown for chickens and ringed teal ducks, Callonetta leucophrys, that single vaccination sufficiently reduced excretion and increased the infective dose required and the transmission between birds was dramatically reduced. However, for golden pheasants, Chrysolophus pictus, even though a single vaccination provided clinical protection, there was no effect on the excretion of challenge virus and no influence on reducing virus transmission (Van der Goot et al., 2007). In some countries, vaccines designed to contain or prevent NAI are specifically banned or discouraged by government agencies because it has been considered that they may interfere with stamping-out control policies. However, most AI control regulations reserve the right to use vaccines in emergencies.

2. Outline of production and minimum requirements for conventional vaccines

The information below is based primarily on the experiences in the USA and the guidance and policy for licensing avian influenza vaccines in that country (United States Department of Agriculture, 1995 [updated 2006]). The basic principles for producing vaccines, particularly inactivated vaccines, are common to several viruses e.g. Newcastle disease (chapter 2.3.14).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

The vaccine production facility should operate under the appropriate biosecurity procedures and practices. If HPNAI virus is to be used in challenge studies, the facility used for such studies should meet the requirements for Containment Group 4 pathogens as outlined in chapter 1.1.3.
a) Characteristics of the seed
   i) Biological characteristics
   For any subtype, only well characterised influenza A virus of proven low pathogenicity, preferably obtained from an international or national repository, should be used to establish a master seed for inactivated vaccines. HPAI viruses should not be used as seed virus for AI vaccine.

   A master seed is established from which a working seed is obtained. The master seed and working seed are produced in SPF or SAN embryonated eggs. The establishment of a master culture may only involve producing a large volume of infective allantoic fluid (minimum 100 ml), which can be stored as lyophilised aliquots (0.5 ml).

   ii) Quality criteria (sterility, purity, freedom from extraneous agents)
   The established master seed should be controlled/examined for sterility, safety, potency and absence of specified extraneous agents.

b) Method of manufacture
   i) Procedure
   For vaccine production, a working seed, from which batches of vaccine are produced, is first established in SPF or SAN embryonated eggs by expansion of an aliquot of master seed to a sufficient volume to allow vaccine production for 12–18 months. It is best to store the working seed in liquid form at below –60°C as lyophilised virus does not always multiply to high titre on subsequent first passage.

   The routine procedure is to dilute the working seed in sterile isotonic buffer (e.g. PBS, pH 7.2), so that about $10^3$–$10^4$ EID$_{50}$ in 0.1 ml are inoculated into each allantoic cavity of 9- to 11-day-old embryonated SPF or SAN chicken eggs. These are then incubated at 37°C. Eggs containing embryos that die within 24 hours should be discarded. The incubation time will depend on the virus strain being used and will be predetermined to ensure maximum yield with the minimum number of embryo deaths.

   The infected eggs should be chilled at 4°C before being harvested. The tops of the eggs are removed and the allantoic fluids collected by suction. The inclusion of any yolk material and albumin should be avoided. All fluids should be stored immediately at 4°C and tested for bacterial contamination.

   In the manufacture of inactivated vaccines, the harvested allantoic fluid is treated with either formaldehyde (a typical final concentration is 1/1000, i.e. 0.1% formalin) or beta-propiolactone (BPL) (a typical final concentration is 1/1000–1/4000, i.e. 0.1–0.025% of 99% pure BPL). The time required must be sufficient to ensure freedom from live virus. Most inactivated vaccines are formulated with non-concentrated inactivated allantoic fluid (active ingredient). However, active ingredients may be concentrated for easier storage of antigen. The active ingredient is usually emulsified with mineral or vegetable oil. The exact formulations are generally commercial secrets.

   ii) Requirements for substrates and media
   The inactivated influenza vaccines prepared from conventional virus are produced in 9- to 11-day-old embryonated SPF or SAN chicken eggs. The method of production is basically the same as for propagating the virus aseptically; all procedures are performed under sterile conditions.

   iii) In-process controls
   For inactivated vaccines, completion of the inactivation process should be tested in embryonated eggs, taking at least 10 aliquots of 0.2 ml from each batch and passaging each aliquot at least twice through SPF or SAN embryos. Viral infectivity must not remain.

   iv) Final product batch tests
   Most countries have published specifications for the control of production and testing of vaccines, which include the definition of the obligatory tests on vaccines during and after manufacture.

   Sterility and purity
   Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

   Safety
   For inactivated vaccines, a double dose is administered by the recommended route to ten 3-week-old birds, and these are observed for 2 weeks for absence of clinical signs of disease or local lesions.

   Batch potency
   Potency of avian influenza vaccine is generally evaluated by testing the ability of the vaccine to induce a significant HI titre in SPF or SAN birds. Conventional potency testing involving the use of three diluted doses and challenge with virulent virus (e.g. chapter 2.3.14) may also be used for vaccines...
prepared to give protection against HPNAI or LPNAI subtypes. For inactivated vaccines to other subtypes, where virulent viruses are not available, potency tests may rely on the measurement of immune response or challenge and assessment of morbidity and quantitative reduction in challenge virus replication in respiratory (oropharyngeal or tracheal) and intestinal (cloaca) tracts. Assessment of haemagglutinin antigen content (Wood et al., 1985) could allow for in-vitro extrapolation to potency for subsequent vaccine batches.

Preservatives
A preservative may be used for vaccine in multidose containers.

c) Requirements for authorisation
i) Safety requirements
Target and non-target animal safety
Most inactivated avian influenza vaccines are licensed for use in chickens and turkeys. Field trials in the target species should be conducted to determine tolerance and safety of the vaccine at full dose. Recently the use of inactivated avian influenza vaccines has been expanded to ducks, geese, other poultry and zoo birds. Any extra-label use of the vaccines should be done cautiously and under the supervision of a veterinarian experienced in disease control through vaccination in the test species. Care must be taken to avoid self-injection with oil emulsion vaccines.

Reversion-to-virulence for attenuated/live vaccines
Only inactivated avian influenza virus vaccines are recommended. Live conventional influenza vaccines against any subtype are not recommended because of the risk for reassortment of gene segments of vaccine virus with field virus, potentially creating more virulent field viruses.

Environmental consideration
None

ii) Efficacy requirements
For animal production
For licensing purposes, avian influenza vaccines should pass an efficacy challenge test using a minimum of 24 SPF chickens per group. The challenge should occur at a minimum of three weeks post-vaccination, using a challenge HPNAI virus dose that causes 90% or greater mortality in the sham population. A standardised challenge dose of $10^8$ mean chicken embryo infectious doses is most widely use. Protection from mortality in the vaccine group should be a minimum of 80%. For LPNAI, mortality is not a feature of challenge models, therefore a statistically significant reduction in virus shedding titre and/or the number of birds shedding virus from oropharynx or cloaca should be observed between sham and test vaccine groups.

In establishing minimum antigen requirements, 50 PD$_{50}$ or 3 µg of haemagglutinin per dose have been suggested (Swayne & Kapczynski, 2008a). Minimum HI serological titres in field birds should be 1:32 to protect from mortality or greater than 1:128 to provide reduction in challenge virus replication and shedding.

For control and eradication
Efficacy should be the same as for animal production.

iii) Stability
When stored under the recommended conditions, the final vaccine product should maintain its potency for at least 1 year. Inactivated vaccines must not be frozen.

3. Vaccines based on biotechnology

a) Vaccines available and their advantages
Recombinant vaccines for AI viruses have been produced by inserting the gene coding for the influenza virus haemagglutinin into a live virus vector and using this recombinant virus to immunise poultry against AI (Swayne, 2004). Recombinant live vector vaccines have several advantages: 1) they are live vaccines able to induce mucosal, humoral and cellular immunity; 2) they can be administered to young birds and induce an early protection, e.g. the fowl poxvirus can be administered at 1 day of age, is compatible with the Marek’s disease vaccine, and provides significant protection 1 week later; 3) they enable differentiation between infected and vaccinated birds, as, for example, they do not induce the production of antibodies against the
nucleoprotein or matrix antigens that are common to all AI viruses. Therefore, only field-infected birds will exhibit antibodies in the AGID test or ELISAs directed towards the detection of influenza group A (nucleoprotein and/or matrix) antibodies. However, these vaccines have limitations in that they will replicate poorly and induce only partial protective immunity in birds that have had field exposure to or vaccination with the vector virus, i.e. fowl poxvirus or infectious laryngotracheitis viruses for currently available recombinant vaccines (Swayne & Kapczynski, 2008a, b). If used in day-old or young birds, the effect of maternal antibodies to the vector virus on vaccine efficacy may vary with the vector type. In the case of fowl poxvirus recombinant vaccine, it has been reported that effective immunisation was achieved when given to 1-day-old chicks with varying levels of maternal immunity (Arriola et al., 1999). However, when very high levels of maternal antibodies are anticipated because of previous infection or vaccination, the efficacy of the fowlpox vector vaccine in such day-old chicks should be confirmed and may require a prime-boost application of recombinant vaccine followed in 2–3 weeks by inactivated AI vaccine boost. In addition, because the vectors are live viruses that may have a restricted host range (for example infectious laryngotracheitis virus does not replicate in turkeys), the use of these vaccines must be restricted to species in which efficacy has been demonstrated.

The use of recombinant vaccines is restricted to countries in which they are licensed and legally available. The recombinant fowlpox-AI-H5 vaccine has been licensed in El Salvador, Guatemala, Mexico, China (the People's Rep. of) and the USA (Swayne & Kapczynski, 2008a). Recombinant fowlpox virus vaccines containing H5 HA have been prepared and evaluated in field trials, but the only field experience with this vaccine has been in Mexico, El Salvador, Guatemala and China (the People's Rep. of) where it has been used in the vaccination campaign against the H5N2 LPNAI and H5N1 HPNAI viruses.

Newcastle disease virus can also be used as a vector for expressing influenza HA haemagglutinin genes. A recombinant Newcastle disease virus vaccine expressing a H5 HA gene was shown to protect chickens against challenge with both virulent Newcastle disease virus and a HPNAI H5N2 virus (Veits et al., 2006). A similar recombinant virus based on Newcastle disease virus vaccine strain La Sota and expressing the Asian lineage H5 HA gene was produced in China (the People’s Rep. of) (Ge et al., 2007) and reported to be efficacious in protection studies with either virus. This latter virus has been licensed in China (the People’s Rep. of) and used widely. Recombinant Newcastle disease virus (rNDV) vaccines are effective in poultry lacking immunity to the Newcastle disease virus vector, but rNDV vaccines are largely ineffective as single dose primary vaccine in poultry with maternal immunity or well-immunised against Newcastle disease. rNDV vaccines are effective if used as a priming vaccine followed by a boost with an inactivated AI vaccine. In addition to these licensed vaccines, various experimental haemagglutinin-based AI vaccines have been described using in vivo or in vitro expression systems including recombinant adenoviruses, salmonella, baculovirus, vaccinia, avian leucosis virus, alphavirus and infectious laryngotracheitis virus (Swayne & Kapczynski, 2008a). DNA encoding H5 haemagglutinin has been evaluated as a potential vaccine in poultry.

b) Special requirements for biotechnological vaccines, if any

Live recombinant vectored vaccines with avian influenza gene inserts should have an environmental impact assessment completed to determine the risk of the vaccine to be virulent in non-target avian species and will not increase in virulence in the target avian species.

4. Surveillance methods for detecting infection in vaccinated flocks and vaccinated birds

A strategy that allows differentiation of infected from vaccinated animals (DIVA), has been put forward as a possible solution to the eventual eradication of NAI without involving mass culling of birds and the resulting economic damage, especially in developing countries (FAO, 2004). This strategy has the benefits of vaccination (less virus in the environment), but the ability to identify infected flocks would still allow the implementation of additional control measures, including stamping out. DIVA strategies use one of two broad detection schemes within the vaccinated population: 1) detection of influenza A virus (‘virus DIVA’), or 2) detection of antibodies against influenza A virus infection (‘serological DIVA’). At the flock level, a simple method consists of regularly monitoring sentinel birds left unvaccinated in each vaccinated flock, but this approach does have some management problems, particularly with regards to identifying the sentinels in large flocks. As an alternative or adjunct system, testing for field exposure may be performed on the vaccinated birds either by detection of field virus or antibodies against the virus. To detect the field virus, oropharyngeal or cloacal swabs from baseline daily mortality or sick birds can be tested, individually or as pools, by molecular methods, such as rRT-PCR or AC-ELISA of the vaccinated populations (Swayne & Kapczynski, 2008a).

To use serological DIVA schemes, vaccination systems that enable the detection of field exposure in vaccinated populations should be used. Several systems have been developed in recent years. These include the use of a vaccine containing a virus of the same haemagglutinin (H) subtype but a different neuraminidase (N) from the field virus. Antibodies to the N of the field virus act as natural markers of infection. This system has been used in Italy following the re-emergence of a LPNAI H7N1 virus in 2000. In order to supplement direct control measures, a ‘DIVA’ strategy was implemented using a vaccine containing H7N3 to combat a H7N1 field infection. Vaccinated and field exposed birds were differentiated using a serological test to detect specific anti-N antibodies (Capua et
al., 2003). The same strategy was used to control LPNAI caused by H7N3 in Italy in 2002–2003 (Capua & Alexander, 2004), in this case with a H7N1 vaccine. In both cases, vaccination combined with stamping out, using the described DIVA strategy resulted in eradication of the field virus. Problems with this system would arise if a field virus emerges that has a different N antigen to the existing field virus or if subtypes with different N antigens are already circulating in the field.

Alternatively the use of vaccines that contain only HA, e.g. recombinant vaccines, allows classical AGID and nucleocapsid protein (NP)- or matrix-based ELISAs to be used to detect infection in vaccinated birds. For inactivated vaccines, a test that detects antibodies to the nonstructural virus protein has been described (Tumpey et al., 2005). This system is yet to be validated in the field.

5. Continued evaluation and updating of vaccine seed strains to protect against emergent variant field virus strains

Historically, H5 LPNAI inactivated vaccine seed strains and recombinant fowl poxviruses with H5 gene inserts have shown broad cross protection in chickens against challenge by diverse H5 HPNAI viruses from Eurasia and North American (Swayne & Kapczynski, 2008a). However, avian influenza vaccines have had limited use in the field until 1995 when the H5N2 HPAI outbreak occurred in Mexico and vaccine use was implemented as part of the control programme (Villareal 2007). The HPAI strains were eradicated by June 1995, but as H5N2 LPNAI viruses have continued to circulate, vaccination was maintained as one of the control tools for these H5N2 LPNAI strains. Within a few years, multiple lineages of antigenically variant H5N2 LPNAI field viruses emerged that escaped from immunity induced by the original 1994 vaccine seed strain used in the conventional inactivated vaccine (Lee et al., 2004). Similarly, emergent H5N1 HPAI field viruses have arisen in China (the People’s Rep. of), Indonesia and Egypt since 2005 that escaped from immunity induced by classical H5 inactivated vaccine seed strains used in commercial vaccines (Chen & Bu, 2009; Grund et al., 2011; Swayne & Kapczynski, 2008b). It is not clear whether the emergence of these antigenic variants is related to use of vaccines or improper use of vaccines.

All avian influenza vaccination programmes should have an epidemiologically relevant surveillance programme to check for emerging variants and representative isolates of AI viruses obtained should be assessed for genetic and antigenic variation. Screening can be done by HI testing using genetic variant field viruses and vaccine seed strains as antigen, and isolates suspected of being antigenic variants should then be analysed by methods to quantify antigenic changes, e.g. antigenic cartography (Fouchier & Smith, 2010). H5 and H7 LPNAI vaccine seed strains used in inactivated vaccines and recombinant vaccine viruses with AI haemagglutinin gene inserts should be re-evaluated and seed strains that are not protective should be discontinued: a) whenever there is evidence of emergence of antigenic variants or vaccine failure (clinical disease in vaccinated flocks with a solid immune response to the vaccine antigen); or b) every 2–3 years for efficacy against circulating field viruses, and the use of seed strains that are not protective should be discontinued. The vaccine seed strain evaluation should include field viruses from all relevant geographical regions and production sectors, and sequence analyses of such viruses to identify genetic variants that can be further evaluated for antigenic change that may reduce the efficacy of the vaccine(s) in use. Strains representative of the major circulating antigenic lineage(s) plus selected antigenic variants should be used in challenge trials against current licensed vaccine seed strains, as well as potential future seed strains. Based on this scientific information, the competent veterinary authority within the country should establish, in consultation with leading veterinary vaccine scientists and international organisations, naturally isolated or reverse genetics LPNAI vaccine seed strains for conventional inactivated vaccines, and H5 and H7 haemagglutinin gene insert cassettes for recombinant vaccines. In some situations, more than one seed strain may be necessary to cover all production sectors within a country. Only high quality and potent vaccines should be licensed and used in AI control programmes. Proper administration of high quality, potent vaccines is critical in inducing protective immunity in poultry populations.

REFERENCES


Chapter 2.3.4. – Avian influenza


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NB: There are OIE Reference Laboratories for Avian influenza (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)).  
Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for avian influenza.
APPENDIX 2.3.4.1.

BIOSAFETY GUIDELINES FOR HANDLING HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUSES IN VETERINARY DIAGNOSTIC LABORATORIES

INTRODUCTION

The spread of highly pathogenic H5N1 avian influenza throughout Asia, Africa and Europe has led to an increase in the number of laboratories performing diagnostics for this pathogen. Highly pathogenic avian influenza viruses (HPAIV), in general, are a serious threat to birds and mortality is often 100% in susceptible chickens. In addition, the agents can also pose a serious zoonotic threat, with over 50% mortality reported in humans infected with HPAIV H5N1. In recognition of the need for guidance on how to handle HPAI viruses safely, the OIE has established the following biocontainment level guidelines for handling specimens that may contain HPAI virus. They are based on biosafety guidelines published in this OIE Terrestrial Manual (2012) and the World Health Organization (2005).

BIOCONTAINMENT LEVELS

Samples for diagnostic testing for HPAI using the following techniques can be processed using the OIE containment level for group 2 pathogens:

- Polymerase chain reaction (PCR)
- Antigen-capture assays
- Serology

Virus isolation and identification procedures for handling specimens that may contain high-titred replication-competent HPAIV should be performed at the OIE containment level for group 3 or group 4 pathogens, which would include the following:

- Personnel protective equipment should be worn, including solid-front laboratory coats, gloves, safety glasses and respirators with greater than or equal to 95% efficiency.
- Specimens from potentially infected birds or animals should only be processed in type II or type III biological safety cabinets (BSC).
- Necropsies of birds should be performed in a Type II BSC while wearing respiratory protection, such as a N95 respirator, or in a Type III biological safety cabinet, or other primary containment devices with 95% efficient air filtration.
- Centrifugation should be performed in sealed centrifuge cups.
- Centrifugation rotors should be opened and unloaded in a BSC.
- Work surfaces and equipment should be decontaminated after specimen processing.
- Contaminated materials should be decontaminated by autoclaving or disinfection before disposal or should be incinerated.

If chickens or other birds or mammals are inoculated with HPAI viruses, inoculation should be done in a containment level for group 4 pathogens and should include:

- Inoculated chickens should be held in isolation cages or other primary containment devices, or non-isolation cages/floor pens in specially designed rooms such as biosafety level 3 agriculture (BSL-3Ag) as designed by the US Department of Agriculture.
- Cages should be in a separate facility that is equipped to handle containment level for group 3 pathogens.
Appendix 2.3.4.1. — Biosafety guidelines for handling HPAI viruses in veterinary diagnostic laboratories

- The room should be under negative pressure to the outside and the cages should be under negative pressure to the room.
- Cages should have HEPA-filtered inlet and exhaust air.
- Biosafety cabinet or other primary containment devices should be available in the animal facility to perform post-mortem examinations and to collect specimens.

REFERENCES


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CHAPTER 2.3.5.

AVIAN MYCOPLASMOSIS
(Mycoplasma gallisepticum, M. synoviae)

SUMMARY

Definition of the disease: Avian mycoplasmosis is caused by several pathogenic mycoplasmas of which Mycoplasma gallisepticum (MG) and M. synoviae (MS) are the most important; they are the only ones listed by the OIE.

Description of the disease: MG causes chronic respiratory disease of domestic poultry, especially in the presence of management stresses and/or other respiratory pathogens. Disease is characterised by coryza, conjunctivitis, sneezing, and by sinusitis, particularly in turkeys and game birds. It can result in loss of production and downgrading of meat-type birds, and loss of egg production. MS may cause respiratory disease, synovitis, or may result in a silent infection. MG and MS strains vary in infectivity and virulence, and infections may sometimes be unapparent.

Identification of the agent: MG and MS can be identified by immunological methods after isolation in mycoplasma media or by detection of their DNA in field samples or cultures. Samples for isolation can be swabs of organs or tissues, exudates, diluted tissue homogenates, aspirates from the infraorbital sinuses or joint cavities, or material from egg yolk or embryos. Clinical signs and lesions will influence the sample selection. Broth and agar are used for isolation, but it is normally necessary to obtain mycoplasma colonies on agar before attempting identification. Basic biochemical tests can be helpful in preliminary classification of isolates but final identification is by immunological tests, the most satisfactory being fluorescent antibody and immunoperoxidase tests.

DNA detection methods based on the polymerase chain reaction are used in specialised laboratories. Once validated, they can be used on swab material or cultures.

Serological tests: Several serological tests are used to detect MG or MS antibodies, but due to variations in specificity and sensitivity, they are recommended for flock screening rather than for testing individuals.

The most commonly used are the rapid serum agglutination (RSA) test, the enzyme-linked immunosorbent assay (ELISA) and the haemagglutination inhibition (HI) test. In the RSA test, sera are mixed with commercially produced stained antigen and sera that react within 2 minutes are heated at 56°C for 30 minutes and retested. Sera that still react, especially when diluted, are considered positive and are tested by either ELISA or HI for confirmation. Several commercial MG and MS antibody ELISA kits are available.

Requirements for vaccines and diagnostic biologicals: Although the preferred method of control is maintenance of MG- and MS-free flocks, both live and inactivated vaccines are used in chickens. Vaccination should be considered only on multi-age sites where infection is inevitable. The normal use is to prevent egg-production losses in commercial layers, although vaccines may also be used to reduce egg transmission in breeding stock or to aid MG eradication on multi-age sites. It is important to vaccinate before field challenge occurs.

Available live vaccines for MG are produced from the F strain, and, more recently, strains ts-11 and 6/85, which are apathogenic strains with improved safety characteristics. Administration of the F strain by the intranasal or eyedrop route is preferred, but aerosol or drinking water administration may be used. The eyedrop method is recommended for ts-11, and a fine spray for 6/85. Pullets are generally vaccinated between 12 and 16 weeks of age. One dose is sufficient and vaccinated birds
remain permanent carriers. Long-term use of the F strain on multi-age sites results in displacement of field strains. The ts-11 strain has been successfully used to eradicate F strain in multi-age commercial layers. A live MS vaccine has been produced from the MS-H strain and should be administered by eyedrop.

Bacterins consist of a concentrated suspension of MG organisms in an oil emulsion. They are administered parenterally to pullets at 12–16 weeks of age, usually subcutaneously in the neck. Two doses are desirable. Bacterins are effective in preventing egg-production losses and respiratory disease, but they do not prevent infection with wild-type MG. A similar bacterin has been licensed in the United States of America for MS, but it is not widely used.

A. INTRODUCTION

Mycoplasma gallisepticum (MG) and M. synoviae (MS) belong to the class Mollicutes, order Mycoplasmatales, family Mycoplasmataceae. It should be noted, however, that M. meleagridis and M. iowae can also cause disease in poultry, but MG and MS are considered to be the most important of the pathogenic mycoplasmas, and both occur world-wide.

MG infection is particularly important in chickens and turkeys as a cause of respiratory disease and decreased meat and egg production (Bradbury, 2001; Ley, 2003). It can also cause upper respiratory disease in game birds. More recently MG has been recognised in North America in house finches as a cause of conjunctivitis (Luttrell et al., 1996). In poultry the infection is spread vertically through infected eggs and horizontally by close contact; the MG nucleic acid has been identified in environmental samples (Marois et al., 2002). Other methods of spread are less well documented.

The clinical signs of MG in infected poultry can vary from subclinical to obvious respiratory signs including coryza, conjunctivitis, coughing and sneezing. Nasal exudate, rales and breathing through the partially open beak may occur. Unilateral or bilateral sinusitis may also be a feature, particularly in turkeys and game birds and the infraorbital sinuses may become so swollen that the eyelids are closed. Conjunctivitis, with frothy ocular exudate is also a common feature in turkeys and game birds, and sometimes in chickens. In turkeys there is often soiling of the wing feathers as the result of attempts to remove exudate from the eyes. Infected finches may reveal ocular and nasal discharge and swollen eyelids in addition to the conjunctivitis.

Mycoplasma gallisepticum may be associated with acute respiratory disease in chickens and turkeys, especially in young birds, with the turkey being more susceptible. The severity of the disease is greatly affected by the degree of secondary infection with viruses such as Newcastle disease and infectious bronchitis, and/or bacteria such as Escherichia coli. In turkeys there is synergism with avian pneumovirus infection. A more chronic form of the disease may occur and can cause egg production in breeders and layers.

Lesions of the respiratory tract take the form initially of excess mucous exudate followed by catarrhal and caseous exudate, which may form amorphous masses in the air sacs. In turkeys and game birds the swollen infraorbital sinuses contain mucoid to caseous exudate.

MG or MS disease in chickens may superficially resemble respiratory disease caused by other pathogens such as mild strains of Newcastle disease (chapter 2.3.14) and avian infectious bronchitis (chapter 2.3.2). These may be present in mixed infection with MG or MS. Infections with Haemophilus paragallinarum (now Avibacterium paragallinarum), and Pasteurella multocida, should also be ruled out. MG in turkeys may be confused with avian pneumovirus infections and the presence of sinusitis may also suggest infection with Pasteurella multocida, Chlamydia (chapter 2.3.1) or MS. Infectious synovitis caused by MS should be differentiated from Staphylococcus aureus infection and from infectious tenosynovitis caused by reovirus.

Chickens with infectious synovitis may exhibit pale combs, lameness and retarded growth. Swellings may occur around joints. Greenish droppings containing large amounts of urates are commonly seen. Joints may contain a viscous, creamy to grey exudate in the joint and along tendon sheaths, along with hepatosplenomegaly and mottled, swollen kidneys (Kleven, 2003). Respiratory signs and lesions are similar to those observed with MG, except that they are generally milder, and, as with MG, there is a synergistic effect with other respiratory agents (Kleven et al., 1972). MS strains exhibit significant variability with respect to their virulence and tissue tropism (Kleven et al., 1973; Landman & Feberwee, 2004; Lockaby et al., 1999).

B. DIAGNOSTIC TECHNIQUES

The presence of MG or MS can be confirmed by isolating the organism in a cell-free medium or by detecting its DNA directly in infected tissues or swab samples. Serological tests are also widely used for diagnosis. When
Chapter 2.3.5. – Avian mycoplasmosis (Mycoplasma gallisepticum, M. synoviae)

results are equivocal the birds are usually resampled, although chicken embryos or chickens may be inoculated with suspect material.

1. Identification of the agent

- Culture

Samples are taken from live birds, fresh carcasses or the carcasses of birds that have been frozen when fresh. From live birds, swabs may be taken from the choanal cleft, oropharynx, oesophagus, trachea, eyes, cloaca and phallus. In the case of dead birds, samples may be taken from the nasal cavity, infraorbital sinus, trachea, or air sacs. Exudates can be aspirated from the infraorbital sinuses and joint cavities.

Samples may also be collected from dead-in-shell embryos or chickens or poults that have broken the shell but failed to hatch. Samples can be taken from the inner surface of the vitelline membrane, and from the oropharynx and air sacs of the embryo.

All samples should be examined as soon as possible after collection. If transportation is necessary, small pieces of tissue should be placed in mycoplasma broth, or swabs should be vigorously agitated in 1–2 ml of mycoplasma broth and then discarded. Alternatively, the swabs can be dipped in mycoplasma broth before the specimens are taken (Zain & Bradbury, 1996) and then replaced in the swab holders for transportation. An ice pack or some other means of chilling should be included as MG and MS die rapidly at room temperature. Serial dilutions of specimens in mycoplasma broth may be of value because the presence of specific antibodies or antibiotics or inhibitory substances in tissues may inhibit mycoplasma growth unless they are diluted out.

Several suitable culture media have been formulated (Freundt, 1983) and those suitable for isolation of avian mycoplasmas can be purchased from Mycoplasma Experience, Reigate, Surrey, United Kingdom. Mycoplasma media generally contain a protein digest and a meat-infusion base supplemented with serum or a serum fraction, yeast factors, glucose and bacterial inhibitors. It is important that each new batch of medium be tested with recently isolated MG cultures of low in-vitro passage because some components, especially the yeast extract and the serum may vary in their ability to support growth.

The medium developed by Frey et al. is widely used in the United States of America (USA) and other countries for isolation of MG and MS (USDA, 2004; Frey et al., 1968). Nicotinamide adenine dinucleotide (NAD) is a growth requirement for the primary isolation of MS, but it may be omitted from the medium for the cultivation of MG.

The following broth and agar media are also satisfactory:

- Part A: Pleuropneumonia-like organism (PPLO) broth base without crystal violet (Difco) (14.7 g); distilled or deionised water (700 ml).

- Part B: Pig serum (heated at 56°C for 1 hour) (150 ml); 25% (w/v) fresh yeast extract (100 ml); 10% (w/v) glucose solution (10 ml); 5% (w/v) thallous acetate (10 ml); 200,000 International Units (IU)/ml penicillin G (5 ml); and 0.1% (w/v) phenol red solution (20 ml). Thallous acetate can be toxic to humans and the precautions for its use should be followed. The pH is adjusted to 7.8. Pig serum may be replaced by horse serum, but it is important to ascertain that it supports the growth of MG.

Part A is autoclaved at 121°C, at 1 atmospheric pressure for 15 minutes and, after cooling, is added to Part B, which has previously been sterilised by filtration.

For the corresponding solid medium, 10 g of purified agar, known to support the growth of MG, is added to part A above. The mixture is autoclaved as before and kept in a water bath at 56°C. The constituents of part B, omitting the phenol red, are mixed separately and then incubated at 56°C. Parts A and B are mixed carefully to avoid the production of air bubbles, and are dispensed into 50 mm dishes using 7–9 ml/dish. Excess surface moisture can be removed by a short incubation at 37°C. Plates are stored in an airtight container at approximately 4°C for up to 2 weeks.

Fresh yeast extract is available commercially, although it is preferable to prepare it ‘in-house’ by taking active dry baker’s yeast (250 g) and suspending it in distilled water (1 litre). This is heated to boiling point, cooled and then centrifuged for 20 minutes at 3000 g. The supernatant fluid is decanted and adjusted to pH 8.0 with 0.1 M NaOH. This is clarified by centrifugation or by filtration, and then sterilised by filtration. The extract is stored at −20°C. Reagent grade glucose (10 g) is dissolved in distilled or deionised water (100 ml) and adjusted to pH 7.8–8.0 with 0.1 M NaOH. It is sterilised by filtration and stored at 4°C. Reagent grade thallous acetate is dissolved (5 g) in distilled or deionised water (100 ml), filter-sterilised and stored at
Chapter 2.3.5. — Avian mycoplasmosis (Mycoplasma gallisepticum, M. synoviae)

--20°C. Penicillin solution (10^6 IU benzyl penicillin in 5 ml distilled water) is stored at 4°C, and has a shelf life of 1 week. For isolation from heavily contaminated samples, penicillin concentration can be increased to 2000 units/ml or ampicillin, 0.5–1.0 mg/ml, maybe used instead. Phenol red (0.1 g) is ground in 0.1 M NaOH (2.8 ml), and then made up to 100 ml in sterile distilled water and autoclaved at 115°C at 1 atmosphere for 30 minutes. It is stored at 4°C. (Note: Thallous acetate is highly toxic and care should be taken, especially when preparing the stock solution.)

Specimens are inoculated on to mycoplasma agar and into broth. Solid medium may help detection of slow-growing mycoplasma colonies, which can be overgrown by saprophytes in broth. It may be necessary to make serial dilutions up to 10^{-6} for successful isolation. Inoculated plates are incubated at 37°C in sealed containers. Increased humidity and CO₂ tension in the atmosphere have been reported to enhance growth; these conditions may be obtained by the inclusion of damp paper or cotton wool, and by flushing the container with 5–10% CO₂ in nitrogen, by placing a lighted candle in the container, or by using a CO₂ incubator or suitable gas-generating system.

The caps of liquid medium containers should be tightly sealed before incubation at 37°C to avoid spurious changes in pH. For the first few days, the plates are examined daily for colonies with a stereoscopic microscope; after that they are examined less frequently. Cultures from field material should not be discarded as negative for at least 20 days.

Broth medium should be examined daily for acidity, indicated by a change from red to orange or yellow in the indicator. Any observable growth is subcultured on to solid medium immediately. Even if no colour change occurs, subculture on to solid medium should be made after 7–10 days or earlier as the presence of an arginine-hydrolysing (alkali-producing) mycoplasma species may mask the acid colour change produced by MG.

Mycoplasma colonies on solid medium can usually be recognised, although they may not have the typical ‘fried egg’ appearance. Bacterial colonies may appear on the first passage, but they are often more pigmented and fail to pass on mycoplasma media.

Biochemical reactions (e.g. fermentation of glucose and failure to hydrolyse arginine) can assist in identification, but they are not specific for MG or MS and necessitate purification of the culture by cloning.

Immunological and DNA detection methods can be used to identify mycoplasma isolates. They include the indirect fluorescent antibody (IFA) and immunoperoxidase (IP) tests, both of which are simple, sensitive, specific and rapid to perform; growth inhibition (GI); and metabolism inhibition (MI). Purified (cloned) cultures are required for the GI and MI tests, but not for the IFA or IP test. IFA and IP can detect the presence of more than one species of mycoplasma, as the colonies specific for the antiserum will react while the others will not. However, M. imitans, a mycoplasma species that is serologically related to MG and that presents the same biochemical properties has been isolated from ducks, geese and sometimes from other nondomestic bird species in some countries. It may be distinguished from MG by use of a PCR-RFLP (polymerase chain reaction/restriction fragment length polymorphism), as described by Kempf (1998).

Alternatively, colonies of the isolate can be examined by immunofluorescence using serial dilutions of antisera to MG and M. imitans in parallel. The homologous antiserum should have a considerably higher titre.

DNA detection methods for identifying MG or MS directly in tissues or for identifying laboratory isolates are discussed below and are usually based on the PCR.

In certain circumstances where results of the above methods are not conclusive, inoculation of chick embryos or bioassays in live chicks may be appropriate. However these techniques are time-consuming and costly and tend to have been replaced by PCR technology, although they remain a useful research tool. The specimens required for inoculation of chicken embryos are the same as those used for the inoculation of artificial media. They are prepared in broth from which thallous acetate is omitted, incubated for 30–60 minutes at 37°C, and then a 0.05–0.1 ml aliquot is inoculated into the yolk sac of several 6–8-day-old chicken embryos derived from mycoplasma-free flocks. The eggs are candied daily and embryos that die within 24 hours of inoculation are discarded. Any further dead embryos are kept refrigerated until cultured and those surviving after 5 days are placed at 4°C for 4 hours to kill them and to reduce haemorrhages on opening. The yolk is subcultured into broth and on to agar. Yolk lipid tends to obscure colonies so it is essential to streak the yolk thinly or, preferably, to dilute it first in mycoplasma broth.

Bioassays may be performed by the inoculation of a homogenate of suspect material into at least four 8–16 week-old susceptible mycoplasma-free chickens. Diagnosis is confirmed by the recovery of the mycoplasma from these birds, demonstration of its DNA and/or the demonstration of specific antibodies (Mallinson et al., 1981).
Chapter 2.3.5. — Avian mycoplasmosis (Mycoplasma gallisepticum, M. synoviae)

- **Immunological methods**
  Immunofluorescence and IP procedures for diagnosis are generally applied to suspect laboratory isolates rather than directly to infected exudates or tissues. This is because the organisms are too small to be conclusively recognized under the light microscope and because the corresponding negative and positive control exudate/tissue is unlikely to be readily available.

  a) **Indirect fluorescent antibody test**
  The recommended technique for the IFA test (Rosendal & Black, 1972) requires an agar culture of the unknown isolate, consisting of numerous small discrete colonies, a known MG or MS culture as a positive control, and a culture of another mycoplasma species, such as M. gallinaceum or M. gallinarum as a negative control. Also required are polyclonal rabbit anti-MG or MS serum, a normal rabbit serum and an anti-rabbit immunoglobulin fluorochrome-conjugated serum. Sera may be prepared in species other than rabbits, but monoclonal antibodies (MABs) should not be used because MG or MS demonstrates variable expression of its surface epitopes and an MAB may fail to recognize the organism. Suitable working dilutions in sterile phosphate buffered saline (PBS; 0.01 M, pH 7.2) of the anti-MG or MS serum and the conjugate are first determined by cross-titration, and are selected for use at two-to-four-fold dilutions less than the actual end-points. These are applied to the colonies of mycoplasmas to be identified that have been previously grown on agar plates as indicated below.

  b) **Indirect immunoperoxidase test**
  This involves a similar principle to the IFA test except that the binding of specific antibodies to colonies in situ is detected by adding an anti-rabbit immunoglobulin that has been conjugated to the enzyme peroxidase. A positive reaction is then developed by adding an appropriate substrate which, on oxidation, produces coloured colonies. An immunobinding procedure can also be used in which the test colonies are blotted on to nitrocellulose (Kotani & McGarrity, 1985) and then reacted in a similar manner. As with IFA, polyclonal sera should be used for serotyping isolates by IP. The advantage of the IP test over immunofluorescence is that the IP test does not require an expensive fluorescence microscope.

  c) **Growth inhibition test**
  In the GI test, the growth of mycoplasmas is inhibited by specific antiserum, enabling species to be identified. It is relatively insensitive and sera must be high-titre, monospecific and prepared in mammalian hosts as poultry sera do not always inhibit mycoplasma growth efficiently. The organism under test must be in pure culture (cloned) and several dilutions should be tested; a concentration of $10^4$ colony-forming units...
Chapter 2.3.5. — Avian mycoplasmosis (Mycoplasma gallisepticum, M. synoviae)

(CFU/ml) is optimal. The rate of growth of the organism may influence growth inhibition, and it is helpful to retard growth initially by incubating at 27°C for 24 hours, followed by incubation at 37°C thereafter. Details of the test and its interpretation are published elsewhere (Clyde, 1983).

- **Nucleic acid detection methods**

An alternative to conventional culture and identification is the use of specific DNA detection methods. MG or MS may be detected by hybridisation with DNA probes, but now it is much more common to use the PCR to amplify specific portions of DNA in the test material. At least one commercial MG DNA test kit uses a PCR directly on material extracted from swabs. One commercial company produces a kit to detect MG field strains and one that identifies the vaccine F strain. Several in-house PCR-based tests have also been published for MG including a multiplex PCR, which is designed to detect all four avian mycoplasma pathogens (Wang *et al*., 1997), but which has not been validated with clinical samples. Several methods are cited by Kempf (1998) and, in addition, a manual published by Lauerman (1998) contains a validated PCR assay for MG, MS, and other avian mycoplasmas based on unique sequences contained in the 16S rRNA gene. This method for MG is presented below. In the USA, a PCR based on the *mgc2* gene of MG (Garcia *et al*., 2005) or the *vha* gene of MS (Hong *et al*., 2004) is becoming more widely used, because preliminary strain identification can be made by sequencing of the PCR product; it must be remembered that unrelated strains may occasionally share the same sequence.

a) **DNA isolation**

DNA is extracted from swab samples (three–five may be pooled) suspended in 1 ml of PCR-grade PBS in a 1.5 ml snap-cap Eppendorf tube. The suspension is centrifuged for 30 minutes at 14,000 g at 4°C. The supernatant is carefully removed with a Pasteur pipette and the pellet is suspended in 25 µl PCR-grade water. The tube and the contents are boiled for 10 minutes and then placed on ice for 10 minutes before centrifugation at 14,000 g for 5 minutes. The DNA is in the supernatant.

b) **Primers**

The MG primers consist of the following sequences.

MG-14F: 5’-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3’

MG-13R: 5’-GCT-TCC-TTG-CGG-TTA-GCA-AC-3’

For MS, the following primers are used:

MS–F: 5’-GAG-AAG-CAA-AAT-AGT-GAT-ATC-A-3’

MS–R: 5’-CAG-TCG-TCT-CCG-AAG-TTA-ACA-A-3’

c) **Polymerase chain reaction**

The reaction mixture should be prepared in a separate clean area using a set of dedicated pipettes. For one 50 µl PCR reaction the mixture is as follows:

- H₂O Ultra-pure: 35.75 µl
- 10 × PCR Buffer: 5.00 µl
- dNTP (10 mM): 1.00 µl
- F Primer (20 pmole/µl): 0.50 µl
- R Primer (20 pmole/µl): 0.50 µl
- Taq (5 U/µl): 0.25 µl
- MgCl₂ (50 mM): 2.00 µl

A 45 µl volume of the reaction mixture is dispensed into each PCR tube. The reaction mixture should be overlaid with a few drops of light weight mineral oil unless the thermocycler is equipped with a heated lid. The tubes are then placed in another clean area where the appropriate DNA sample (5 µl) is added to each tube. Positive and negative controls should be used in each run.

The tubes are then placed in a thermal cycler for the following cycles: 40 cycles: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, 1 cycle (final extension): 72°C for 5 minutes and soak at 4°C.

d) **Electrophoresis**

PCR products are detected by conventional 2% agarose gel electrophoresis, incorporating appropriate size markers, followed by examination under UV light. The PCR product for MG is 185 bp. Visualisation of the PCR products should be carried out in a separate laboratory area, well separated from all other steps in the PCR procedure.
PCR tests still tend to be carried out by specialist laboratories and should probably be regarded as useful adjuncts to the present diagnostic methods once their validity is firmly established. Great care needs to be taken to avoid contamination of samples with MG or MS DNA from nearby post-mortem rooms, culture laboratories or from positive amplificates from previous PCR runs (see Chapter 1.1.5 Principles and methods of validation of diagnostic assays for infectious diseases, for appropriate safeguards). However one commercial kit referred to above is now licensed by the United States Department of Agriculture (USDA) as a diagnostic method and approved for use in the National Poultry Improvement Plan (NPIP). It should be noted that PCR tests are not validated for testing day-old birds for accurate detection of infection.

Molecular methods are also available for differentiation of MG and MS strains (Kempf, 1998), but their use tends to be restricted at present to specialist laboratories. A rapid and accurate method for DNA fingerprinting uses arbitrary primed PCR or random amplified polymorphic DNA (RAPD). This technique uses short, arbitrary PCR primers, which generate reproducible patterns in agarose gels (Fan et al., 1995). This method is rapid and simple, and has proven to be very useful for rapid identification of strains of MG for epidemiological studies. However, there may be problems with reproducibility, so strains to be compared must be run on the same gel. Also, interpretation of banding patterns which appear to be similar can be difficult.

Gene-targeted sequencing (GTS), using PCR primers for the mgc2, gapA, pvpA, and MGA_0309 genes of MG can be used to provide an accurate and reproducible method of typing of strains, which will allow rapid global comparisons between laboratories (Ferguson et al., 2005). Preliminary strain identification with diagnostic PCR primers for the mgc2 gene of MG or the vlnA gene of MS by sequencing of the PCR product allows for preliminary strain identification without the need for prior isolation of the organism (Hong et al., 2004; 2005). However, unrelated strains sometimes have identical sequences using these primers, and further characterisation may be necessary.

2. Serological tests

The serological tests in common use may lack specificity and/or sensitivity; their use is strongly recommended for monitoring flocks rather than for testing individual birds. Diagnosticians wishing to use such tests are advised to establish the test sensitivity and specificity (see chapter 1.1.5) under their own laboratory conditions. It should also be noted that these tests have not been validated for use with sera from day-old birds or from game birds (Bradbury, 2005).

The most commonly used tests are RSA, ELISA and HI although several others have been described such as radioimmunoassay, microimmunofluorescence and IP assay. The number of sera to be tested within a flock depends on the level of detection and the confidence limits required. Minimal requirements may be laid down for international trade and the frequency of testing may also be stipulated as, for example, in the European Communities Council Directive 90/539/EEC. Minimal requirements and approved tests are also set out for members of the NPIP of the USA.

Poultry companies using ELISA technology for screening large numbers of sera for virus antibodies may find this type of assay convenient also for mycoplasma testing. The ELISA technology will not be described in detail here because several MG kits are available commercially. Instead, the details of the HI test are provided as the reagents needed for this test are not widely available commercially.

a) Rapid serum agglutination test

Sera are collected from a sample of the flock and, if not tested immediately, are stored at 4°C and not frozen. The test should be carried out at room temperature (20–25°C) within 72 hours of serum collection and the reagents should also be at room temperature. Prior centrifugation will reduce nonspecific reactions. The RSA antigens are available commercially, but they may vary in specificity and sensitivity from different manufacturers and from batch to batch. They must be stored according to the manufacturer’s instructions. Suitable RSA-stained antigens may also be prepared ‘in-house’ using culture methods as described in Section B.1.; these are then stained with crystal violet dye. Quality control standards for mycoplasma antigens for serological tests are described below.

- **Test procedure** (Allan & Gough, 1974)
  i) Drop one volume (approximately 0.02 ml) of serum on to a clean white tile or glass plate followed by one volume of stained MG or MS antigen. Do not allow the serum to dry out before addition of the antigen. It is important to shake the antigen bottle vigorously and frequently during use to keep the correct amount of antigen in suspension.
  ii) Use a stirring rod to spread the mixture over a circular area of approximately 1.5 cm diameter. Rock the tile or plate for 2 minutes. Agglutination is indicated by flocculation of the antigen within 2 minutes.
  iii) Include known positive and negative controls in the test.
iv) Retest serial dilutions of any sera that agglutinate after heating at 56°C for 30 minutes. If they still react strongly, they are considered to be positive on dilution (1/4 or more).

In the USA, MG and MS positive reference antisera can be obtained from the USDA National Veterinary Services Laboratories (NVSL), and in Europe from AFSSA Ploufragan1, France. MG and MS and control sera produced in chickens or in turkeys and with a range of titres can be purchased. Sets of antisera can be purchased also from the University of Georgia Department’s of Avian Medicine, subject to availability.

There are no international standards for interpreting these tests, but a high proportion of positive sera in a flock (10% or more) indicates MG infection, especially if confirmed by HI test or ELISA. For further confirmation, the flock should be retested within a month. Inconclusive results make it necessary to attempt to isolate the organism or to demonstrate the presence of its DNA. Doubtful results for MG or MS should be investigated by performing tests with MS antigen (and vice versa) as infection with these organisms sometimes causes cross-reactions.

Tests can be conducted on yolk as well as sera although the yolk must first be diluted or extracted.

b) Haemagglutination inhibition test

MG and MS are capable of haemagglutinating avian red blood cells (RBCs), and specific antibodies in sera cause inhibition. A strain should be selected that grows well and haemagglutinates reliably. The HI test requires a satisfactory haemagglutinating MG and MS antigen, washed fresh chicken or turkey RBCs, as appropriate, and the test sera. The antigen can be either a fresh broth culture or a concentrated washed suspension of the mycoplasma cells in PBS. It may be difficult to sustain a supply of high-titred broth culture antigen; however, the use of concentrated antigen (usually containing 25–50% glycerol and stored at −70°C), increases the likelihood of nonspecific reactions. In the USA, MG and MS haemagglutination (HA) antigen can be purchased from the NVSL.

The HI test follows well-known procedures (Allan & Gough, 1974). The HA titre of the antigen is first determined in doubling dilutions, the HA unit being defined as the least amount of antigen giving complete HA in the test system employed. The HI test should be performed using 4 HA units by the following method or a method having equivalent sensitivity as determined by tests with known positive sera.

All HA titrations and HI tests are best performed in multiwell plastic plates with V-shaped wells and using constant volumes of 50 µl. A positive and a negative control serum are incorporated into each test. One row of eight wells is required for each serum under test.

- Test procedure
  i) Add 50 µl of PBS to the first well in each row.
  ii) Add 8 HA units of antigen in 50 µl volumes to the second well in each row and add 50 µl of 4 HA units of antigen to each of wells 3 to 8.
  iii) Add 50 µl of a previously-prepared 1/5 dilution of the serum under test to the first well, mix, and transfer 50 µl to the second well, and so on, and discard 50 µl from the last well. The first well is the serum control well.
  iv) Six wells are required for the antigen control. Add 50 µl of PBS to wells 2 to 6, inclusive, and add 50 µl of the 8 HA unit antigen to wells 1 and 2. Mix the contents of well 2 and transfer 50 µl to well 3, mix and repeat up to well 6, and discard 50 µl.
  v) Two wells are required for the RBC control. Add 50 µl of PBS to each of these.
  vi) Add 50 µl of a 0.5% suspension of RBCs (chicken cells for chicken serum and turkey for turkey serum) to all wells.
  vii) Shake the plate lightly to ensure thorough mixing of the well contents, and read after standing for approximately 50 minutes at room temperature or when the antigen titration is reading 4 HA units. For reading, the plate should be tilted and only those wells in which the RBCs ‘stream’ at the same time as those in the RBC control wells should be considered to be inhibited. The serum control should show a clear button of RBCs and the positive and negative controls should react as expected. The HI titre is the highest serum dilution exhibiting complete inhibition of HA.

Sera giving nonspecific HA must be adsorbed to remove all nonspecific haemagglutinins so that a clear button is obtained in the control well without HA antigen. The adsorption is carried out by incubating 1 ml of the serum dilution with 6–8 drops of packed washed chicken or turkey RBCs. The cells are removed after incubation at 37°C for 10 minutes, and the supernatant is tested for haemagglutinating activity.

1 Agence française de sécurité sanitaire des aliments (AFFSA) Ploufragan, Mycoplasmology Bacteriology Unit, 22440 Ploufragan, France.
There is no official definition of positive and negative results for international trade but the NPIP of the USA states that titres of 1/80 or above are considered to be positive and titres of 1/40 are strongly suspicious.

c) Enzyme-linked immunosorbent assay

Several commercial MG and MS antibody ELISA kits are marketed. The sensitivity is determined to some extent by the manufacturer’s recommendations for the cut-off levels for positive and suspicious reactions. Sensitivity may sometimes be ‘damped down’, to avoid the well-known cross-reaction between MG and MS. One ELISA uses an MAb that recognises an epitope on a 56 kDa polypeptide of MG (Czifra et al., 1993). In this system, ELISA plates are coated with whole cell MG antigen and the sera under test are added as in the conventional indirect ELISA, but the reaction is assessed by the extent of blocking that occurs when the conjugated MAb is added. A similar ELISA has also been marketed for MS. One advantage is that the system can be used for sera from any avian species without adaptation.

**Quality control of Mycoplasma gallisepticum and M. synoviae antigens**

i) *Mycoplasma gallisepticum* antigens

Antigens are usually prepared from the S6 strain or the A5969 strain of MG. Antigens prepared from other strains may also be used when necessary.

**MG antigen for the RSA test:** The methods of quality control described below apply solely to suspensions of MG stained with a suitable dye and containing preservative and intended for use in the rapid plate agglutination test with serum. Such antigens are available commercially.

On microscopic examination, the antigen should appear as a homogeneous suspension without floccules or precipitates and the suspending liquid should be free from residual dye. It must be free from contamination with bacteria and fungi. The pH must be between 6.5 and 7. It must be stored at 5±3°C and be warmed to room temperature before use.

The sensitivity and specificity of the antigen is determined with respect to its reaction with known positive sera of high and low titre and known negative sera. A positive reaction is recognised by the formation of coloured floccules and the clearing of the suspending medium. The criteria described above continue to apply until the expiry date declared by the manufacturer.

**MG antigen for the HI test:** The test is preferably performed with live, actively growing cultures. The antigen must be free from contamination with bacteria and fungi.

**MG antigen for the ELISA:** It may be difficult to prepare satisfactory antigen for use in the indirect ELISA without considerable prior experimentation and confirmation of sensitivity and specificity. Use of a reliable commercial kit is probably the best approach for most diagnostic laboratories. Some kits are now USDA-licensed and approved for use in the NPIP in the USA.

ii) *Mycoplasma synoviae* antigens

Antigens prepared from the WVU 1853 strain or other suitable strains should be used.

**Mycoplasma synoviae antigen for the RSA test:** the specifications apply as for MG antigen for the RSA test.

**Mycoplasma synoviae antigen for the HI test:** the same specifications apply as for MG antigen for the HI test.

iii) Additional comments

Sera giving nonspecific reactions to the RSA test do not usually give a positive reaction in the HI test using live HA antigen. Positive RSA reactions can be confirmed by the HI test with sera taken after the first 2–3 weeks of infection (the time taken for HI antibodies to develop). However, the HI test tends to be strain specific (Kleven et al., 1988) and therefore may lack sensitivity. ELISA may be a useful alternative.

Samples of serum should not be frozen before use in RSA tests. They should be free from haemolysis and contamination to avoid nonspecific reactions. The use of inactivated vaccines for other diseases may result in nonspecific reactions. Samples should be tested as soon as possible (within 72 hours) because mycoplasma antibodies may deteriorate on storage. Sera may be inactivated in a water bath at 56°C for 30 minutes.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

The preferred method of control is to maintain MG- and MS-free flocks. Vaccination should be considered only in situations where field exposure is inevitable, such as on multi-age sites. Potential exposure of neighbouring poultry flocks should also be carefully considered.
Two types of vaccines are available for the control of MG. These are mild to avirulent MG strains used as live vaccines, or inactivated oil-emulsion bacterins. The subject of MG vaccination has been reviewed by Whithear (Whithear, 1996). Although there is antigenic variability among MG strains, it is thought that vaccination with a single strain is sufficient.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

- **Live vaccines: methods of use**

The use of live vaccines is equivalent to ‘controlled exposure’. The objective is to infect the flock with a mild, immunogenic MG strain at an age when little or no significant damage occurs. Such exposure results in resistance to challenge later in life, such as on multi-age commercial sites. Successfully vaccinated birds are resistant to respiratory disease, airsacculitis, and egg production drops caused by MG. Vaccination also results in reduced levels of egg transmission in breeders.

The F strain of MG has been the most commonly used vaccine strain (Carpenter et al., 1981). It is a naturally occurring strain of mild to moderate virulence for chickens, but it is virulent for turkeys. It ordinarily spreads slowly from bird to bird. When administered to healthy chickens via the upper respiratory tract, little or no respiratory reaction is observed. However, when administered by aerosol or in the presence of other respiratory disease agents, such as Newcastle disease or infectious bronchitis virus, respiratory signs and airsacculitis may result. Vaccinated chickens are permanent carriers, so a single dose is adequate. Use of F strain vaccine in each replacement flock on a multi-age site will eventually result in displacement of the field strain with the vaccine strain. Strains ts-11 and 6/85 are avirulent and spread to unvaccinated birds does not occur or occurs very poorly when birds are in very close contact (Ley et al., 1997).

Commercial pullets are usually vaccinated between 12 and 16 weeks of age, but vaccination of younger or older birds is permissible. It is essential that vaccination occurs before the flock is naturally infected. Vaccination in cases of probable early field exposure can be carried out in birds as young as 2–4 weeks of age. For the F strain, intranasal or eyedrop administration is preferred. Administration in the drinking water may result in some birds being missed unless the procedure is carried out properly. Aerosol administration should also be done carefully, so that all birds are exposed. A respiratory reaction should be expected at approximately 5–7 days after vaccination if aerosol administration is used. Vaccinated flocks should be tested with the agglutination test approximately 3–4 weeks post-vaccination to be sure that all birds were properly exposed. It is desirable that birds be vaccinated at an age when there is no reaction to other respiratory vaccines. Strain ts-11 should be administered by eyedrop, and 6/85 is given as a fine spray. Vaccination with ts-11 results in a low but distinctive serological response by serum plate agglutination, HI, and ELISA, but vaccination with 6/85 does not ordinarily result in a serological response. No post-vaccination reaction should be observed with 6/85 or ts-11. Flocks vaccinated with F strain or ts-11 are culture positive for the life of the flock, but 6/85 may be difficult to recover later than 4–6 weeks after vaccination.

Commercial live vaccines should be used within 1–2 hours after reconstitution. Lyophilised vaccine should be stored at 4°C. Some manufacturers supply the vaccine frozen. Such vaccine should be stored in liquid nitrogen, dry ice, or at −70°C or colder. Live MG vaccine is not stable for long periods at ordinary freezer temperatures. Storage for more than a few days at −20°C should be avoided.

Strains 6/85 and ts-11 are inherently safer than F strain, although the level of protection may be somewhat less, and may be useful as the primary vaccine strain on a multi-age site or as a ‘second generation vaccine’ on sites previously using F strain vaccine. They may also be preferred in situations where inadvertent exposure of neighbouring poultry flocks is of concern. F strain displaces wild-type MG more efficiently than either ts-11 or 6/85, but ts-11 has been used to eradicate F strain MG from a multi-age commercial egg-production site (Turner & Kleven, 1998). Multi-age sites where strain 6/85 is consistently used often test MG-negative, suggesting that it has displaced the wild-type strain.

Live vaccines have also been used in some countries in broiler breeder pullets. In Australia, ts-11 live vaccine is being extensively used in broiler breeder pullets as well as in commercial layers. F strain vaccine has been used in broiler breeder pullets raised under multi-age conditions in some Latin American countries for several years; more recently there has been limited use of strains ts-11 and 6/85. There has been limited use of the 6/85 strain as a vaccine for commercial turkeys in the USA, but no good data on its effectiveness are available. Generally, vaccination of turkeys with live vaccines is not recommended and vaccination of broilers with either live or inactivated vaccines has not been successful. None of the vaccines has been validated for use in game birds.

A live vaccine for MS is available in several countries for use in broiler breeder and layer chickens. It is produced from a temperature-sensitive mutant, MS-H (Markham et al., 1998). Its characteristics and method of use are similar to those for the MG vaccine, ts-11.
Inactivated vaccines: method of use

MG bacterins are prepared from a concentrated suspension of whole cells that is emulsified into an oil adjuvant. A high antigen content is essential.

Bacterins are ordinarily used in commercial pullets to provide protection against egg-production drops that occur after MG exposure on multi-age layer sites (Hildebrand et al., 1983). They may also be used to reduce the level of egg transmission in breeder pullets. Use of bacterins in broilers is limited by the fact that birds vaccinated before 1–2 weeks of age are not protected. Although bacterins may provide protection against respiratory signs, airsacculitis, and egg-production losses, vaccinated flocks are readily infected. The duration of immunity is not known, but most flocks are exposed within 1–2 months after vaccination.

Administration is by the intramuscular or subcutaneous route, usually with a dose of 0.5 ml per bird. There is a risk that a persistent reaction at the site of vaccination will require trimming of carcasses of spent fowl vaccinated by the intramuscular route, so subcutaneous administration in the upper dorsal part of the neck is the most commonly used route. Two doses are preferred, but cost and labour considerations may dictate the use of a single dose, usually between 16 and 18 weeks of age for commercial pullets. A multidose syringe may be used. All equipment should be cleaned and sterilised between flocks, and vaccination crews should exercise proper methods of biosecurity when travelling between flocks. Vaccine should be stored at 2–8°C up to the time of use. It should not be frozen or exposed to strong light.

A similar bacterin for MS is also licensed in the USA, but it has received limited use.

1. Seed management

a) Characteristics of the seed

- Live vaccine
  The vaccine strain should be immunogenic, must readily colonise the upper respiratory tract, and cause minimal damage to the respiratory system. A strong antibody response does not necessarily correlate with immunity.

  The seed culture should be free from all extraneous agents. The culture should be cloned to ensure purity. If desired, restriction endonuclease patterns of the mycoplasmal DNA on agarose gels can be run to be sure of the identity and purity of the strain.

  The seed culture should be stable with no tendency to revert to virulence. This can be confirmed with ten back passages in susceptible chickens. Contact chickens can be introduced at weekly intervals. If necessary, tracheal swabs can be taken from infected chickens and can then be inserted into the trachea of contact chickens. Transmission of the organism should be proven. The resulting isolate can then be used to challenge susceptible chickens.

- Killed vaccine

  For killed vaccines the most important characteristics are high yield and good antigenicity. It is assumed, but not proven, that virulent strains are desirable. The seed culture should be free from all extraneous organisms.

b) Method of culture

The seed culture may be propagated in a medium similar to that described above (Section B.1) for live vaccines, the broth culture is lyophilised or frozen at –70°C or colder. For bacterins the culture must be concentrated and resuspended in a small volume of saline or PBS before the emulsion is prepared.

c) Validation as a vaccine

Data on efficacy should be obtained before bulk manufacture of vaccine begins. Chickens should be vaccinated by the same route that will be used in the field. Vaccinated birds should be challenged, and protection should be determined against respiratory signs, nasal discharge, and/or airsacculitis. Ideally, protection against egg-production losses should be evaluated, but such challenge trials are expensive and cumbersome.

Efficacy test: Groups of 20 specific pathogen free (SPF) chickens or at least mycoplasma-free chickens, 2 weeks of age or older, are vaccinated by eyedrop or other route of administration with one field dose of live vaccine, or subcutaneously or intramuscularly with one dose (usually 0.5 ml) of bacterin. A similar group of
unvaccinated chickens is maintained separately as controls. All chickens should be challenged with a 24-hour broth culture of a virulent strain of MG, 2–3 weeks post-vaccination. A simple challenge method is inoculation of 0.1 ml of the challenge culture into the posterior thoracic air sac. All birds are necropsied 7–10 days post-challenge, and air sac lesions are scored. Alternative methods are to challenge by inoculating 0.1 ml into the infraorbital sinus and examining the birds for nasal discharge from 7 to 14 days post-challenge or to challenge by aerosol and measure the thickness of the tracheal mucosa on microscopic sections at four to six equidistant predetermined points (Whithear, 1996).

2. Method of manufacture

The vaccine must be manufactured in suitable clean and secure accommodation, well separated from diagnostic facilities or commercial poultry. Special care must be taken to avoid MG contamination of other products manufactured in the same facility.

Production of vaccine should be on a seed-lot system, using a suitable MG strain of known origin, passage history, and purity. The growth medium is similar to that given above. The serum used in the growth medium should be inactivated at 56°C for 1 hour to prevent contamination with any mycoplasmal organism that may be present, and filter sterilised. A source of SPF serum is desirable.

Broth medium is inoculated, with a rapidly growing inoculum, at a rate of approximately 5% (v/v). Incubation is at 37°C. Production can be in batches using large flasks or in a fermenter. In batch cultures, harvest is approximately 24 hours after inoculation. Live vaccines are preserved by lyophilisation or by freezing at –70°C, in liquid nitrogen, or on dry ice.

For bacterin production, the antigen must be concentrated, usually by centrifugation, ultrafiltration, or other suitable method. Bacterins are made as water-in-oil emulsions, typically 80% mineral oil, 20% aqueous, with suitable emulsifying agents.

3. In-process control

**Antigen content:** At harvest, the titre should be from $10^8$ to $10^9$ CFU/ml. The antigen concentration of bacterins is difficult to standardise but may be based on packed cell volume, which is typically 1% (v/v) packed cells in the final product.

**Inactivation of killed vaccines:** Inactivation is frequently done with either beta-propiolactone or formaldehyde. The inactivating agent and the inactivation procedure must be shown under the conditions of vaccine manufacture to inactivate the vaccine organism and potential contaminants.

Prior to inactivation, care should be taken to ensure a homogeneous suspension free from particles that may not be penetrated by the inactivating agent. A test for inactivation should be carried out by culture in mycoplasma broth on each batch of both the bulk harvest after inactivation and the final product. No evidence of growth of mycoplasma should be observed.

**Sterility of killed vaccines:** Oil used in the vaccine must be sterilised by heating at 160°C for 1 hour, or by filtration, and the procedure must be shown to be effective. Tests appropriate to oil-emulsion vaccines are carried out on each batch of final vaccine as described, for example, in the British Pharmacopoeia (Veterinary) 1985.

4. Batch control

a) **Sterility**

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

b) **Safety**

- **Live vaccine safety test**

  The birds vaccinated in the efficacy test given above can be used to evaluate the safety of the vaccine.

- **Killed vaccine safety test**

  Birds vaccinated in the efficacy test described above may be observed for adverse local or systemic effects.

c) **Potency**

Potency tests for both live and killed vaccine can be conducted by the procedures given above for the efficacy test. The titre of live vaccines should be sufficient to induce infection by the route recommended;
10^5 CFU/dose is sufficient for eyedrop administration of live F strain vaccine. The recommended dose of ts-11 is \( \geq 10^7.7 \) colour changing units (CCU)/dose and for 6/85 a dose of \( 10^7\text{–}10^8 \) CFU was effective in challenge trials. For MS-H, doses of \( \geq 4.8 \times 10^5 \) were shown to be effective.

d) Duration of immunity (killed vaccine)
Because flocks are generally exposed within 1–2 months after vaccination, duration of immunity is not a primary consideration. After field challenge, resistance is considered to be permanent.

e) Stability
Evidence should be provided on three batches of vaccine to show that the vaccine passes the batch potency test at 3 months beyond the requested shelf life.

f) Preservatives
A preservative is normally required for vaccine in multidose containers. The concentration of the preservative in the final vaccine and its persistency throughout the shelf life should be checked.

A suitable preservative that has already been established for such purposes should be used. Mycoplasmas are susceptible to many antibacterials except for penicillins; such antibiotics should not be included as preservatives.

g) Precautions (hazards)
Oil-emulsion vaccines cause serious injury to the vaccinator if accidentally injected into the hand or other tissues. In the event of such an accident, the person should go at once to a hospital, taking the vaccine package with him or her. Each vaccine bottle and package should be clearly marked with a warning of the serious consequences of accidental self-injection. Such wounds should be treated by the casualty doctor as a 'grease gun injury'.

Personnel vaccinating birds with live virus vaccines by aerosol spray should wear protective clothes and masks.

5. Tests on the final product

a) Safety
See Section C.4.b.

b) Potency
See Section C.4.c.

REFERENCES


Chapter 2.3.5. — Avian mycoplasmosis (Mycoplasma gallisepticum, M. synoviae)


Chapter 2.3.5. — Avian mycoplasmosis (Mycoplasma gallisepticum, M. synoviae)


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NB: There are OIE Reference Laboratories for Avian mycoplasmosis (Mycoplasma gallisepticum and M. synoviae) (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Avian mycoplasmosis (Mycoplasma gallisepticum and M. synoviae).
CHAPTER 2.3.6.
AVIAN TUBERCULOSIS

SUMMARY

Avian tuberculosis is an important disease which affects companion, captive exotic, wild and domestic birds. The disease is most often caused by Mycobacterium avium (serotypes 1, 2 and 3) and M. genavense. The most significant cause of poultry disease is M. avium.

Clinical signs of the disease vary depending on the organs involved. The classical presentation is characterised by chronic and progressive wasting and weakness. Diarrhoea is common. Some birds may show respiratory signs and occasionally sudden death occurs. Some birds may develop granulomatous ocular lesions.

Mycobacterium tuberculosis is less commonly the cause of infection in birds, often as a result of transmission from pet bird owners, and clinical signs differ from those caused by the more commonly occurring species of mycobacteria.

Mycobacterium avium complex and M. intracellulare can also infect an extensive range of different animal species such as swine, cattle, deer, sheep, goats, horses, cats, dogs, and exotic species. Mycobacterium genavense has also been reported in a dog and an immunocompromised cat.

Disease onset in birds is normally more rapid with M. genavense than with M. avium.

In humans, M. avium and M. genavense are capable of inducing a progressive disease that is refractory to treatment, mostly in immunocompromised hosts. All manipulations involving the handling of open live cultures or of material from infected birds must be carried out with adequate biohazard containment.

Diagnosis of tuberculosis in birds depends on the demonstration of Mycobacterium spp. in the dead bird, or the detection of an immune response, cellular or humoral, in the live bird.

Identification of the agent: Where clinical signs of tuberculosis are seen in the flock, or typical lesions of tuberculosis are present in birds at necropsy, the demonstration of acid-fast bacilli in smears or sections made from affected organs is sufficient for a positive diagnosis. If acid-fast bacilli are not found, but typical signs or lesions are present in the birds, culture of the organism must be attempted. Any acid-fast organism isolated should be identified by biochemical, nucleic-acid-based tests, serological or chromatographical (e.g. high performance liquid chromatography [HPLC]) criteria.

Tuberculin test and serological tests: These tests are normally used to determine the prevalence of disease in a flock, or to detect infected birds. When used to detect the presence of tuberculosis in a flock, they should be supported by the necropsy of any birds that give positive reactions.

In domestic fowl, the tuberculin test in the wattle has been the test of choice. This test is less useful in other species of bird. A better test, especially for waterfowl, is the whole blood stained-antigen agglutination test (Rozanska). It is more reliable and has the advantage that it will give a result within a few minutes, while the bird is still being held. The tests are not reliable in caged birds.

Requirements for vaccines and diagnostic biologicals: No vaccines are available for use in birds. An antigen preparation stained with 1% malachite green is available for the whole blood agglutination test. Avian tuberculin purified protein derivative is the standard preparation for use in the tuberculin test of domestic poultry.
Chapter 2.3.6. – Avian tuberculosis

A. INTRODUCTION

Several mycobacterial species can be involved in the aetiology of avian tuberculosis. The disease is most commonly produced by infection with Mycobacterium avium complex (serotypes 1, 2 and 3) and M. genavense (Tell et al., 2001). Other species, such as M. intracellulare, M. scrofulaceum, M. fortuitum, M. tuberculosis and M. bovis are less common causes of avian tuberculosis (Tell et al., 2001). Mycobacterium avium complex and M. intracellulare are capable of infecting an extensive range of different animal species such as swine, cattle, deer, sheep, goats, horses, cats, dogs, and exotic species (Thorel et al., 1997; 2001).

Mycobacterium avium complex consists of three subspecies: M. avium subsp. avium, M. avium subsp. sylvaticum, and M. avium subsp. paratuberculosis (Thorel et al., 1990). The latter is the causal agent of Johne’s disease, or paratuberculosis, in ruminants and other mammalian species (see Chapter 2.1.11 Paratuberculosis [Johne’s disease]). Although successful experimental infections with M. a. paratuberculosis in poultry have been reported (Larsen & Moon, 1972), there is no evidence that this organism is involved in the aetiology avian tuberculosis.

Most M. a. avium isolates from birds have a repetitive sequence IS901 in their genome and produce a characteristic three-band pattern in IS1245 restriction fragment length polymorphism (RFLP) (Ritacco et al., 1998). There is convincing evidence that the presence of IS901 correlates with pathogenicity in birds (Dvorska et al., 2003; Pavlik et al., 2000). This repetitive sequence is also present in M. a. sylvaticum that is capable to produce tuberculosis in birds. IS901 has only been detected in M. avium strains with serotypes 1, 2 and 3 (Pavlik et al., 2000; Ritacco et al., 1998) that are apparently more pathogenic to birds than other serotypes (Tell et al., 2001). On the basis of genetic and phenotypic differences it has recently been proposed to divide M. a. avium into two subspecies: M. a. hominissuis for human and porcine isolates and M. a. avium for bird-type isolates (Mijs et al., 2000). The isolates designated as M. a. hominissuis had the conserved three-band pattern in IS1245 RFLP and were unable to grow at 24 and 45°C (Mijs et al., 2002). It is worth noting that the typical features of bird-isolates, the three-band pattern in IS1245 RFLP and presence of IS901, have also been found in cervine and bovine isolates of M. a. avium (O’Grady et al., 2000).

Tuberculosis in birds is most prevalent in chickens and in wild birds raised in captivity. Turkeys are quite susceptible, but duck and geese are comparatively resistant. The practices of allowing poultry to roam at large on the farm (free range) and of keeping the breeders for several years are conducive to the spread of tuberculosis among them. Infected individuals and contaminated environment (water and soil) are the main source of infection (Tell et al., 2001). Mycobacteria can survive for several months in the environment (Tell et al., 2001).

In most cases, infected birds show no clinical signs, but they may eventually become lethargic and emaciated. Many affected birds show diarrhea and comb and wattles may regress and become pale. Affected birds are usually older than one year. Some show respiratory signs and sudden death may occur, dyspnoea is less common, and granulomatous ocular lesions (Pocknell et al., 1996) and skin lesions have been reported. Under intensive husbandry conditions, sudden death may occur, often associated with severe lesions in the liver; such lesions are easily observed at post-mortem examination (Tell et al., 2001).

The primary lesions of tuberculosis in birds are nearly always in the intestinal tract. Such lesions take the form of deep ulcers filled with caseous material containing many organisms, and these are discharged into the lumen and appear in the faeces. Before the intestinal tract is opened, the ulcerated areas appear as tumour-like masses attached to the gut wall, but when the intestine is opened, the true nature of the mass becomes evident. Typical caseous lesions are nearly always found in the liver and spleen, and these organs usually are greatly enlarged because of the formation of new tuberculous tissue. The lungs and the other tissues are ordinarily free from lesions even in advanced cases.

In most species of affected bird, tuberculous-like lesions are mostly found in the intestinal tract, liver and spleen. Lesions in other organs are less common. Exceptions include pigeons, waterfowl, and some finches, in which the disease begins primarily in the respiratory tract.

It is essential to bear in mind that M. avium, M. intracellulare and M. genavense are capable of giving rise to a progressive disease in humans that is refractory to treatment, especially in immunocompromised individuals (Tell et al., 2001). All manipulations involving the handling of open live cultures or of material from infected birds must be performed with adequate biohazard containment (see Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

If there is a characteristic history of tuberculosis in the flock and typical lesions are found in birds at post-mortem, the detection of acid-fast bacilli in smears or sections from affected organs, stained by the Ziehl–Neelsen method,
is normally sufficient to establish the diagnosis. Occasionally a case will occur, presumably as a result of large infecting doses giving rise to acute overwhelming disease, in which affected organs, most obviously the liver, have a ‘morocco leather’ appearance with fine greyish or yellowish mottling. In such cases acid-fast organisms may not be found, but careful inspection will reveal parallel bundles of brownish refractile bacilli. Prolongation of the hot carbol fuchsin stage of Ziehl–Neelsen staining to 10 minutes will usually reveal that these are indeed acid-fast bacilli, with unusually high resistance to penetration of the stain. Recently, DNA probes and polymerase chain reaction (PCR) techniques have been used to identify the agent. Traditionally, *M. avium* is separated from common nonchromogenic slow-growing organisms by their ability to grow at 42°C (*M. a. avium*) and biochemical tests such as the Tween hydrolysis test, pyrazinamidase, growth on thiopehn-2-carboxylic acid hydrazide (TCH)-containing media and tellurite reduction. *Mycobacterium genavense* is particularly fastidious and has special requirements for growth and identification.

### Culture

If there is a characteristic flock history and suggestive lesions are found at necropsy, but no acid-fast bacilli are seen in smears or sections, an attempt must be made to isolate the causative organism from the necropsy material. Liver or spleen are the best organ to use, but if the carcass is decomposed, bone marrow may prove more satisfactory as it could be less contaminated. As with culture of *M. bovis*, non-sterile specimens need to be processed with detergent, alkali or acid to eliminate rapidly growing microorganisms before culture (see Chapter 2.4.7 Bovine tuberculosis). *Myocobacterium avium* grows best on media such as Lowenstein–Jensen, Herrold’s medium, Middlebrook 7H10 and 7H11 or Coletsos, with 1% sodium pyruvate added. It may occasionally be necessary to incorporate mycobactin, as used for the isolation of *M. paratuberculosis* and *M. silvaticum*. Growth may be confined to the edge of the water of condensation. Cultures should be incubated for at least 8 weeks. Typically *M. avium* produces ‘smooth’ colonies, within 2–4 weeks; rough variants do occur. Shorter incubation times can be achieved using the liquid culture BACTEC system.

For *M. genavense*, use of the BACTEC system with no additives but with pH 6.0 and a lowered oxygen tension is recommended (Realini *et al.*, 1997; 1998). The optimal solid medium is Middlebrook 7H11 medium acidified to pH 6 and supplemented with blood and charcoal (Realini *et al.*, 1999).

Typing of mycobacteria to the species and subspecies level requires a specialised laboratory. Conventional biochemical tests for species identification are lengthy and fail to distinguish between *M. avium* and *M. intracellulare*. Thus, a miscellaneous group of mycobacteria that includes both species is usually classified under the denomination of *M. avium* complex (MAC). Seroagglutination, which is based on sugar residue specificity of surface glycopeptidolipids, allows classification of MAC organisms into 28 serovars. More sophisticated typing methods directed at cell-wall-specific targets are currently available, such as enzyme-linked immunosorbent assays with monoclonal antibodies to major serovars, and high performance liquid chromatography (HPLC). Serovars 1 to 6, 8 to 11 and 21 are currently ascribed to *M. avium*, and serovars 7, 12 to 20 and 25 to *M. intracellulare*. However, no consensus was achieved on other serovars, and some isolates cannot be typed (Inderlied *et al.*, 1993). Tuberculosis in birds is usually caused by *M. avium* types 1, 2, or 3. If one of these is found, it may be assumed to be the cause of the disease. If the isolate is not one of these, further identification tests must be carried out. However, it should be borne in mind that superficial tuberculosis lesions in caged birds, especially psittacines, may be caused by *M. tuberculosis*. Hence, if rough colonies of mycobacteria are isolated from such birds, they should be tested for growth at 42°C. If the isolate will not grow at 42°C, *M. tuberculosis* should be suspected.

### Nucleic acid recognition methods

Specific and reliable genetic tests for speculation are currently available (Saito *et al.*, 1990). Commercial nucleic acid hybridisation probes have become a ‘gold standard’ for distinction between *M. avium* and *M. intracellulare* cultures. *M. genavense* can also be distinguished with these tests. A further probe that covers the whole MAC was also developed, as genuine MAC strains have been described that fail to react with specific *M. avium* and *M. intracellulare* probes (Soini *et al.*, 1996). These tests use a chemiluminescent-labelled, single-stranded DNA probe that is complementary to the ribosomal RNA of the target organism. The labelled DNA–RNA hybrids are measured in a luminometer. Various in-house molecular methods have been reported for the identification of mycobacterial cultures, including MAC. A multiplex PCR method for differentiating *M. avium* from *M. intracellulare* and *M. tuberculosis* complex has some advantages (Cousins *et al.*, 1996). 16S RNA sequencing (Kirschner *et al.*, 1993) or PCR amplification followed by either hybridisation with species-specific probes or restriction enzyme analysis (Devallois *et al.*, 1996; Telenti *et al.*, 1993; Trueba *et al.*, 2004) may also be used. Even though some of these methods would theoretically detect the agent directly in tissue samples, none of them has been validated for this use. Therefore, molecular identification of MAC is currently performed on organisms previously isolated by culture. Sequencing of hsp65 to distinguish between subsets of *M. avium* has also been found useful (Turenne *et al.*, 2006).
Regarding intraspecies genotyping, pulsed-field gel electrophoresis of large DNA restriction fragments has proved to be highly sensitive (Mazurek et al., 1993). Also, a number of DNA mobile elements have been identified that may be exploited for this purpose. Insertion sequence IS1245, which is virtually \textit{M. avium} specific, was shown to be the most discriminative for the analysis of strain relatedness (Bono et al., 1995; Guerrero et al., 1995). A standardised method consisting of IS1245 restriction fragment length polymorphism (RFLP) analysis was recently proposed (Van Soolingen et al., 1998). Bird infection was found to be caused by a particular subset of \textit{M. avium} strains that are characterised by specific, highly conserved RFLP patterns with IS1245 and IS901, in addition to serovars 1, 2 or 3 (Ritacco et al., 1998).

Recently O’Grady et al. performed RFLP investigation using probes derived from IS901, IS1245 and IS1311 to study the molecular epidemiology of \textit{M. avium} and \textit{M. intracellulare} infection, in particular to gain an understanding of the sources of infection in humans (O’Grady et al., 2000).

If specialised typing facilities are not available, the likelihood that the organism isolated is the cause of the disease may be established by pathogenicity tests. It is preferred that these be carried out on the species of bird being investigated, but failing that, domestic fowl or Japanese quail may be used. Young adult birds are best. An disease may be established by pathogenicity tests. It is preferred that these be carried out on the species of bird

or, by that time, the bird will have extensive lesions filled with acid-fast bacilli.

Finally, sufficient sterile normal saline solution is added to suspend the culture at 0.1 mg/ml. Birds are reweighed. Finally, sufficient sterile normal saline solution is added to suspend the culture at 0.1 mg/ml. Birds are then inoculated intravenously with 1 ml of the suspension. If the organism is virulent, the bird will die in 5–6 weeks or, by that time, the bird will have extensive lesions filled with acid-fast bacilli.

2. Immunological methods

Tests used for export depend on the importing requirement of individual countries. In the main, the tuberculin test or the haemagglutination (stained antigen) test are most frequently used for export testing of poultry.

a) Tuberculin test

The most widely used test in domestic fowl, and the only test for which an international standard for the reagent exists, is the tuberculin test. The tuberculin is the standard avian purified protein derivative (PPD). Birds are tested by intradermal inoculation in the wattle with 0.05 ml or 0.1 ml of tuberculin (containing approximately 2000 International Units [IU]), using a very fine needle of approximately 10 mm × 0.5 mm. The test is read after 48 hours and a positive reaction is any swelling at the site, from a small firm nodule approximately 5 mm in diameter to gross oedema extending into the other wattle and down the neck. With practice, even very small wattles on immature birds can be inoculated successfully. However, in immature birds the comb may be used, although results are not so reliable. Tuberculin testing of the wattle in turkeys is much less reliable than in the domestic fowl. Inoculation in the wing web has been recommended as being more efficient, but this is still not as good as for domestic fowl. Other birds may also be tested in the wing web, but results are not generally satisfactory. The bare ornamental skin areas on Muscovy ducks and some species of pheasant can be used, but reliability is doubtful and interpretation difficult. Testing in the foot web of waterfowl has also been described; the test is not very sensitive and is often complicated by infections of the inoculation site.

In pheasants, the tuberculin test can be performed in either of two ways. In the first, 0.05 ml or 0.1 ml of tuberculin is injected into the skin of the lower eyelid. A positive result is indicated by marked swelling at the site of injection after 48 hours. Alternatively, 0.25 ml of tuberculin is injected into the thoracic muscles and the birds are observed for 6–10 hours. Infected birds will show signs of depression and keep aside from the flock, and there may be cases of sudden death. No clinical signs will be provoked in uninfected birds.

b) Stained antigen test

- Preparation of the antigen

An antigen stained with 1% malachite green is used for the rapid whole blood plate agglutination test (Rozanska, 1965). The strain used for preparation of the stained antigen must be smooth and not autoagglutinate in saline suspension. It must conform to the characteristics of the \textit{M. avium} species.

A strain that will detect infection with any serotype is recommended to be used instead of the specific serotype that is most likely to be encountered (in Europe serotype 2 for domestic fowl, serotype 1 for waterfowl). It may be preferable to use a strain that is highly specific for the serotype it detects. The specificity of strains can be determined only by testing them as antigens, although in general a serotype 2 antigen will always detect serotype 3 infection and vice versa. Serotype 1 strains appear to detect more often a wide spectrum of infection, and will often also detect infections with mycobactin-dependent mycobacteria or \textit{M. silvaticum}. There is no reason not to use a culture containing more than one strain of
**Chapter 2.3.6. – Avian tuberculosis**

_M. avium_, provided that it shows the desired properties of sensitivity and specificity. Consistency of results between batches will be easier with the use of pure cultures.

The organism should be grown in a suitable liquid medium, such as Middlebrook 7H9 containing 1% sodium pyruvate for better growth. Good growth should be obtained in approximately 7 days. The liquid culture is used as seed for bulk antigen preparation.

Antigen for agglutination tests is best grown on solid medium, such as Löwenstein–Jensen or 7H11, containing 1% sodium pyruvate instead of glycerol, using Roux flasks or large bottles. The use of solid medium maximises the chance of detecting any contamination, and antigens grown in some liquid media are not agglutinated by specific antibody. Liquid seed culture should be diluted (on the basis of experience) to give discrete colonies on the solid medium. This will usually give the best yield, and again increases the chance of detecting contamination. About 10 ml of inoculum will usually be enough to allow it to wash over the whole surface, and provide sufficient moisture to keep the air in the bottle near 100% humidity.

The bottles are incubated at 37°C, and good growth should be obtained in 14–21 days with most strains. The antigen is harvested by the addition of sterile glass beads and twice the volume of sterile normal saline (containing 0.3% formalin) as was used to inoculate the bottle. The bottle is then shaken gently to wash off all the growth and the washing is collected into a sterile bottle and reincubated at 37°C for 7 days. The killed bacilli are then washed twice in sterile normal saline with 0.2% formalin by centrifugation and resuspension. This sequence is safer than the original method in which the washing was carried out before the incubation that kills the organisms. Finally the organisms are again centrifuged and resuspended in sterile normal saline containing 0.2% formalin and 0.4% sodium citrate, to a concentration of about 10^{10} bacteria per ml. This corresponds to a concentration ten times that which matches tube No. 4 on McFarland’s scale.

Cultures for antigen should be inspected for contamination daily for the first 5 days of incubation. The suspension made from the culture washings is also re-examined microscopically (for likely contaminants such as yeasts), and rechecked by culture to ensure that the formalin has killed the mycobacteria.

- **Validation of the antigen**

Cultures should be checked by Gram staining for the presence of organisms other than mycobacteria.

One or more batches for agglutinating antigen must be tested for efficacy in naturally or artificially infected tuberculous birds by comparison with a standard preparation of known potency. The potency relative to that of the standard preparation must not differ significantly from that declared on the label. Each bottle of antigen must be tested with normal chicken serum (to detect autoagglutination) and _M. avium_ positive chicken serum of low and high antibody content. This should be done, where possible, alongside a previous batch of stained antigen. Those bottles that give satisfactory agglutination reactions with the antisera can now be pooled and the antigen stained. This is done by the addition of 3 ml of 1% malachite green solution per 100 ml of suspension. If possible, the stained antigen should now be checked using whole blood just as the unstained antigen was tested with serum. The agglutinating antigen should keep for at least 6 months in the refrigerator at 4°C, and much longer if frozen at –20°C or below. If a batch has not been used for a long time it should be rechecked, especially for autoagglutination.

The only safety test needed is the culture test of the unwashed antigen after 7 days of incubation, to ensure that all the bacilli are dead.

- **Test procedure**

The stained-antigen agglutination test has been used with good results, especially in both domestic and ornamental waterfowl. A drop (0.05–0.1 ml) of the antigen is mixed with the same volume of fresh whole blood, obtained by venipuncture, on a white porcelain or enamel tile. The mixture is rocked for 2 minutes and examined for agglutination. The agglutination may be coarse, in which case it is obvious, or quite fine, in which case it may be most clearly seen as an accumulation of the malachite-green-stained antigen around the edge of the drop, leaving the centre a normal blood-red colour. This test is especially useful for screening large flocks for immediate culling, and therefore has advantages over the tuberculin test for the control of the disease, even in domestic fowl. It has also been claimed that in domestic fowl it is more reliable than the tuberculin test.

**Note on limitation of use**

Neither the tuberculin test with avian tuberculin nor the stained-antigen agglutination test is likely to be of any value in cases of _M. tuberculosis_ infection in caged birds.
C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No vaccines are available.

Avian tuberculin is a preparation made from the heat-treated products of growth of *M. avium*. It is used by intradermal injection to reveal delayed hypersensitivity as a means of identifying birds infected with or sensitised to the same species of tubercle bacillus.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

Strains of *M. avium* used to prepare seed cultures should be identified as to species by appropriate tests. They should be shown to be free from contaminating organisms and to be capable of yielding a product of satisfactory quality. The strains recommended by the European Union (EU), for example, are D4ER and TB56. Reference may also be made to the World Health Organization (1987).

b) Method of culture

The seed material is kept as a stock of freeze-dried cultures. If the cultures have been grown on solid media, it will be necessary to adapt the organism to grow as a floating culture. This is most easily accomplished by incorporating a piece of potato in the flasks of liquid medium (e.g. Watson Reid’s medium). When the culture has been adapted to liquid medium, it can be maintained by passage at 2–4-week intervals (Angus, 1978; Haagsma & Angus, 1995).

The production culture substrate must be shown to be capable of producing a product that conforms to the standards of the European Pharmacopoeia (2000) or other international standards. It must be free from ingredients known to cause toxic or allergic reactions.

c) Validation

The strains of *M. avium* used as seed cultures must be shown to be free from contaminating organisms.

Seed lots must be shown to be efficacious in producing tuberculin with sufficient potency. The necessary tests are described in Section C.4 below.

2. Method of manufacture

Avian tuberculin may be made by the following three methods:

a) Old tuberculin

The organism is cultivated in glycerol broth medium, killed by heating in flowing steam, and filtered to remove cells. The filtrate is concentrated by heat and sterilised by filtration.

b) Heat-concentrated synthetic-medium tuberculin

As for old tuberculin but the glycerol broth medium is replaced by a synthetic medium (modified Dorset-Henley’s synthetic medium).

c) Purified protein derivative

As for heat-concentrated synthetic-medium (HCSM) tuberculin but, instead of being concentrated by heat, the protein in the filtrate is precipitated chemically (ammonium sulphate or trichloroacetic acid [TCA] are used), washed and resuspended. PPD tuberculin is recommended as it gives fewer false-positive reactions and can be standardised more precisely. An antimicrobial preservative that does not give rise to false-positive reactions, such as phenol (not more than 0.5% [w/v]), may be added. Mercurial derivatives should not be used. Glycerol (not more than 10% [w/v]) or glucose (2.2% [w/v]) may be added as a stabiliser. The product is dispensed aseptically into sterile neutral glass containers, which are then sealed to prevent contamination. The product may be freeze-dried.
3. **In-process control**

The production flasks, inoculated from suitable seed cultures, are incubated for the appropriate time period. Any flasks showing contamination or grossly abnormal growth should be discarded after autoclaving. As incubation proceeds, the surface growth of many cultures becomes moist and may sink into the medium or to the bottom of the flask. In PPD tuberculins, the pH of the dissolved precipitate (the so-called concentrated tuberculin) should be pH 6.6–6.7. The protein level of the PPD concentrate is determined by the Kjeldahl method. Total nitrogen and trichloroacetic acid precipitable nitrogen are usually compared.

4. **Batch control**

a) **Sterility**

Sterility testing is generally performed according to the European Pharmacopoeia or other guidelines (see also Chapter 1.1.7 Tests for sterility and freedom from contamination of biological materials).

b) **Safety**

Tuberculin PPD can be examined for freedom from living mycobacteria using the culture method described previously. This culture method, which does not require use of animals, is used in many laboratories and its use is encouraged over the use of animals for this purpose. The following is the previously described method, using experimental animals, to evaluate safety of PPD. Two guinea-pigs, each weighing not less than 250 g and that have not been treated previously with any material that will interfere with the test, are injected subcutaneously with 0.5 ml of the tuberculin under test. No abnormal effects should occur within 7 days.

c) **Residual infectivity**

Tests on tuberculin for living mycobacteria may be performed either on the tuberculin immediately before it is dispensed into final containers or on samples taken from the final containers themselves. A sample of at least 10 ml must be taken and this must be injected intraperitoneally or subcutaneously into at least two guinea-pigs, dividing the volume to be tested equally between the guinea-pigs. It is desirable to take a larger sample, 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea-pigs are observed for at least 42 days, and are examined macroscopically at post-mortem. Any lesions found are examined microscopically and by culture.

Each filled container must be inspected before it is labelled, and any showing abnormalities must be discarded.

d) **Sensitising effect**

To test the sensitising effect, three guinea-pigs that have not previously been treated with any material that could interfere with the test are each injected intradermally on each of three occasions with the equivalent of 500 IU of the preparation under test in a 0.1 ml volume. Each guinea-pig, together with each of three control guinea-pigs that have not been injected previously, is injected intradermally 15–21 days after the third injection with the same dose of the same tuberculin. The reactions of the two groups of guinea-pigs should not be significantly different when measured 24–28 hours later.

e) **Potency**

The potency of avian tuberculin is determined in guinea-pigs sensitised with M. avium, by comparison with a standard preparation calibrated in IU.

Use no fewer than nine albino guinea-pigs, each weighing 400–600 g. Sensitise the guinea-pigs by administering to each, by deep intramuscular injection, a suitable dose of inactivated or live M. avium. The test is performed between 4 and 6 weeks later as follows: Shave the guinea-pigs’ flanks so as to provide space for three-to-four injections on each side. Prepare at least three dilutions of the tuberculin under test and at least three dilutions of the standard preparation in isotonic buffer solution containing 0.0005% (w/v) polysorbate 80 (Tween 80). Choose the dilutions so that the reactions produced have diameters of not less than 8 mm and not more than 25 mm. Allocate the dilutions to the injection sites randomly according to a Latin square design. The dilutions are injected intradermally in volumes of 0.1 or 0.2 ml.

Between 24 and 28 hours, the diameters of the reactions are measured and the results are calculated using standard statistical methods, taking the diameters to be directly proportional to the logarithms of the concentrations of the tuberculins. The estimated potency must be not less than 75% and not more than 133% of the potency stated on the label. The test is not valid unless the fiducial limits of error (\( \rho = 0.95 \)) are
not less than 50% and not more than 200% of the estimated potency. If the batch fails a potency test, the test may be repeated one or more times provided that the final estimate of potency and of fiducial limits is based on the combined results of all the tests.

It is recommended that avian tuberculin should contain the equivalent of at least 25,000 IU/ml, giving a dose for practical use of 2500 IU/0.1 ml.

f) Specificity

One or more batches of tuberculin may be tested for specificity together with a standard preparation of bovine tuberculin by comparing the reactions produced in guinea-pigs sensitised with *M. bovis* using a procedure similar to that described in Section C.4.d. In guinea-pigs sensitised with *M. bovis*, the potency of the preparation of avian tuberculin must be shown to be not more than 10% of the potency of the standard preparation of bovine tuberculin used in the potency test.

g) Stability

During storage, liquid avian tuberculin should be protected from the light and held at a temperature of 5°C (±3°C). Freeze-dried preparations may be stored at higher temperatures (but not exceeding 25°C) protected from the light. During use, periods of exposure to higher temperatures or to direct sunlight should be kept at a minimum.

Provided the tuberculins are stored at a temperature of between 2°C and 8°C and protected from light, they may be used up to the end of the following periods subsequent to the last satisfactory potency test: Liquid PPD tuberculins: 2 years; lyophilised PPD tuberculins: 8 years; HCSM tuberculins diluted: 2 years.

h) Preservatives

Antimicrobial preservatives or other substances that may be added to a tuberculin, must have been shown not to impair the safety and effectiveness of the product. The maximum permitted concentrations for phenol is 0.5% (w/v) and for glycerol it is 10% (v/v). The pH should be between 6.5 and 7.5.

i) Precautions (hazards)

Experience both in humans and animals led to the observation that appropriately diluted tuberculin injected intradermally results in a localised reaction at the injection site without generalised manifestations. Even in very sensitive persons, severe, generalised reactions are extremely rare and limited.

5. Tests on the final product

a) Safety

A test for the absence of toxic or irritant properties must be carried out according to the specifications of the European Pharmacopoeia (see also Section C.4.b).

b) Potency

The potency of tuberculins must be estimated by biological methods. These methods must be used for HCSM and PPD tuberculins; they are based on the comparison of the tuberculins to be tested with standard tuberculins (see also Section C.4.d).

REFERENCES


Chapter 2.3.6. – Avian tuberculosis


* *

NB: There is an OIE Reference Laboratory for Avian tuberculosis
(see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and diagnostic biologicals for avian tuberculosis
CHAPTER 2.3.7.

DUCK VIRUS ENTERITIS

SUMMARY

Duck virus enteritis (DVE) or duck plague is an acute contagious infection of ducks, geese and swans (order Anseriformes) caused by an alpha-herpesvirus. Diagnosis is based on a combination of assessing the clinical signs, gross pathology and histopathology supported by identification of the virus by either isolation or polymerase chain reaction.

Identification of the agent: The virus may be isolated from the liver, spleen and kidneys of birds dying from this infection. Virus may be recovered by infecting susceptible ducklings, in which the disease can be reproduced; by inoculating embryonated Muscovy duck eggs on the chorioallantoic membrane; or by inoculating primary cell cultures of duck embryo or Muscovy duck embryo origin. The identity of the virus can be confirmed by neutralisation tests using specific antiserum to inhibit pathological changes in the duck embryos or the cytopathological effects in the cell cultures, or by direct or indirect immunofluorescence tests on infected cell cultures. Alternatively the viral DNA may be detected by the polymerase chain reaction from the oesophagus, liver and spleen of DVE virus infected birds as well as from Muscovy duck embryos or cells used for virus isolation.

Serological tests: Immunological tests have little value in the diagnosis of acute infection. Serum neutralisation tests in ovo and in vitro have been used to monitor exposure to the virus in wildfowl.

Requirements for biological products: A live attenuated virus vaccine is available to control DVE in birds over 2 weeks of age. Ducks are vaccinated subcutaneously or intramuscularly for active immunity. Vaccine virus is not thought to spread from vaccinated to unvaccinated stock. An inactivated vaccine has been reported to be efficacious in laboratory tests, but has not been developed or licensed for large-scale use.

A. INTRODUCTION

Duck virus enteritis (DVE) is an acute, sometimes chronic, contagious virus infection that occurs naturally only in ducks, geese and swans, all members of the family Anatidae of the order Anseriformes. The disease is a potential threat to commercially reared, domestic and wild waterfowl. The aetiological agent, anatid herpesvirus-1 or DVE virus (DEV), is a member of the Alphaherpesvirinae subfamily of the Herpesviridae. DVE may also be referred to as duck plague, anatid herpes, eendenpest, entenpest and peste du canard. The infection has not been reported in other avian species, mammals or humans.

In domestic ducks and ducklings, DVE has been reported in birds ranging from 7 days of age to mature breeders. In susceptible flocks the first signs are often sudden, high and persistent mortality with a significant drop in egg production in laying flocks. In domestic ducks the incubation period ranges from 3–7 days. Mortality usually occurs 1-5 days after the onset of clinical signs and is often more severe in susceptible adult breeder ducks. In chronically infected partially immune flocks only occasional deaths occur. Recovered birds may be latently infected carriers and may shed the virus in the faeces or on the surface of eggs over a period of years (Richter & Horzinek, 1993; Shawky & Schat, 2002). DVE limited solely to Muscovy ducks has been observed in the USA (Campagnolo et al., 2001; Davison et al., 1993).

Clinical signs and gross pathology associated with a DVE outbreak vary with the species, immune status, age and sex of the affected birds, and with the virulence of the virus. Similarly, as infection progresses within a flock, more clinical signs are typically observed. In breeder ducks the range of signs include ‘sudden deaths’, photophobia associated with partially closed, pasted eye-lids, polydipsia, loss of appetite, ataxia, and nasal discharge. Birds often have ruffled feathers, watery diarrhoea and soiled vents. Sick birds may maintain an upright stance by using their wings for support, but their overall appearance is one of weakness and depression. In ducklings 2–7 weeks of age, losses may be lower than in older birds and the signs associated with DVE include dehydration, loss of weight, conjunctivitis and serous ocular discharge, a blue colouration of the beaks and blood-stained vents.
At necropsy, adult ducks that have died are typically in good body condition. In mature males, prolapse of the penis may occur. In mature females, haemorrhages may be observed in ovarian follicles. The gross lesions are characterised by vascular damage, with tissue haemorrhages, free blood in the body cavities and intestinal lumen and a range of lesions affecting the digestive tract mucosa. These latter lesions progress with the course of the disease and include initial mucosal haemorrhages and eruptions and intense annular congestion, leading to pseudo-membranous or diphtheritic mucosal lesions. Necrotic degenerative changes are evident in the lymphoid and parenchymatous organs. In the liver this manifests as irregularly distributed pinpoint haemorrhages and white foci giving a speckled appearance. In ducklings lesions of the lymphoid tissues tend to be more prominent than visceral haemorrhages. Collectively, these lesions are pathognomonic for DVE. The pathology and histopathology of DVE in white Pekin ducks has been reviewed (Sandhu & Metwally, 2008). Microscopic lesions are characterised by vascular damage and its consequences in visceral organs. Eosinophilic intranuclear inclusions and cytoplasmic inclusions in epithelial cells of the digestive tract are typically present. Birds that recover from natural infection are suggested to be immune to re-infection, but latency (in the trigeminal ganglion) and reactivation of virus is recognised.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Primary isolation of the virus is best achieved from samples of liver, spleen or kidney tissue, which have been homogenised in buffered saline containing antibiotics and clarified by low-speed centrifugation (1800 g). Isolation may be attempted by inoculating such homogenates into cell cultures, ducklings or duck embryos.

a) Cell cultures

Cell culture is reported as the method of choice for isolation of DVE virus, but may not always be successful. If attempted, isolations may be made in primary duck embryo fibroblasts (DEF) (Wolf et al., 1976) or, preferably primary Muscovy duck embryo fibroblasts (MDEF) (Gough & Alexander, 1990; Kocan, 1976). Muscovy duck embryo liver (MDEL) cells are thought to be even more sensitive (R.E. Gough, pers. comm.). Cell monolayers grown in Eagle’s minimal essential medium (MEM) containing 10% fetal calf serum (FCS), 2 mM glutamine, and 0.17% sodium bicarbonate and gentamicin are washed with serum-free MEM and then inoculated with the clarified sample homogenate suspected to contain DVE virus. After incubation for 1 hour at 37°C to allow for virus adsorption, the cultures are maintained on MEM containing 2% FCS, 2 mM glutamine, and 0.17% sodium bicarbonate and gentamicin, and incubated in an atmosphere containing 5% CO₂. The cytopathic effect (CPE) is characterised by the appearance of rounded clumped cells that enlarge and become necrotic 2–4 days later. Cultures should be stained with a fluorescent antibody conjugate using a direct or indirect method to identify the virus (see Section B.1.d). Cells can also be fixed and then stained with haematoxylin and eosin to show syncytial formation, intranuclear inclusions and marked cytoplasmic granulation. It has been reported (Burgess & Yull, 1981) that the isolation of DEV in MDEF cells is favoured by incubation at temperatures between 39.5°C and 41.5°C. However, an elevated temperature does not appear to be essential for isolation, which is often carried out at 37°C. More than one passage in cell culture may be necessary to isolate the virus. This virus isolation method in cell cultures may be modified to a plaque assay by overlaying the cell monolayer with maintenance medium containing 1% agarose. As the virus can be cell associated, sequential passaging should be carried out by trypsinising potentially infected cells and replanting them, as well as inoculating fresh cells with infected culture supernatant from the previous passage.

b) Ducklings

Inoculation of live ducklings should be used only when no other methods are available for meaningful diagnosis. The requirements of the OIE Terrestrial Animal Health Code (Chapter 7.8 Use of animals in research and education) must be followed. When inoculated intramuscularly, 1-day-old susceptible ducklings die within 3–12 days; uninoculated ducklings, housed separately, should be maintained as controls at the same time. Muscovy ducklings (Cairina moschata) are more susceptible than white Pekin ducklings. Both macroscopic and microscopic lesions typical of DVE should be seen on post-mortem examination. The diagnosis may be confirmed either by vaccinating ducklings against DVE and challenging them subsequently with the virus isolate or by immunofluorescence. However, virulent strains of the virus exist, against which the vaccine may be ineffective (Kisary & Zsak, 1983). In the author’s experience of natural infections occurring in Muscovy ducks, this method of virus isolation has proved more sensitive than cell culture methods.

c) Duck embryos

Primary virus isolations can be made by inoculation on to the chorioallantoic membrane (CAM) of 9–14 day embryonated Muscovy duck eggs. Before harvest, inoculated embryonated eggs should be chilled at 4°C for 4 hours or overnight to kill the embryos before further manipulations. The embryos may die, showing
characteristic extensive haemorrhages 4–10 days after inoculation. Two to four serial blind passages of the homogenised CAMs may be necessary before isolation can be effected. This method is not as sensitive as that using susceptible day-old ducklings.

Embryonated chicken eggs are not very susceptible to infection with field strains of DEV. The virus can nevertheless be adapted to chicken embryos by serial passages. Pekin duck embryos vary in their susceptibility to strains of DVE virus.

d) Immunological methods

Serological methods used to confirm the identity of newly isolated virus include neutralisation assays performed in either embryonated eggs or cell cultures. A plaque assay for DEV in duck embryo cell cultures has been described (Dardiri & Hess, 1968). In the author's laboratory a microtitre assay using primary MDEF or MDEL cells is used. Provided a hyperimmune antiseraum of sufficiently high titre is used, a fluorescent antibody test (direct or indirect) for DEV in DEF, MDEF or MDEL cells is the next most sensitive assay after isolation in 1–9-day old ducklings (Erickson et al., 1974). A reverse passive haemagglutination test for DEV has been described (Deng et al., 1984), but it is reported to be less sensitive than immunofluorescence and plaque assays. An avidin–biotin–peroxidase method of immunoperoxidase staining to detect DEV antigen in formalin-fixed, paraffin-embedded sections of liver and spleen from experimentally infected birds has been described (Islam et al., 1983). The identity of the virus may also be confirmed by negative stain electron microscopy, but this alone is not positive confirmation that the herpesvirus observed is DEV virus. Immuno-electron microscopy has been developed recently using DVE hyperimmune serum (Pearson & Cassidy, 1997).

e) Nucleic acid recognition methods

Detection of DVE virus by polymerase chain reaction (PCR), including quantitative real-time PCR, has been reported (Hansen et al., 1999; 2000; Plummer et al., 1998; Pritchard et al., 1999; Qi et al., 2009; Wu et al., 2011; Yang et al., 2006). Primers have been identified that are able to amplify DNA from DVE virus present in various tissues, including oesophagus, liver and spleen, from an original outbreak and after passage from Muscovy duck embryos. The following is an example protocol for PCR methods for detection of DVE virus; other protocols exist. Recently, a LAMP-based nucleic acid amplification method for the detection of DVE DNA has been published (Ji et al., 2009).

- PCR method

This DNA extraction procedure can be used on disrupted cell suspensions from DEV -infected tissue culture, 10% ground tissue suspensions, or cloacal swab material in transport medium. This method is used to prepare duck plague DNA for the known positive PCR controls.

- Extraction of viral DNA

Note: All product transfers in steps i to v are performed in a biological safety cabinet.

i) For a 10% ground tissue suspension, add 400 µl to a 1.5 ml microfuge tube and microfuge at 16,000 g for 5 minutes. Transfer the supernatant to a new tube and go to step ii.

ii) For tissue culture suspensions and cloacal swab material, add 400 µl of the sample, or supernatant from step i above, to a 1.5 ml tube and microfuge at 16,000–20,000 g for 45 minutes to pellet the virus.

iii) Discard the supernatant and resuspend the pellet with 200 µl of Tris/ethylene diamine tetra-acetic acid (EDTA) buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA).

iv) Add 10 µl of a 5 µg/µl proteinase K solution to give a final concentration of 0.2 µg/µl, mix thoroughly, and incubate at 56°C for 1 hour.

v) Add 25 µl of 10% sodium dodecyl sulfate (SDS) solution to give a final SDS concentration of 1%, mix thoroughly, and incubate at 37°C for 1 hour.

vi) Add 15 µl of 5 M NaCl to give a final concentration of 0.3 M and mix thoroughly.

vii) Add 300 µl of fresh phenol buffered with Tris/HCl, pH 8.0, to the tube, and mix by inverting 50 times.

viii) Microfuge the tube at 16,000 g for 5 minutes and transfer the top aqueous phase (sample) to a new tube.

1 Provided by Dr W.R. Hansen, US Geological Survey, Biological Resources Division, National Wildlife Health Center, 6006, Schroeder Road, Madison, WI 53711, USA. This procedure uses the following commercial items: GeneAmp PCR Reagent Kits containing dNTPs, 10× amplification buffer for hot start PCR, Taq DNA polymerase, Lambda PCR control reagents, and Ampliwax beads (Applied Biosystems), and a 100 base pair molecular size ladder (Invitrogen)
ix) Repeat the phenol extraction steps vii and viii once more.

x) Add 500 µl of ether to the tube, mix thoroughly, and microfuge at 16,000 \( g \) for 1 minute.

xi) Discard the top aqueous phase (ether) and repeat the ether extraction step (step x) once more.

xii) Heat the tube with the lid open at 56°C for about 15 minutes or until the smell of ether is gone.

xiii) Split the tube contents in two and add 2.25 times the sample volume of 100% ethanol to each tube, mix the tube contents by inverting the tube several times, and leave at room temperature (22°C) for 30 minutes.

xiv) Microfuge the tube at 16,000 \( g \) for 45 minutes and discard the supernatant.

xv) Add 200 µl of 70% ethanol to gently wash the pellet and then microfuge at 16,000 \( g \) for 15 minutes.

xvi) Discard the supernatant and dry the pellet at 56°C for 30–45 minutes with the tube lid open.

xvii) Resuspend the DNA in 30 µl distilled water that is RNAase and DNAase free.

xviii) Store the sample tube at 4°C until tested (few days) or at –20°C for long-term storage.

• Polymerase chain reaction

Lower reaction mixtures for the DEV PCR and the lambda control are prepared in advance in a biosafety cabinet using the kit manufacturer’s recommended methods for a hot start PCR. The lower reaction mixture is dispensed into tubes, sealed with Ampliwax at 80°C, as recommended by the manufacturer, and stored at 4°C for 1–2 months.

PCR primers for DEV DNA-directed DNA polymerase gene

Primer 1 sequence: 5'-GAA-GGC-GGG-TAT-GTA-ATG-TA-3' (forward)

Primer 2 sequence: 5'-CAA-GGC-TCT-ATT-CGG-TAA-TG-3' (reverse)

i) The upper reaction mixture is prepared according to the kit manufacturer’s recommendations the day of the test, and distributed to each sample tube including DEV and lambda control tubes.

ii) Add 10 µl of DNA suspension from the stored sample tubes to the PCR lower reaction tubes with corresponding labels.

iii) Place known DEV DNA diluted to 1 pg/10 µl into one control tube and 10 µl of distilled water into the no DNA control tube. Add 10 µl of lambda DNA supplied in the kit and 10 µl of water to the corresponding lambda control tubes.

iv) Place all the tubes in a thermal cycler that is programmed as follows:

One cycle: Hold 94°C for 2 minutes

Hold 37°C for 1 minute

Hold 72°C for 3 minutes

35 cycles: Hold 94°C for 1 minute

Hold 55°C for 1 minute

Hold 72°C for 2 minutes

One cycle: Hold 72°C for 7 minutes

Hold 4°C until stored

PCR tubes are stored at 4°C until the samples are examined for amplification products.

• Electrophoretic analysis of PCR products

i) A fresh 1 × TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3) is prepared from a 10× stock for agarose preparation and for use in the electrophoresis chamber.

ii) A 1% agarose solution is prepared in TAE buffer, heated to dissolve the agarose, and, when cool, poured into a gel former with a comb.

iii) The solidified gel is placed into the electrophoresis chamber and TAE running buffer is added.

iv) PCR test samples, including the DEV and lambda controls, are mixed 1/10 with 1 µl of loading buffer (0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol, 0.01 M Tris/HCl, pH 8.0, and 50% [v/v] glycerol) and 10 µl of each is added to individual wells of the gel. The 100 bp molecular size markers are added to each side of the gel.

v) Run the gel for 1 hour at 120 volts and then stain in a 1% ethidium bromide solution for 20 minutes (alternative, safer products may be used to visualise PCR products, e.g. RedSafe\textsuperscript{TM} or GelRed\textsuperscript{TM}).
stain the gel for 45 minutes in deionised water and view the gel on a UV-illuminated light box. Photograph the gel to record results.

- **Interpretation of the results**

A 500 bp amplification band in the lambda control sample indicates the PCR ran successfully. A 446 bp band in the DEV known DNA control indicates the DEV primers are working. A 446 bp band in the unknown test sample indicates DVE viral DNA was present. No amplification products will be present in the DEV or lambda no DNA controls. If bands appear in these negative control products, cross-contamination occurred during the test set-up and the test must be repeated.

f) **Strain variation**

Although strains of DEV differ considerably in virulence, there is little reported evidence of serological variation.

2. **Serological tests**

Serological tests have little value in the diagnosis of acute DEV infections, but assays based on serum neutralisation in embryonated eggs and cell cultures have been used to monitor antibodies following exposure to DEV in wildfowl. The humoral response to natural infection with DVE virus is often low and antibodies may be short-lived (Docherty & Franson, 1992); it is assumed that cell-mediated immunity also plays a role in the infection (Richter & Horzinek, 1993). However, detection of neutralising antibodies to DVE virus in serum is possible. Virus neutralisation (VN) (Thayer & Beard, 1998) assays using a constant-serum/varying-virus method may be performed in chicken or duck embryos by using embryo-adapted virus, or in cell cultures. For laboratories lacking duck embryos, serological diagnosis is possible by virus neutralisation, using a chicken embryo fibroblast adapted DVE strain and primary chicken embryo fibroblasts (CEF). Neutralisation indices (NI) (Thayer & Beard, 1998) between 0 and 1.5 were detected in domestic and wild waterfowl that had not been exposed to DEV; a NI of 1.75 or greater was considered to be evidence of prior exposure to DVE virus (Dardiri & Hess, 1967). Alternatively, sera may be screened using a constant-virus/varying-serum method. In the author’s laboratory a microtitre neutralisation assay using primary MDEF or DEF is used. Serial twofold dilutions of each serum sample (heat-inactivated at 56°C) are prepared in 50 µl of serum-free MEM in microtitre plates. Approximately $10^{2.0}$ TCID$_{50}$ (50% tissue culture infective dose) of DVE virus in 50 µl of MEM is added to each well and the mixtures are allowed to react at 37°C for 1 hour. A suspension of primary MDEF or DEF in MEM supplemented with 2 mM L-glutamine, 0.17% sodium bicarbonate and 10% FCS, are adjusted to contain $3 \times 10^6$ cells per ml. Cells are next added to the plates at 100 µl per well and the plates are then incubated for up to 96 hours at 37°C in a humidified 5% CO$_2$ atmosphere. Following incubation, cells are observed daily by light microscopy and finally fixed with 10% formol-buffered saline and stained with 1% crystal violet. The plates are read macroscopically. The titre for virus neutralising activity is expressed as the reciprocal of the highest dilution of serum at which a monolayer grew, i.e. there is no evidence of CPE and therefore complete virus neutralisation has occurred. A titre of less than 3 log$_2$ is usually considered to be negative. A VN titre of 8 or greater is considered to be significant and is evidence of exposure to DVE virus (7). VN antibody may also be detected using cell cultures by mixing sera at a single dilution, e.g. 1/10, with 100–200 TCID$_{50}$ virus and then testing inoculated cell cultures for non-neutralised virus by immunofluorescence. Although this method is not quantitative, it can be useful for screening large numbers of sera. These latter methods, using constant-virus/varying-serum, are much more economical on sera than the NI methods.

C. **REQUIREMENTS FOR VACCINES**

1. **Background**

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

a) **Rationale and intended use of the product**

A live attenuated virus vaccine can be used to control DVE in birds over 2 weeks of age (Richter & Horzinek, 1993). The live vaccine virus is not thought to spread by contact from vaccinated to unvaccinated ducklings. Fattening or breeding ducks may be vaccinated subcutaneously or intramuscularly to produce an active immunity. Maternally-derived immunity in ducklings is reported to decline rapidly and progeny of breeders vaccinated with a live attenuated vaccine are fully susceptible.
A live attenuated vaccine propagated in a duck embryo fibroblast cell line has been reported to be successful (Mondal et al., 2010).

An inactivated vaccine has been reported to be as efficacious as modified live vaccine (Shawky & Sandhu, 1997). This vaccine has been tested only under laboratory conditions; it has not been tested on a large scale and is not licensed.

2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed

i) Biological characteristics

DVE vaccine can be prepared from a strain of the virus that has been attenuated by serial passage in embryonated chicken eggs. In the USA the vaccine strain seed was originally imported from Holland and has been serially passaged 41–46 times.

The seed virus should be prepared in 8–11-day-old specific pathogen free (SPF) embryonated chicken eggs by inoculating on to the CAM followed by incubation at 37°C. The seed may be stored at –70°C or lower in the form of a homogenate of the embryo CAM in buffered saline.

ii) Quality criteria (sterility, purity, freedom from extraneous agents)

The seed virus should be shown to be free from extraneous viruses pathogenic to ducks, chickens and turkeys. It should also be free from bacterial, fungal and mycoplasmal contaminants.

The identity of the virus should be confirmed by a VN test conducted with specific antiserum using the constant-serum/varying-virus method. This test should be performed in embryonated chicken eggs. The antiserum should reduce the virus titre by at least $10^{1.75}$ ELD$_{50}$ (50% embryo lethal dose).

b) Method of manufacture

i) Procedure

The vaccine is produced in 8–11-day-old SPF embryonated chicken eggs inoculated on to the CAM and incubated at 37°C. Most embryo deaths occur between 48 and 96 hours after inoculation. The embryos, their CAMs and chorioallantoic fluids are harvested, pooled and homogenised in buffered saline and clarified by low-speed centrifugation (1800 g). The preparation is diluted as appropriate, and a stabiliser is incorporated. It is then dispensed into vials and preferably frozen rapidly to –70°C or lower.

ii) Requirements for substrates and media

All reagents should be sterile and eggs obtained from a specific pathogen-free source.

iii) In-process controls

Any embryo dying within the first 24 hours of inoculation should be discarded as nonspecific deaths.

iv) Final product batch tests

Sterility and purity

Tests for sterility and freedom from contamination of biological materials may be found in the chapter 1.1.7.

Safety

A group of 1-day-old ducklings susceptible to DEV should be inoculated subcutaneously or intramuscularly with the vaccine at 10 times the recommended dose, and observed for 7–14 days for any signs of adverse reactions.

Batch potency

The virus titre of the vaccine should be determined in 9- to 11-day-old embryonated chicken eggs inoculated on to the CAM and incubated at 37°C. The vaccine should contain a minimum of $10^{3.0}$ ELD$_{50}$ per dose at time of use.

The immunogenicity of the vaccine can be assessed in DVE-susceptible ducks or ducklings by inoculating the recommended vaccine dose intramuscularly and challenging intramuscularly 21 days later with virulent DVE virus. The vaccinated birds should survive challenge while unvaccinated control...
birds should die. This test should be carried out on the master seed but need not be done routinely on each vaccine batch produced. For release of subsequent batches, the titre of the virus should be a sufficient indication of vaccine potency.

c) Requirements for authorisation

i) Safety requirements
A group of 1-day-old ducklings susceptible to DVE should be inoculated subcutaneously or intramuscularly with the vaccine at 10 times the recommended dose, and observed for 7–14 days for any signs of adverse reactions.

Target and non-target animal safety
The vaccine is intended solely for use to protect ducklings and ducks against DVE virus.

Reversion-to-virulence for attenuated/live vaccines
There are no reports of reversion to virulence by the DVE vaccine

Environmental consideration
None.

ii) Efficacy requirements

For animal production
Immunity in vaccinated ducks should last throughout a breeding season. Annual re-vaccination is recommended (Sandhu & Metwally, 2008).

For control and eradication
The vaccine virus is not thought to spread by contact from vaccinated to unvaccinated ducks, as the unvaccinated birds remain susceptible to infection.

iii) Stability
When stored at −70°C or lower the vaccine is stable for at least 1 year. Potency testing should be repeated after this time on an aliquot of vaccine to determine whether virus titre has been maintained. Once thawed the vaccine should not be refrozen, it should be maintained at 4°C in a refrigerator but for no longer than 1 week. Lyophilised vaccine should be stored at 4–8°C and used before the stated expiry date.

3. Vaccines based on biotechnology

a) Vaccines available and their advantages
None

b) Special requirements for biotechnological vaccines, if any
None

REFERENCES


Chapter 2.3.7. – Duck virus enteritis


Chapter 2.3.7. – Duck virus enteritis


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CHAPTER 2.3.8.

DUCK VIRUS HEPATITIS

SUMMARY

Hepatitis in ducks can be caused by at least three different viruses. The more common and internationally widespread is duck hepatitis virus (DHV) type I, a member of the Picornaviridae in the new genus Avihepatovirus, which causes a highly lethal, acute, contagious infection in ducklings under 6 weeks of age and, frequently, under 3 weeks of age. It does not occur in older birds. This infection is often referred to simply as duck virus hepatitis. Three genotypes that may also be serotypes of DHV type I have been identified and designated duck hepatitis A virus (DHAV) types 1, 2 and 3.

DHV type II has been reported in the United Kingdom only. It occurred in ducklings from 10 days to 6 weeks of age, and caused pathological changes similar to those of DHV type I. From electron microscopy and molecular studies it is a member of the Astroviridae and is known as duck astrovirus type 1 (DAstV-I).

DHV type III has been reported only in the United States of America. It causes similar liver lesions in young ducklings, but is less virulent than DHV type I. From molecular investigations it is a member of the Astroviridae and designated duck astrovirus type II (DAstV-II). It is considered distinct from DAstV-I.

Diagnosis of hepatitis in ducklings is based on the characteristic disease pattern in the flock, gross pathological changes, the recovery of virus from dead ducklings, and the reproduction of the disease in susceptible ducklings.

Identification of the agent: It is not possible to distinguish among DHV types I, II and III on the basis of clinical findings and pathology, but distinctions can be made from the responses of ducklings, embryonated eggs and cell cultures to the isolated viruses. Alternatively DHV type I RNA may be detected by a one-step reverse-transcriptase polymerase chain reaction from duckling liver and also from allantoic fluid and embryo liver from inoculated duck eggs. Reverse-transcriptase polymerase chain reaction (RT-PCR) identifications by nucleic acid sequence analysis have been described for DAstV-1 and DAstV-II.

Serological tests: Serological tests have little value in the diagnosis of the acute infections caused by DHV types I, II and III.

Serum neutralisation tests in ovo have been used with all three viruses and in-vitro tests have been developed for DHV type 1. These tests have been used for virus identification, assay of immune responses to vaccination and epidemiological surveys.

Requirements for vaccines: DHV type I infections can be controlled by the use of live attenuated virus vaccines and an inactivated virus vaccine. They are administered to breeder ducks to confer passive immunity to ducklings. Live attenuated virus vaccines may also actively immunise DHV type-I-susceptible day-old ducklings.

Ducklings susceptible to DHV type I may also be passively protected with a chicken egg yolk antibody preparation.

DHV type III infections can be controlled by the use of a live attenuated virus vaccine given to breeder ducks to confer passive immunity to ducklings.
A. INTRODUCTION

Duck hepatitis (DH) is caused by at least three different small RNA viruses, namely duck hepatitis virus (DHV) types I, II and III. The most common is DHV type I. The genome sequences of five strains of DHV type I have been released on public databases (strain-DRL-62 – DQ219396, strain-R85952 – DQ226541, strain 03D – DQ249299, strain H – DQ249300 & strain 5886 – DQ249301) (Kim et al., 2006; Tseng et al., 2007).

Analyses of these sequences show them to be closely related picornaviruses, but unrelated to any members of the currently defined genera. It has therefore been proposed that they should form a novel genus within the Picornaviridae (Kim et al., 2006; Tseng & Tsai, 2007). It has also been suggested that DHV type I be subdivided and renamed as two additional genotypes and possibly serotypes have been detected; thus duck hepatitis A virus type 1 (DHAV-1 the original DHV type I), DHAV-2 and DHAV-3 have been created (Wang et al., 2008). DHAV-2 is also documented as N-DHV (Tseng & Tsai, 2007) and DHAV-3 has been described (Kim et al., 2007a). DHAV-2 was originally isolated from a mule duckling and a gosling but mixed DHAV-1 and DHAV-2 infections are common in Taiwan (Tseng & Tsai, 2007). All the DHAV-3 isolates are from Korea (Kim et al., 2007a).

The International Committee on Taxonomy of Viruses have created a new genus Avihepatovirus in the family Picornaviridae. This genus contains the species, Duck hepatitis A virus, which includes three (sero)types – duck hepatitis virus I (DHV-I) and two new duck hepatitis virus (N-DHV) genotypes; the three types would be known as duck hepatitis A virus (DHAV): DHAV-1, DHAV-2 and DHAV-3.

Until recently DHV type I had only been associated with causing disease in mallard and Pekin ducklings but it has now been reported to cause pancreatitis and encephalitis in Muscovy ducks (Guerin et al., 2007).

DHV type II is an astrovirus also known as duck astrovirus-1, (DAstV-1), and DHV type III is now classified as an astrovirus, duck astrovirus-2 (DAstV-2), distinct from DAstV-1 (Todd et al., 2009).

These viruses, which cause acute infections, should not be confused with duck hepatitis B virus, an Avihepadnavirus classified in the same group as mammalian hepatitis B virus. The significance of this infection for the duck is not fully understood.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) DHV type I (DHAV-1, DHAV-2, DHAV-3)

Limited information on the pathogenicity of DHAV-2 and DHAV-3 indicates that the clinical presentation is similar to DHAV-1 but serologically there is no cross neutralisation between DHAV-1 and DHAV-2 (Tseng & Tsai, 2007) and limited cross neutralisation between DHAV-1 and DHAV-3 (Kim et al., 2007a).

DHV type I causes a highly contagious infection of ducks. It is of no known public health significance. The disease is an acute, rapidly spreading, often fatal virus infection of young ducklings. It usually affects ducklings under 6 weeks of age and often much younger. The clinical disease is characterised by lethargy and ataxia followed by opisthotonos and death. Ducklings lose their balance, fall on their sides and kick spasmodically prior to death. The whole disease sequence is rapid and can take as little as 1–2 hours. Practically all mortality in a flock will occur within 3–4 days, with most deaths on the second day. Gross pathological changes appear chiefly in the liver, which is enlarged and displays distinct petechial and ecchymotic haemorrhages. Spleen enlargement and swelling of the kidneys with some congestion of renal blood vessels may also be apparent. Microscopic changes in the liver are characterised by extensive hepatocyte necrosis and bile duct hyperplasia, together with varying degrees of inflammatory cell response and haemorrhage.

The clinical and pathological observations are highly indicative of DHV type I infection. The virus can readily be recovered from liver tissue by homogenisation as a 20% (w/v) suspension in buffered saline. The suspension is clarified, and can then be treated further (if desired) with 5% chloroform (v/v) for 10–15 minutes at ambient temperature. DHV type I is resistant to this treatment.

The presence of DHV type I is usually confirmed by one or more of the following procedures:

i) By subcutaneous or intramuscular inoculation of the isolate into ducklings between 1 and 7 days of age that are susceptible to DHV type I. The characteristic clinical disease should follow, with deaths
Chapter 2.3.8. — Duck virus hepatitis

occurring within 18–48 hours of inoculation, often in less than 24 hours. The ducklings should show the gross pathology attributable to DHV type I. Virus should be re-isolated from the livers.

ii) By inoculation of serial dilutions of the liver homogenate into the allantoic sac of embryonated duck eggs (10–14 days) or chicken eggs (8–10 days). Duck embryos die between 24 and 72 hours later, whereas chicken embryos are more variable and erratic in their response and usually take 5–8 days to die. Gross pathological changes in the embryos include stunting and subcutaneous haemorrhages over the whole body, with oedema particularly of the abdominal and hind limb regions. The embryo livers may be red and yellowish, swollen and may show some necrotic foci. In embryos that take longer to die, the greenish colour of the allantois is more pronounced, and both the liver lesions and stunting become more evident.

iii) By inoculation of primary cultures of duck embryo liver (DEL) cells, which are particularly sensitive (Woolcock, 1986). Dilutions of the liver homogenate containing DHAV type 1 cause a cytopathic effect (CPE), which is characterised by cell rounding and necrosis. When overlaid with a maintenance medium containing 1% agarose (w/v), the CPE gives rise to plaques approximately 1 mm in diameter.

• Immunological tests

Such tests have not been used extensively for the routine identification of DHV type I infection. Various virus neutralisation (VN) assays have been described, which may assume greater significance if DHV types II and III infections become more widespread. The tests that have been described (Chalmers & Woolcock, 1987; Woolcock, 1986; 1991; 2008a) include:

i) Passive subcutaneous immunisation of 1–7-day-old ducklings susceptible to DHAV type 1 with 1–2 ml specific hyperimmune serum or specific egg yolk antibody. These ducklings are then challenged intramuscularly or subcutaneously, 24 hours later with at least 10^{3.0} LD_{50} (50% lethal dose) of the virus isolate. A control group of uninoculated ducklings is similarly challenged. Identification of infection is based on 80–100% survival in the passively immune ducklings and 80–100% mortality in the controls.

ii) 1–7-day old DHAV type-1 susceptible and DHAV type 1 maternally immune ducklings are challenged intramuscularly or subcutaneously with at least 10^{3.0} LD_{50} of the virus isolate. Identification is based on 80–100% losses in the susceptible ducklings and 80–100% survival in the maternally immune ducklings.

iii) Serial tenfold dilutions of the virus isolate are mixed with equal volumes of DHAV type-1 specific hyperimmune serum diluted between 1/5 and 1/10. The mixtures are allowed to react at room temperature for 1 hour and are then inoculated (0.2 ml) subcutaneously into susceptible ducklings, also via the allantoic cavity (0.2 ml) of embryonated duck eggs and on to primary DEL cell monolayer cultures. Controls in each case consist of the virus isolate mixed with control serum.

There is evidence for antigenic variation among DHV type I isolates. A variant, DHV type Ia, isolated in the United States of America (USA) only partially reacts with the classical type I virus in cross serum neutralisation tests (Sandhu et al., 1992; Woolcock, 2008b). Other variants have been reported from India and Egypt, but nothing further is known about them. Recent reports of disease in Muscovy ducks from France (Guerin et al., 2007; Sandhu et al., 1992; Woolcock, 2008) and two new genotypes (DHAV-2 and DHAV-3) that may also be serotypes (Kim et al., 2007a; Tseng & Tsai, 2007; Wang et al., 2008) suggest a greater diversity among DHV type I than was originally thought.

• Nucleic acid recognition methods

Several publications on the molecular structure of DHV type I have been published emphasising genotypic variation among isolates, particularly from China (Ding & Zhang, 2007; Fu et al., 2008; Jin et al., 2008; Liu et al., 2008), Chinese Taipei (Tseng et al., 2007; Tseng & Tsai, 2007) and Korea (Kim et al., 2006; 2008). A one-step reverse transcriptase-PCR assay using primers to the conserved 3D gene has been described for DHV-I (Kim et al., 2007b). Anchun et al. 2009 also report an RT-PCR to detect Chinese isolates but it is not clear whether these are DHAV-1 or DHAV-2. The development of a one-step real-time Taqman RT-PCR assay again based on primers to a conserved region in the 3D gene has been reported (Yang et al., 2008) but it is not clear whether this is for DHAV-1 or DHAV-2; this report does not provide a clear step by step protocol used for the method they developed.

• Polymerase chain reaction

This method has been extracted from Kim et al., (2007b). It is based on primers specific to amplify a region of the 3D gene of DHAV-1.
Chapter 2.3.8. – Duck virus hepatitis

• Detection of DHV-I from duck and chicken embryo organs and nucleic acid extraction

Supernatants prepared from duckling livers infected with DHV type I are collected and filtered (0.2 µm). The allantoic cavities of each of five 11-day-old duck and 9-day-old chicken embryonated eggs are inoculated with 0.2 ml viral supernatant. The allantoic fluid and liver samples are collected from embryos inoculated with two reference strains and each liver sample is ground in a tissue grinder and phosphate buffered saline is added to make 10% suspensions. Liver sample suspensions and allantoic fluid are centrifuged at 2000 g for 30 minutes, the supernatants are treated with the Viral Gene-spin™ viral DNA/RNA extraction kit following the manufacturer’s instructions (iNtRON Biotechnology, Seongnam, Korea). The nucleic acids are used for one-step RT-PCR. After measuring RNA concentrations using the NanoDrop ND-1000 (NanoDrop, Wilmington, DE), the samples are stored at –20°C.

• Oligonucleotide primers

DHV-1 ComF (5'-AAG-AAG-GAG-AAA-ATY-[C or T]-AAG-GAA-GG-3') and

DHV-1 ComR (5'-TTG-ATG-TCA-TAG-CCC-AAS-[C or G]-ACA-GC-3')

Flanked by a 467 bp DNA sequence in the 3D gene.

• One-step RT-PCR

The one-step RT-PCR is conducted using the Maxime RT-PCR PreMix kit (iNtRON Biotechnology). The 20-µl reaction mixtures contain 1 U of OptiScript reverse transcriptase, 2.5 mM dNTPs, 2.5 U i-StarTaq DNA polymerase, and RT-PCR buffer (50 mM Tris/HCl and 75 mM KCl). In addition, the following components are included in the reaction: 4 µl (50 ng) RNA or DNA template, 1 µl (10 pmol/µl) of each specific primer (DHV-1 ComF and DHV-1 ComR), and DEPC-treated dH2O to a total reaction volume of 20 µl.

A T-gradient thermal cycler (Biometra, Gottingen, Germany) is used for one-step RT-PCR. Reverse transcription is performed at 45°C for 30 minutes, after which the enzyme is inactivated at 94°C for 5 minutes. PCR amplification is conducted using an initial denaturation for 20 seconds at 94°C; followed by 40 cycles of annealing for 30 seconds at 52°C, extension for 30 seconds at 72°C, and denaturation for 20 seconds at 94°C; and a final extension for 5 minutes at 72°C. Reactions are stored at 4°C.

• Detection of one-step RT-PCR products

PCR products (10 µl) are separated by electrophoresis (100 V) in horizontal 1.5% agarose gels (iNtRON Biotechnology) and Tris-acetate buffer (40 mM Tris-acetate, 1 mM ethylenediamine tetra-acetic acid). Gels are stained with ethidium bromide (0.5 µg/ml), visualised under ultraviolet light, and photographed.

• Interpretation of results

A DNA fragment of 467 bp is amplified by one-step RT-PCR using RNA extracted from the livers of ducklings infected with reference DHV type I strains. Negative control RNA is obtained from an uninfected duckling liver and does not amplify under the same conditions.

b) DHV type II (duck astrovirus type 1 [DAstV-1])

DHV type II, also named duck astrovirus type 1, infection of ducks has only been reported from the United Kingdom (Asplin, 1965; Gough et al., 1985). It is an acute, fatal infection of ducklings producing clinical and pathological signs similar to DHV type I. Affected birds may show signs of polydypsia and usually die within 1–2 hours of appearing sick.

Gross pathological changes include multiple haemorrhages, both punctate and confluent bands in the liver, swollen pale kidneys with congested blood vessels, and enlarged spleens. The alimentary tract is often empty although the small intestine may contain mucus, and haemorrhagic areas are occasionally seen. Petechial haemorrhages are also occasionally seen on the heart. Histologically, changes in the liver are similar to those seen in DHV type I infections; the extent of bile duct hyperplasia may be greater than with DHV type I, but this is relative. DHV type II has astrovirus-like morphology and virions are 28–30 nm in diameter. It is classified in the family Astroviridae as duck astrovirus 1 (DAstV-1) (Gough et al., 1985; Koci & Schultz-Cherry, 2007).

The virus may be recovered in 20% (w/v) homogenised liver suspensions in buffered saline. This can be used to inoculate:
Chapter 2.3.8. – Duck virus hepatitis

i) Susceptible ducklings, in which the response can be variable. A mortality rate of up to 20% may occur within a period of 2–4 days. The gross pathology is similar to that observed in field cases (Gough et al., 1985). This is in contrast to the findings with DHAV type 1 infection, which is more virulent and rapid in its effect.

ii) Embryonated chicken or duck eggs, either via the amniotic cavity or yolk sac. These may respond, erratically, after four passages, but no deaths may be seen during earlier passages. Embryos take 6–10 days to show evidence of infection; when this occurs there is stunting with green necrotic livers.

Growth of DHV-II in primary chicken embryo liver cell cultures has been reported (Baxendale & Mebatsion, 2004); plaque formation was detected at 5 days post-infection after 4 or 5 serial passages.

• Immunological tests

Immunological tests have not been employed routinely as the serological response to infection of both ducklings and duck embryos is poor. However, a neutralisation assay has been applied (Gough et al., 1985) for virus identification by inoculating chicken embryos via the amniotic cavity with constant-serum/varying-virus mixtures.

Cross protection tests have been performed in 2–4-day old ducklings (Gough et al., 1985); these are inoculated with antisera to types I or II, then challenged 3 days later with the virus isolate. This technique could distinguish DHV type II from types I and III.

• Polymerase chain reaction

Confirmation that DHV-II is an astrovirus has been made using nucleic acid sequence data (Todd et al., 2009).

c) DHV type III (duck astrovirus type 2 [DAstV-2])

DHV type III has been reported in the USA only. Losses of up to 20% occur in ducklings immune to DHV type I (Haider & Calnek, 1979; Toth, 1969). DHV type III causes an acute infection of young ducklings with clinical signs similar to those seen in type I infections.

The gross pathology is also similar to type I infection. The liver surface is pale and mottled with many red bands and some petechial haemorrhages. The spleen is paler, but not noticeably enlarged, and the kidneys may show patchy congestion.

The virus can be recovered from homogenised liver suspensions and is resistant to treatment with 5% chloroform. The virus can be isolated by:

i) Inoculating the isolate intramuscularly into susceptible ducklings. The mortality rate may reach 20% with 60% morbidity. No deaths occur in the first 24 hours and all losses ensue between day 2 and day 4 after inoculation. Intravenous inoculation is more effective; type III infection is less virulent than type I.

ii) Inoculating the isolate on to the chorioallantoic membrane (CAM) of 10-day-old embryonated duck eggs. The response is erratic, but some embryo mortality always occurs within 7–10 days. The membranes assume a dry crusty appearance, beneath which they are oedematous. The embryos may be stunted and oedematous with skin haemorrhages. The liver, kidneys and spleen are enlarged.

Attempts to cultivate the virus in hens’ eggs have not been successful.

Attempts to induce a CPE with the virus in tissue cultures have not been successful, but the virus has been detected by direct immunofluorescence in experimentally infected DEL and duck embryo kidney (DEK) cell monolayer cultures (Haider & Calnek, 1979).

• Polymerase chain reaction

DHV type III has now been identified as an astrovirus (DAstV-2) by nucleic acid sequence data. It is considered distinct from DHV type II (DAstV-1) (Todd et al., 2009).

2. Serological tests

These do not apply to diagnosis as the clinical disease is too acute.
All three DHV types have been used in virus neutralisation tests in ovo, but their success depends on the expression of the virus in the assay system used; with type II and III viruses this can be a problem. In vitro tests have been developed for DHV type I; these include a plaque reduction assay and a microtitre assay (Woolcock, 1986; 1991). The plaque reduction assay may be performed using either primary DEK or DEL cells. Primary cell culture monolayers are prepared in Eagle’s minimal essential medium (MEM) containing 5–10% fetal calf serum (FCS), 2 mM glutamine, 0.17% sodium bicarbonate and gentamicin. Trypsinised cells are seeded into 5 cm diameter Petri dishes, then incubated at 37°C in a 5% CO₂ atmosphere. Monolayers should be nearly confluent at 24–48 hours post-seeding. The monolayers are washed twice with serum-free MEM or Hank’s balanced salt solution to remove all traces of FCS before infecting with DHV type I. Equal volumes of DHV type I suspended in serum-free MEM, adjusted to 200 plaque-forming units (PFU) per 0.1 ml, are mixed with equal volumes of serially diluted duck sera (twofold dilutions in MEM). The serum samples should be heat inactivated at 56°C for 30 minutes before testing. The virus/serum mixtures are incubated at 37°C for 1 hour; then 0.1-ml aliquots are added to the confluent cell monolayers, three dishes per dilution. The plates are left for 30 minutes at room temperature (20–22°C), then overlaid with agarose maintenance medium (MEM containing 2% chicken serum and 0.1–0.2% FCS to which agarose had been added to a final concentration of 1% [w/w]). The plates are then placed at 37°C in a 5% CO₂ atmosphere. The number of plaques produced is recorded after 48 hours’ incubation. Plaques may be observed using an oblique light source, or alternatively monolayers may be fixed with 10% formol-buffered saline and stained with 1% crystal violet. Serum antibody titres are expressed as the reciprocal of the highest serum dilution that reduces the plaque count by 50%.

A microtitre neutralisation assay may be performed using primary DEK cells. Serial twofold dilutions of each serum sample (heat-inactivated) are prepared in 50 µl of serum-free Eagle’s basal medium (BME) in microtitre plates. Approximately 10².0 TCID₅₀ (50% tissue culture infective dose) of DHV type I in 50 µl of BME is added to each well and the mixtures are allowed to react at 37°C for 1 hour. Primary DEK cells are suspended in BME supplemented with 10% tryptose phosphate broth, 2 mM L-glutamine, 0.17% sodium bicarbonate and 2–4% chicken serum, and are adjusted to contain 3 × 10⁵ cells/ml. Cells are next added to the plates at 100 µl per well and the plates are then incubated for up to 96 hours at 37°C in a humidified 5% CO₂ atmosphere. Following incubation, cells are fixed with 10% formol-buffered saline and stained with 1% crystal violet. The plates are read macroscopically. The titre for virus neutralising activity is expressed as the reciprocal of the highest dilution of serum at which a monolayer grew, i.e., there is no evidence of CPE and therefore complete virus neutralisation has occurred. A titre of less than 4 log₂ is considered to be negative.

These neutralisation tests have been used to assay humoral immune responses to vaccination and for epidemiological surveys, as well as for virus identification.

C. REQUIREMENTS FOR VACCINES

1. Background

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

a) Rationale and intended use of the product

DHV type I can be controlled by the use of a live attenuated virus vaccine. This is given to breeder ducks so that immunity is transferred via the yolk to newly hatched birds. Live vaccine virus can also be used to actively immunise newly hatched DHV type-I-susceptible ducklings (Crighton & Woolcock, 1978). An inactivated DHV type I vaccine is also effective when administered to breeder ducks that have been primed with live vaccine or previously field exposed to live DHV type I; progeny from these breeders have maternal immunity (Woolcock, 1991). Ducks may also be passively protected by inoculation of antibodies in chicken egg-yolk.

The development and evaluation of a vaccine to protect ducklings against DHA-V-3 in Korea has been described (Kim et al., 2009). The methods used are comparable to those described in this text for DHV type I (also named DHA-V-1).

An attenuated live virus DHV type II vaccine has been used to protect ducklings only under experimental conditions (Gough et al., 1985).

DHV type III infections have been controlled by the use of attenuated live virus vaccines given to breeder ducks, so that the immunity is transferred via the yolk sac to the hatching ducklings.
2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed
   i) Biological characteristics
      The type I virus vaccine seed used most commonly in Europe is derived from an isolate passaged in embryonated chicken eggs 53–55 times; that in the USA for live and inactivated vaccines has been passaged 84–89 times.
      The type II virus vaccine seed originated from an isolate attenuated by 25 serial passages in embryonated chicken eggs (Asplin, 1965), and has been employed only experimentally under field conditions (R.E. Gough, pers. comm.).
      The type III vaccine seed has been attenuated by 30 serial passages in embryonated duck eggs inoculated via the CAM.
   ii) Quality criteria (sterility, purity, freedom from extraneous agents)
      All seed viruses should be shown to be free from extraneous viruses that are pathogenic for ducks, chickens or turkeys. The seeds should be free from all microbiological and fungal contamination.
      The identity of the virus type should be confirmed by a VN test conducted with specific antiserum by a constant-serum/varying-virus method. In the case of types I and II viruses, the tests are performed in embryonated chicken eggs; with type III virus the tests are done in embryonated duck eggs. The antiserum should reduce the titre of the respective virus by at least 10^{2.0} ELD_{50} (50% embryo lethal dose).

b) Method of manufacture
   i) Procedure
      DHV types I and II viruses are treated similarly. The vaccine is produced in 9–10-day-old SPF embryonated chicken eggs inoculated via the allantoic route, and incubated at 37°C. Most embryo deaths occur within 2–3 days in the case of DHV type I, but with type II, the deaths do not occur until 6–10 days after inoculation, although they are harvested at 3–5 days for maximum virus yield. The embryo harvests are homogenised in buffered saline and clarified by low-speed centrifugation. The preparation is diluted as appropriate and dispensed into vials, which are preferably frozen rapidly at −70°C or below. Subsequently, they may be stored satisfactorily between −20°C and −40°C. DHV type I attenuated vaccine is also available as a lyophilised preparation that may be stored at 2–8°C. The reconstituted vaccine may be used with or without the incorporation of aluminium hydroxide in the diluent.
      In the case of inactivated DHV type I vaccine, the embryo harvests are homogenised and clarified by low-speed centrifugation and then further purified by treatment with chloroform (final concentration 10% [v/v]). This preparation is then inactivated with freshly prepared binary ethylenimine (BEI). The inactivated virus is then blended with an adjuvant such as LES-STM; 0.2% (v/v) formalin is added as a preservative (Woolcock, 1991).
      The type III vaccine is prepared in 10-day-old SPF duck eggs inoculated via the CAM with attenuated DHV type III and incubated at 37°C. Most embryo deaths occur between 6 and 10 days. Eggs containing dying embryos, together with their CAMs, are harvested and homogenised in buffered saline and clarified by low-speed centrifugation. The preparation is diluted as appropriate and dispensed into vials, which are preferably frozen rapidly at −70°C or below.

• Egg yolk antibody
   Virulent DHV type I prepared from duckling livers or attenuated virus may be used to hyperimmunise SPF chickens for egg-yolk antibody production. Eggs are collected from the hyperimmunised birds and stored at 4°C until time of production. The yolks are separated, pooled and blended with an antifoaming agent. The mixture is diluted with buffered saline containing no more than 0.2% (v/v) formalin as a preservative. The dispensed product is stored at 4°C and has a shelf life of 1 year. Tests are carried out for sterility in the usual way for the absence of contaminants.

ii) Requirements for substrates and media
   All reagents should be sterile and eggs obtained from a specific pathogen-free source.

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1 A preparation of *Salmonella typhimurium* (STM), a B cell mitogen, in a lipid emulsion system (LES). Available from Ribi Immunochem Research, Hamilton, Montana 59840, USA.
iii) **In-process controls**

Any embryo deaths within the first 24 hours of inoculation should be discarded as nonspecific deaths.

iv) **Final product batch tests**

**Sterility and purity**

Tests for sterility and freedom from contamination of biological materials may be found in the chapter 1.1.7.

**Safety**

A group of 1–3-day-old ducklings susceptible to the type of virus concerned, should be inoculated subcutaneously or intramuscularly (in the case of types I and II), or subcutaneously (in the case of type III), with the attenuated vaccine at ten times the recommended dose, and kept under observation for between 10 and 21 days for any adverse reactions. Attenuated live vaccines should be stable and not revert to virulence on back passages in susceptible ducklings.

A safety test on the inactivated DHV type I vaccine is performed by inoculating the recommended dose (0.5 ml) intramuscularly into a group of day-old ducklings; no adverse effects should be observed during the period of testing.

Safety tests on yolk antibody are done by inoculating 1 ml subcutaneously into each of a group of ducklings, which are then kept under observation for 3 days for signs of adverse effects.

**Batch potency**

For DHV types I and II viruses, the virus titre of the vaccine should be determined in 9–10-day-old embryonated chicken eggs inoculated into the allantoic cavity and incubated at 37°C. The immunogenicity, of the vaccine for ducklings susceptible to type I or II virus can be assessed by inoculating subcutaneously a minimum of \(10^{2.5}\) ELD\(_{50}\) per duckling of the vaccine virus and challenging subcutaneously 72 hours later with \(10^{3.0}\) LD\(_{50}\) per duckling of virulent DHV virus type I or II (Crighton & Woolcock, 1978). At least 80% of the vaccinated birds should survive and, in the case of type I, at least 80% of the controls should die; in the case of type II, a 20% mortality in the controls is more realistic.

The immunogenicity of the inactivated vaccine is considered to be satisfactory if a four-fold or greater increase in neutralising antibody titre can be demonstrated following administration to ducklings that have been previously primed with live attenuated DHV type I.

For type III virus, the titre of the vaccine should be determined in 10-day-old embryonated duck eggs inoculated on to the CAM. Immunogenicity tests in ducklings have proved difficult because of the variable pathogenicity of the challenge virus for ducklings.

Potency tests on yolk antibody are done by determining the neutralising index (NI) for the product in embryonated hens’ eggs using the constant-yolk/varying-virus method. A minimum NI of \(10^{3.0}\) is considered to be satisfactory. The efficacy of the product is determined by inoculating a group of susceptible ducklings with the recommended dose of egg yolk antibody. A second group is left untreated. After 24 hours each group is challenged with virulent DHV type I virus. The product is adjudged efficacious if at least 80% of the treated ducklings survive and at least 80% of the controls die.

c) **Requirements for authorisation**

i) **Safety requirements**

A group of 1–3-day-old ducklings susceptible to the type of virus concerned, should be inoculated subcutaneously or intramuscularly (in the case of types I and II), or subcutaneously (in the case of type III), with the attenuated vaccine at ten times the recommended dose, and kept under observation for between 10 and 21 days for any adverse reactions. Attenuated live vaccines should be stable and not revert to virulence on back passages in susceptible ducklings.

A safety test on the inactivated DHV type I vaccine is performed by inoculating the recommended dose (0.5 ml) intramuscularly into a group of day-old ducklings; no adverse effects should be observed during the period of testing.

Safety tests on yolk antibody are done by inoculating 1 ml subcutaneously into each of a group of ducklings, which are then kept under observation for 3 days for signs of adverse effects.

**Target and non-target animal safety**

The vaccines and egg yolk are intended solely for use to protect ducklings against DHV and to immunise breeder ducks so that maternal antibodies may be transferred to progeny.
Reversion-to-virulence for attenuated/live vaccines

Reversion to virulence on serial passage in ducklings has been reported (Woolcock & Crighton, 1979; 1981).

Environmental consideration
None.

Efficacy requirements

For animal production
In the case of newly hatched ducklings, attenuated live DHV type I replicates rapidly and results in an immunity within 48–72 hours of vaccination. This immunity persists throughout the susceptible period of life (Crighton & Woolcock, 1978). However in ducklings protected by vaccination of their parents, the level of maternally derived immunity decreases over the first 2 weeks of life, but such ducklings can be actively re-immunised with attenuated virus given subcutaneously or orally at about 7–10 days of age (Hanson & Tripathy, 1976; Tripathy & Hanson, 1986). Alternatively, the immunity can be enhanced by the administration of either specific hyperimmune serum or egg yolk antibody prepared from eggs laid by chickens actively hyperimmunised against DHV type I.

Breeder ducks primed with live DHV type I at 12 weeks of age and then given, intramuscularly, a single dose of inactivated type I vaccine at 18 weeks of age should produce maternally immune progeny through a complete laying cycle (Woolcock, 1991).

For control and eradication
Breeder ducks given live attenuated DHV type I vaccine two or three times at 12, 8 and 4 weeks before coming in to lay, and breeder ducks given live attenuated DHV type III vaccine twice at 12 and 4 weeks before coming in to lay should produce passively immune progeny throughout a breeding season. However, it is usually recommended to revaccinate every 3 months with DHV type I vaccine and every 6 months with DHV type III vaccine after the onset of lay. DHV type I attenuated vaccine can also be supplied as a lyophilised preparation that is blended with a diluent containing aluminium hydroxide, just before administration. This is given at 7 weeks of age with a second dose 2 weeks before onset of lay. This should provide maternally immune progeny through a complete laying cycle. No information on the use of DHV type II vaccine in breeder ducks is available.

Live attenuated DHV type I or type II vaccine given subcutaneously or intramuscularly to 1-day-old ducklings protects against the disease for the duration of their susceptibility. No information is available on the use of DHV type III vaccine to actively immunise 1-day-old ducklings.

Breeder ducks primed with live DHV type I at 12 weeks of age and then given a single dose of inactivated DHV type I vaccine intramuscularly at 18 weeks of age, should produce maternally immune progeny through a complete laying cycle (Woolcock, 1991).

Egg-yolk antibody offers passive immunisation in the face of an outbreak. The duration of its efficacy is short-lived.

Stability

Aqueous preparations of live attenuated DHV type I, II and III vaccines when stored frozen at −70°C or lower should remain stable for at least 1 year. Once thawed these vaccines should be held at 4°C and used within 1 week. Live lyophilised vaccines may be stored at 2–8°C and should retain their potency for at least 1 year.

The inactivated DHV type I vaccine is blended with adjuvant and can be stored at 4°C for at least 20 months without loss of immunogenicity.

Egg-yolk antibody can be stored for up to 1 year at 4°C.

3. Vaccines based on biotechnology

a) Vaccines available and their advantages
None

b) Special requirements for biotechnological vaccines, if any
Not applicable
REFERENCES


Chapter 2.3.8. — Duck virus hepatitis


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OIE Terrestrial Manual 2012 499
Fowl cholera (avian pasteurellosis) is a commonly occurring avian disease that can affect all types of birds and is distributed worldwide. Fowl cholera outbreaks often manifest as acute fatal septicaemia. Diagnosis depends on isolation and identification of the causative bacterium, Pasteurella multocida. Presumptive diagnosis may be based on the occurrence of typical signs and lesions and/or on the microscopic demonstration of myriad bacteria in blood smears, or impression smears of tissues such as liver or spleen. Mild or chronic forms of the disease also occur where the disease is endemic, with localised infection primarily of the respiratory and skeletal systems.

**Identification of the agent:** Pasteurella multocida is readily isolated, often in pure culture, from visceral organs such as lung, liver and spleen, bone marrow, gonads or heart blood of birds that succumb to the acute bacteraemic form of the disease, or from the caseous exudate characteristic of chronic fowl cholera lesions. It is a facultative anaerobic bacterium that grows best at 37°C. Primary isolation is usually accomplished using media such as dextrose starch agar, blood agar, and trypticase–soy agar. Isolation may be improved by the addition of 5% heat-inactivated serum. Colonies range from 1 to 3 mm in diameter after 18–24 hours of incubation and are discrete, circular, convex, translucent, and butyrous. The cells are coccobacillary or short rod-shaped, 0.2–0.4 × 0.6–2.5 µm in size, stain Gram negative, and generally occur singly or in pairs. Bipolar staining is evident with Wright or Giemsa stains.

Identification of *P. multocida* is based on the results of biochemical tests, which include carbohydrate fermentation, enzyme production, and selected metabolite production.

Serological characterisation of strains of *P. multocida* includes capsular serogrouping and somatic serotyping. DNA fingerprinting can differentiate among *P. multocida* having the same capsular serogroup and somatic serotype. These characterisations require a specialised laboratory with appropriate diagnostic reagents.

**Serological tests:** Serological tests are rarely used for diagnosis of fowl cholera. The ease of obtaining a definitive diagnosis through isolation and identification of the causative organism generally precludes the need for serodiagnosis.

**Requirements for vaccines and diagnostic biologicals:** The *P. multocida* vaccines in general use are bacterins, containing aluminium hydroxide or oil as adjuvant, prepared from multiple serotypes. Two doses of the killed vaccine are typically required. Live culture vaccines tend to impart greater protective immunity, but are used less frequently because of potential post-vaccinal sequelae such as pneumonitis and arthritis. Multivalent vaccines typically incorporate somatic serotypes 1, 3, and 4 as they among the more commonly isolated avian serotypes. Safety and potency testing of bacterins usually use the host animal. Final containers of live cultures are tested for potency by bacterial counts.

**A. INTRODUCTION**

Fowl cholera is a contagious bacterial disease of domesticated and wild avian species caused by infection with *Pasteurella multocida*. It typically occurs as a fulminating disease with massive bacteraemia and high morbidity and mortality. Chronic infections also occur with clinical signs and lesions related to localised infections. The pulmonary system and tissues associated with the musculoskeletal system are often the seats of chronic infection. Common synonyms for fowl cholera are avian pasteurellosis and avian haemorrhagic septicaemia. Fowl cholera is not considered to have zoonotic potential as avian isolates are generally nonpathogenic in mammals.
Diagnosis depends on isolation and identification of the causative organism. Bacterial colonisation with necrosis, fibrinosuppurative exudate, and degrees of fibroplasia is readily cultured from cases of fowl cholera. Infections often involve joints, foot pads, tendon sheaths, sternal bursa, conjunctivae, wattles, pharynx, lungs, air sacs, middle ears, bone marrow, and meninges. Lesions resulting from these infections are usually characterised by mild ascites and pericardial oedema. Birds that survive the acute septicaemic stage or those infected with organisms of low virulence may develop chronic fowl cholera, characterised by localised infections. These mild forms of the disease may occur.

All avian species are susceptible to *P. multocida*, although turkeys may be the most severely affected. The first sign of disease is dead birds. Other signs include: fever, anorexia, depression, mucus discharge from the mouth, diarrhoea, ruffled feathers, drop in egg production coupled with smaller eggs, increased respiratory rate, and cyanosis at the time of death. Lesions that are often observed include: congested organs with serosal haemorrhages, enlarged liver and spleen, multiple small necrotic areas in the liver and/or spleen, pneumonia, and mild ascites and pericardial oedema. Birds that survive the acute septicaemic stage or those infected with low virulence may develop chronic fowl cholera, characterised by localised infections. These infections often involve joints, foot pads, tendon sheaths, sternal bursa, wattles, pharynx, lungs, air sacs, middle ears, bone marrow, and meninges. Lesions resulting from these infections are usually characterised by bacterial colonisation with necrosis, fibrinosuppurative exudate, and degrees of fibroplasia. Diagnosis depends on isolation and identification of the causative organism.

### 1. Identification of the agent

*Pasteurella multocida* is a facultative anaerobic bacterium that grows best at 35–37°C. Primary isolation is usually accomplished using media such as blood agar, trypticase–soy agar or dextrose starch agar, and isolation may be improved by supplementing these media with 5% heat-inactivated serum. Maintenance media usually do not require supplemental serum. Colonies range from 1 to 3 mm in diameter after 18–24 hours of incubation. They usually are discrete, circular, convex, translucent, and butyrous. Capsulated organisms usually produce larger colonies than those of noncapsulated organisms. Watery mucoid colonies, often observed with mammalian respiratory tract isolates, are very rare with avian isolates. The cells are coccobacillary or short rod-shaped, usually 0.2–0.4 × 0.6–2.5 µm in size, stain Gram negative, and generally occur singly or in pairs. Recently isolated organisms or those found in tissue smears show bipolar staining with Wright or Giemsa stains or methylene blue, and are usually encapsulated.

Isolation of the organism from visceral organs, such as liver, bone marrow, spleen, or heart blood of birds that succumb to the acute form of the disease, and from exudative lesions of birds with the chronic form of the disease, is generally easily accomplished. Isolation from those chronically affected birds that have no evidence of disease other than emaciation and lethargy is often difficult. In this condition or when host decomposition has occurred, bone marrow is the tissue of choice for isolation attempts. The surface of the tissue to be cultured is seared with a hot spatula and a specimen is obtained by inserting a sterile cotton swab, wire or plastic loop through the heat-sterilised surface. The specimen is inoculated directly on to agar medium or into tryptose or another broth medium, incubated for a few hours, transferred to agar medium, and incubated again.

Identification is based primarily on the results of biochemical tests. Carbohydrate fermentation reactions are essential. Those carbohydrates that are fermented include: glucose, mannose, galactose, fructose, and sucrose. Those not fermented include: rhamnose, cellobiose, raffinose, inulin, erythritol, adonitol, m-inositol, and salicin. Mannitol is usually fermented. Arabinose, maltose, lactose, and dextrin are usually not fermented. Variable reactions occur with xylose, trehalose, glycerol, and sorbitol. *Pasteurella multocida* does not cause haemolysis, is not motile and only rarely grows on MacConkey agar. It produces catalase, oxidase, and ornithine decarboxylase, but does not produce urease, lysine decarboxylase, beta-galactosidase, or arginine dihydrolase. Phosphatase production is variable. Nitrate is reduced; iodide and hydrogen sulphide are produced, and methyl red and Voges–Proskauer tests are negative. Detection of hydrogen sulphide production may require lead acetate-laden paper strips suspended above a modified H₂S liquid medium (Rimler et al., 1998). Commercial biochemical test kits are available.

Differentiation of *P. multocida* from other avian *Pasteurella* spp. and *Riemerella (Pasteurella)* anatipestifer can usually be accomplished using the tests and results indicated in Table 1. Laboratory experience has shown that
Chapter 2.3.9. – Fowl cholera

*P. multocida* is most easily identified by its colony morphology and appearance in Gram stains. Positive reactions to indole and ornithine decarboxylase are the most useful biochemical indications.

### Table 1. Tests used to differentiate *Pasteurella multocida* from other avian *Pasteurella* species and *Riemerella anatipestifer*

<table>
<thead>
<tr>
<th>Test*</th>
<th><em>Pasteurella multocida</em></th>
<th><em>Pasteurella gallinarum</em></th>
<th><em>Riemerella anatipestifer</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemolysis on blood agar</td>
<td>–</td>
<td>–</td>
<td>v</td>
</tr>
<tr>
<td>Growth on MacConkey’s agar</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Indole production</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Gelatin liquefaction</td>
<td>–</td>
<td>–</td>
<td>+u</td>
</tr>
<tr>
<td>Catalase production</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Urease production</td>
<td>–</td>
<td>–</td>
<td>v</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Lactose fermentation</td>
<td>–u</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sucrose fermentation</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Maltose fermentation</td>
<td>–u</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Test reaction results: – = no reaction; + = reaction; v = variable reactions; –u = usually no reaction; +u usually a reaction.

Antigenic characterisation of *P. multocida* is accomplished by capsular serogrouping and somatic serotyping. Capsular serogroups are determined by a passive haemagglutination test (Carter, 1955; 1972). Capsular serogroups, determined by a passive haemagglutination test, are A, B, D, E, and F. All but serogroup E have been isolated from avian hosts. A nonserological disk diffusion test that uses specific mucopolysaccharidases to differentiate serogroups A, D, and F has been developed (Rimler & Glisson, 1997).

Somatic serotypes are usually determined by an agar gel immunodiffusion (AGID) test (Heddleston, 1962; Heddleston et al., 1972). Serotypes 1 through 16 have been reported; all 16 serotypes have been isolated from avian hosts (Rimler et al., 1998). The most effective characterisation involves determination of both serogroup and serotype. These determinations require a specialised laboratory with appropriate diagnostic reagents. To determine the serotype, the laboratory prepares the unknown bacterial culture as antigen for the AGID test and then must test it against all 16 serotype-specific antisera. Antigens present in a single isolate may react with multiple serotype-specific antisera resulting in bi- or trinomial serotypes, as illustrated by the 3, 4 and 3, 4, 12 strains (Rimler et al., 1998).

- **Somatic typing procedure using the gel diffusion precipitin test**
  1. Inoculate a dextrose starch agar (DSA) plate (20 × 150 mm containing 70 ml of medium or two 15 × 100 mm plates containing 20 ml of medium per plate) with cells from a pure culture of *Pasteurella multocida* by using a sterile cotton swab. Swab the entire surface of the plate(s). Incubate the plate(s) in a 37°C incubator for 18–24 hours. This procedure is used to produce antigen for positive control purposes or to prepare antigen from diagnostic cultures.
  2. Harvest the cells from the plate(s) using 2.5 ml of 0.85% saline with 0.6% formaldehyde and a sterile hockey stick. Place the cells in a tube using a sterile pipette.
  3. Autoclave the cells at 100°C for 1 hour.
  4. Centrifuge the cell suspension mixture at 13,300 g for 20 minutes.
  5. Remove the supernatant and place in a sterile tube.
  6. Prepare the agar gel for use in the gel diffusion precipitin test (GDPT) by placing 17.0 g of NaCl, 1.8 g of agar noble, and 200 ml of distilled water into a 500 ml flask. Microwave the contents of the flask with the cap loose for 2.5 minutes. Swirl the contents of the flask and microwave again for 2.5 minutes. Allow the agar to cool slightly for 10–15 minutes. Do not prepare less than 200 ml of agar in a microwave. Dehydration during the microwave process can increase the agar concentration and negatively impact or inhibit diffusion.
Chapter 2.3.9. – Fowl cholera

vii) Place 5 ml of melted agar onto the surface of a 75 × 25 mm plain glass microscope slide. It is important that the slides are level prior to dispensing the agar. Allow the agar to cool (approximately 30 minutes) completely.

viii) Wells are cut in the agar bed. The wells are 3 mm in diameter and 3 mm apart from edge-to-edge. Frequently an Ouchterlony template is used to create two or three replicates of wells per slide. Each replicate has a centre well and is surrounded by four wells located at 90° angles (from centre).

ix) Reference antiserum is always placed in the centre well (of a replicate). Antigen from a diagnostic or reference culture is placed in one of the surrounding wells within a replicate. Each well is filled to capacity.

x) The slides are incubated within a moist chamber in a 37°C incubator for 48 hours. Precipitin lines of a reaction can be best observed with subdued lighting from underneath the slide. When present, reactions should occur between the centre and surrounding well(s) as an arc of precipitin. Sometimes these reactions are close to the edge of a well. Slides should be carefully examined. Diagnostic cultures can react to more than one reference somatic antiserum.

xi) Positive controls should be used. Reference antiserum should be tested against reference antigen each time the test is performed.

DNA fingerprinting of *P. multocida* by restriction endonuclease analysis (REA) has proved valuable in epidemiological investigations fowl cholera in poultry flocks. Isolates of *P. multocida* having both capsular serogroup and somatic serotype in common may be distinguished by REA. Ethidium-bromide-stained agarose gels are analysed following electrophoresis of DNA digested with either *Hha*I or *Hpa*I endonuclease (Wilson *et al.*, 1992).

2. Serological tests

Serological tests for the presence of specific antibodies are not used for diagnosis of fowl cholera. The ease of obtaining a definitive diagnosis by isolation and identification of the causative organism precludes the need for serodiagnosis. Serological tests, such as agglutination, AGID, and passive haemagglutination, have been used experimentally to demonstrate antibody against *P. multocida* in serum from avian hosts; none were highly sensitive. Determinations of antibody titres using enzyme-linked immunosorbent assays have been used with varying degrees of success in attempts to monitor seroconversion in vaccinated poultry, but not for diagnosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Fowl cholera may be caused by any of 16 Heddleston serotypes of *P. multocida*, although certain serotypes appear to be more often associated with disease. The *P. multocida* vaccines in general use are bacterins, containing aluminium hydroxide or oil adjuvant, prepared from inactivated cells of serotypes selected on the basis of epidemiological information. Commercial bacterins are usually composed of serotypes 1, 3, and 4. Vaccination plays a significant role in the control of this disease. Live vaccines containing modified *P. multocida* are not generally used except in North America.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

Bacterin is normally administered by intramuscular injection in the leg or breast muscles, or subcutaneously at the back of the neck. Two doses are typically administered at 2–4-week intervals. As with most killed vaccines, full immunity cannot be expected until approximately 2 weeks after the second dose of a primary vaccination course. Live vaccines are typically administered in the drinking water. Vaccination of diseased birds or those in poor nutritional status should be avoided as a satisfactory immune response may not be generated in such circumstances.

1. Method of manufacture

The general method for production of *P. multocida* bacterins is presented here. Production cultures of each bacterial isolate to be included in the final product are prepared. The cultures are typically started in small vessels and subpassaged into progressively larger volumes of media until the desired production volume is achieved. Each production culture is inactivated by formalin or other acceptable means. All of the component cultures are mixed, and usually blended, with an adjuvant prior to filling sterile final containers.
Chapter 2.3.9. — Fowl cholera

The following section is based on the requirements for *P. multocida* bacterins and vaccines as found in Title 9, United States Code of Federal Regulations (CFR). Other countries may have slightly different requirements.

2. **Master seed management**

   a) **Characteristics of the seed**

   All strains of *P. multocida* to be incorporated into a bacterin or vaccine must be well characterised, of known serotype, pure, safe and immunogenic. The culture(s) that is evaluated and characterised is designated by lot number and called a master seed. All cultures used in the production of licensed bacterins or vaccines must be derived from an approved master seed(s) and must be within an accepted number of passages from the master seed lot.

   b) **Validation as a vaccine**

      i) **Efficacy**

      Products prepared from candidate master seeds must be shown to be effective against challenge infection. Efficacy must be demonstrated in each animal species (chickens, turkeys, ducks, psittacines) and by each route of administration for which the product will be recommended, and protection must be demonstrated against each challenge serotype for which protection is claimed. The lot of product used to demonstrate efficacy must be produced from the highest allowable passage of master seed.

      For live avian *Pasteurella* vaccines, 20 vaccinates and 10 controls are used in each efficacy trial. Birds are challenged not less than 14 days after vaccination and are observed for 10 days after challenge. A satisfactory test requires that at least eight of the controls die and at least 16 of the vaccinates survive.

      The arithmetic mean count of colony-forming units in the lot of product that is used to demonstrate efficacy is used as the minimum standard (immunogenicity standard) for all subsequent production lots of vaccine.

      Efficacy of bacterins must be demonstrated similarly prior to licensure. However, no immunogenicity standards are derived from the lot that was used to demonstrate initial efficacy; each production lot is satisfactorily tested in a vaccination-challenge trial prior to release for sale and distribution.

      ii) **Safety**

      The safety of master seeds used in the production of live vaccines must be evaluated prior to licensing. Safety must be tested in each animal species (chickens, turkeys, ducks, psittacines) for which the product is recommended. Each of 10 birds is given an equivalent of 10 vaccine doses and observed for 10 days. At least 8 of 10 birds must show no unfavourable reactions attributable to the master seed.

      Additionally, the master seeds must be tested for reversion to virulence and evaluated for excretion from the host and transmission to other target species.

      The safety of each production lot is tested by methods described in Section C.4.c.

3. **In-process control**

   The purity of the cultures is determined at each stage of production prior to inactivation. This may be achieved by microscopic examination (e.g. phase–contrast microscopy, Gram strain) and/or by culture. Killed cultures are tested for completeness of inactivation. Analytical assays to determine the levels of formaldehyde or other preservatives are done on bulk vaccine and must be within specified limits. During manufacturing, production parameters must be tightly controlled to ensure that all serials (batches) are produced in the same manner as that used to produce the serials used for immunogenicity studies.

4. **Batch control**

   a) **Sterility**

   Sterility tests are done on filled vaccine. Each lot must pass sterility requirements, for example those detailed in the 9 CFR Part 113.26 or 113.27 (CFR, 2001). (See also Chapter 1.1.7 *Tests for sterility and freedom from contamination of biological materials*.)

   b) **Safety**

   Safety testing is conducted on each bulk or filled vaccine lot. Live vaccines are tested according to the method described in C.1.c.ii, except that only one representative animal species is required. Bacterins are administered according to label recommendations, and the birds are observed for 14 days; at least 18 of 20 birds must show no unfavourable reactions attributable to the bacterin.
c) **Potency**

Each production lot of bacterin or live vaccine must be tested for potency by a test that is related to, and considered predictive of, efficacy. Potency tests are performed on the product in its final form.

Bacterins are tested for potency in a vaccination-challenge trial. Separate groups of birds (20 vaccinates, 10 controls) must be challenged with each of the serotypes of *P. multocida* for which protection is claimed. Bacterins are administered according to the dose and route recommended on the label. Two doses are administered 3 weeks apart, and all birds are challenged 2 weeks after the second dose. The birds are observed for 14 days after challenge. For a satisfactory test, at least 14 of 20 vaccinates must survive and at least 8 of 10 controls must die.

The potency of live vaccine lots is determined by a bacterial count performed on reconstituted lyophilised product in its final container. The mean bacterial count of any vaccine lot at the time of preparation must be sufficiently high to ensure that at any time prior to product expiration, the count is at least twice the immunogenicity standard. (The European Pharmacopoeia requires a count that is at least equal to the immunogenicity standard.)

d) **Stability**

The acceptability of the shelf life of a vaccine is confirmed by testing the product for potency at the end of the approved shelf life. At least three lots of vaccine are tested and must pass established potency requirements. Vaccines are stored at 2–7°C and protected from freezing. Partly used packs should be discarded at the end of a day’s operations.

e) **Preservatives**

Any preservatives must be added within specified limits. Preservatives are generally added to vaccines to limit the growth of any contaminants introduced when the rubber cap is pierced with a needle. Ideally, multidose vaccination equipment should be used whereby the vaccine pack is entered only once with a sterile needle.

f) **Precautions (hazards)**

Vaccines prepared with aluminium-based adjuvants may cause temporary nodules at the site of injection. Operator self-injection poses no immediate problems, but medical advice should be sought as there is a risk of infection via a contaminated needle.

Vaccines prepared with oil based adjuvants may cause more severe reactions at the site of injection, which may manifest as large nodules. Care should be taken to administer these vaccines correctly. Operator self injection requires immediate medical attention, involving prompt incision and irrigation of the site.

5. **Tests on final product**

a) **Safety**

See Section C.4.b.

b) **Potency**

See Section C.4.c.

**REFERENCES**


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CHAPTER 2.3.10.

FOWLPOX

SUMMARY

Fowlpox is a disease of chickens and turkeys caused by a DNA virus of the genus Avipoxvirus of the family Poxviridae. Its distribution is world-wide. It is slow-spreading and characterised by the formation of proliferative lesions and scabs on the skin, and diphtheritic lesions in the upper parts of the digestive and respiratory tracts. In the case of the cutaneous form, the mortality rate is usually low and affected birds are more likely to recover than those with the diphtheritic form. In the diphtheritic form, proliferative lesions involving the nasal passages, larynx or trachea can result in respiratory distress and death from suffocation.

Fowlpox causes a transient drop in egg production and a reduced growth rate in young birds.

Identification of the agent: Fowlpox should be suspected where skin eruptions occur on exposed areas. Histological examination of cutaneous or diphtheritic lesions reveals epithelial hyperplasia with intracytoplasmic inclusions in affected cells. Elementary bodies may be detected in smears from lesions by the use of the Gimenez method. Electron microscopy of lesions will detect virus particles with the characteristic poxvirus morphology by negative staining or in ultrathin sections of the lesion.

The diphtheritic form of fowlpox involving the trachea must be differentiated from infectious laryngotracheitis, which is caused by a herpesvirus and is characterised by the presence of intranuclear inclusion bodies.

Virus isolation is done by inoculation on to chorioallantoic membranes of 9–12-day-old developing chicken embryos or avian cell cultures.

Serological tests: Immune responses to fowlpox virus may be demonstrated by the use of virus neutralisation, agar gel immunodiffusion, immunofluorescence, or passive hemagglutination tests, enzyme-linked immunosorbent assay and by immunoblotting.

Requirements for vaccines and diagnostic biologicals: Modified live fowlpox or pigeon pox virus vaccines of chicken embryo or avian cell culture origin are available commercially. The use of vaccines is indicated in areas where the disease is endemic, or on premises where infection has been diagnosed.

A. INTRODUCTION

The morphology of the fowlpox virus is like that of other viruses of the poxviridae family. The mature virus (elementary body) is brick shaped and measures about 330 × 280 × 200 nm. The outer coat is composed of random arrangements of surface tubules. The virion consists of an electron-dense centrally located biconcave core or nucleoid with two lateral bodies in each concavity and surrounded by an envelope. The 288 kbp fowlpox virus genome encodes for over 250 genes.

Fowlpox has a world-wide distribution and is caused by a DNA virus of the genus Avipoxvirus of the family Poxviridae (Tripathy, 1993; Tripathy & Reed, 2003). Its incidence is variable in different areas because of differences in climate, management and hygiene or the practice of regular vaccination. It can cause drops in egg production, or retarded growth in younger birds.

Fowlpox is a slow-spreading virus disease of chickens and turkeys, characterised in the cutaneous form (dry pox) by the development of proliferative lesions, ranging from small nodules to spherical wart-like masses on the skin of the comb, wattle and other unfeathered areas. In the diphtheritic form (wet pox), slightly elevated white opaque
nodules develop on the mucous membranes. They rapidly increase in size to become a yellowish diphtheritic membrane. Lesions occur on the mucous membranes of the mouth, oesophagus, larynx or trachea. The mortality rate is higher in the diphtheritic form than in the cutaneous form, sometimes nearing 50% particularly in young birds. Integration of reticuloendotheliosis virus (REV) sequences has been observed in the genome of fowlpox virus (Singh et al., 2000; 2003b). It is interesting that this insertion event occurred over 50 years ago (Kim & Tripathy, 2001). While most field strains contain REV provirus, vaccine strains have only remnants of long terminal repeats (Singh et al., 2003b). Virulence is enhanced by the presence of REV provirus in the genome of field strains of fowlpox virus. Complete sequence of the genome of a vaccine-like strain of fowlpox virus has been determined (Afonso et al., 2000). The functions of the majority of the genes are not known at this time. It is however, interesting that the virus tends to persist in the poultry environment for extended periods of time where other viruses may not survive. In this regard the presence of photolyase gene and A-type inclusion body gene in the virus genome appear to protect the virus from environmental insults (Srinivasan et al., 2001; Srinivasan & Tripathy, 2005). Antigenic cross-reactivity is observed among avianpox viruses and it appears that many genes are conserved. Limited studies on antigenic, genetic and biologic comparison of fowlpox virus with other avianpox viruses especially those that infect the wild birds are available. Recently, complete sequence of canarypox virus genome has become available.

B. DIAGNOSTIC TECHNIQUES

Identification of the agent

Fowlpox virus multiplies in the cytoplasm of epithelial cells with the formation of large intracytoplasmic inclusion bodies (Bollinger bodies) that contain smaller elementary bodies (Borrel bodies). The inclusions can be demonstrated in sections of cutaneous and diphtheritic lesions by the use of haematoxylin and eosin (H&E), acridine orange or Giemsa stains (Tripathy et al., 1973). The elementary bodies can be detected in smears from lesions, for example by the Gimenez method (Tripathy & Hanson, 1976), which is described below. Electron microscopy can be used to demonstrate viral particles of typical poxvirus morphology by negative staining or in ultrathin sections of infected tissues (Doane & Anderson, 1987).

**a) A smear technique for fowlpox**

i) Place a drop of distilled water and the lesion (cutaneous or diphtheritic) on a clean slide. Prepare a thin smear by pressing the lesion with another clean slide and rotating the upper slide several times.

ii) Air dry and gently fix the smear over a flame.

iii) Stain the smear for 5–10 minutes with freshly prepared primary stain (8 ml stock solution\(^1\) of basic fuchsin mixed with 10 ml of phosphate buffer\(^2\), pH 7.5, and filtered through Whatman filter paper No. 1).

iv) Wash thoroughly with tap water.

v) Counterstain with malachite green (0.8% in distilled water) for 30–60 seconds.

vi) Wash the smear with tap water and then dry.

vii) Examine the smear under oil immersion. The elementary bodies appear red and are approximately 0.2–0.3 µm in size.

**b) Virus isolation**

Fowlpox virus can be isolated by the inoculation of suspected material into embryonated chicken eggs. Approximately 0.1 ml of tissue suspension of skin or diphtheritic lesions, with the appropriate concentration of antibiotics, is inoculated on to the chorioallantoic membranes (CAMs) of 9–12-day-old developing chicken embryos. These are incubated at 37°C for 5–7 days, and then examined for focal white pock lesions or generalised thickening of the CAMs. Histopathological examination of the CAM lesions will reveal eosinophilic intracytoplasmic inclusion bodies following staining with H&E (Tripathy et al., 1973; Tripathy & Reed, 1998).

Primary chicken embryo fibroblasts, chicken embryo kidney cells, chicken embryo dermis cells, or the permanent quail cell line QT-35, can also be used to propagate fowlpox virus (Ghildyal et al., 1989; 1992).

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\(^1\) Stock solution: A solution of basic fuchsin (5 g) in 95% ethanol (100 ml) is slowly added to a second solution of crystalline phenol (10 g) in distilled water (900 ml). This stock solution, kept in a tightly screw-capped glass bottle, is incubated for 48 hours at 37°C, and then stored at room temperature.

\(^2\) Phosphate buffer, pH 7.5: \(\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}\) (2.47 g) and \(\text{Na}_2\text{HPO}_4\) (11.65 g) are added to distilled water (1000 ml) and stored at 4°C.
Chapter 2.3.10. – Fowlpox

Schnitzlein et al., 1988). The adaptation of virus strains to cell cultures is an important requirement for plaque formation, as not all strains will form plaques initially.

c) Molecular methods

Restriction fragment length polymorphism (RFLP) analysis can be used for comparison of field isolates and vaccine strains of fowlpox virus (Ghildyal et al., 1989; Schnitzlein et al., 1988). However, this procedure is not used in routine diagnosis.

Cloned genomic fragments of fowlpox virus can be used effectively as nucleic acid probes for diagnosis of fowlpox. Viral DNA isolated from lesions can be detected by hybridisation either with radioactively or nonradioactively labelled genomic probes. This method is especially useful for differentiation of fowlpox from infectious laryngotracheitis when tracheal lesions are present (Fatunmbi et al., 1995).

Genomic DNA sequences of various sizes can be amplified by the polymerase chain reaction (PCR) using specific primers (Fallavena et al., 2002; Lee & Lee, 1997). This technique is useful when there is only an extremely small amount of viral DNA in the sample.

2. Serological tests

Although both cell-mediated immunity (CMI) and humoral immunity play an important role in poxvirus infections, routine use of the CMI test is not convenient. Therefore, serological tests, such as virus neutralisation (VN), agar gel immunodiffusion (AGID), passive haemagglutination and fluorescent antibody tests as well as the enzyme-linked immunosorbent assay (ELISA), are used to measure specific humoral antibody responses. Evidence of successful immunisation with vaccine can be determined by examining a flock 7–10 days after vaccination for ‘takes’. A take consists of a swelling of the skin or a scab at the site where the vaccine was applied and its presence is evidence of successful immunisation.

a) Virus neutralisation

After virus/serum interaction, the residual virus activity may be assayed in embryonating chicken eggs or in cell cultures (Morita, 1973). This technically demanding test may not be convenient for routine diagnosis. Only some selected strains of the virus have plaque-forming ability in chicken embryo cells. Neutralising antibodies develop within 1–2 weeks of infection.

b) Agar gel immunodiffusion

Precipitating antibodies can be detected by reacting test sera against viral antigens. The antigen can be derived by sonication and homogenisation of infected skin or CAM lesions as well as by treatment of infected cell cultures as described in Section B.2.f below. The lysed suspension is centrifuged and the supernatant is used as antigen. Gel-diffusion medium is prepared with 1% agar, 8% sodium chloride and 0.01% thiomersol. The viral antigen is placed in the central well and the test sera are placed in the peripheral wells. It is important to include a positive and negative control serum. The plates are incubated at room temperature. Precipitation lines develop in 24–48 hours after incubation of the antigen with antibody to homologous or closely related strains. The test is less sensitive than the ELISA (Buscaglia et al., 1985) or the passive haemagglutination test (Winterfield & Hitchner, 1965).

c) Passive haemagglutination

Tanned sheep or horse red blood cells are sensitised with a partially purified fowlpox viral antigen (Tripathy et al., 1970). The antigen is prepared from infected CAMs or cells as described in Section B.2.f below. Passive haemagglutination is more sensitive than AGID. The test will give cross-reactions among avian pox viruses.

d) Fluorescent antibody tests

Direct or indirect immunofluorescence tests will reveal specific intracytoplasmic fluorescence in infected cells. The latter test is commonly used and involves two steps: the antibody against fowlpox virus is reacted with the antigen in the infected cells, followed by a secondary fluorescein-isothiocyanate-labelled antibody against chicken gamma globulin (e.g. goat anti-chicken). Such labelled antibodies are available commercially. In this regard, formalin-fixed tissue sections can be used effectively for fluorescent antibody tests.

e) Immunoperoxidase

Specific staining of cytoplasmic inclusions is achieved when horseradish-peroxidase-conjugated specific polyclonal antibody against fowlpoxvirus is reacted with the hydrated sections of fowlpox-infected fixed
tissues (CAM and skin) or cell culture. Similar results are obtained when either polyclonal or monoclonal antibodies are used in an indirect test. An advantage of the technique is that the sections can be examined with the light microscope and can be stored for an extended period without loss of colour (Tripathy et al., 1973).

f) Enzyme-linked immunosorbent assay

ELISAs have been developed to detect humoral antibodies to fowlpox virus. They are capable of detecting antibody 7–10 days after infection (Buscaglia et al., 1985), but commercial kits for this test are not available.

Fowlpox virus antigens are prepared either from infected QT-35 cell monolayers or CAM lesions. Infected QT cells are pelleted (700 g for 10 minutes at 4°C), washed with isotonic buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 5 mM ethylene diamine tetra-acetic acid [EDTA]) followed by lysis in hypotonic buffer (10 mM Tris, pH 8.0, 10 mM KCl, 5 mM EDTA) containing 0.1% Triton X-100 and 0.025% beta-mercaptoethanol. Nuclei and cellular debris are removed by low-speed centrifugation (500 g for 5 minutes at 4°C) and the resulting supernatant is used as a source of fowlpox virus antigens for ELISA or immunoblotting. To isolate viral antigen from CAM lesions, initial grinding of the lesions with subsequent detergent treatment as described earlier would be required. Virus propagated in chicken embryo fibroblasts and chicken embryo dermis cells has also been used for antigen. The antigen preparation is as described for QT cells.

Wells of microtitre plates are coated with 1 µg of soluble fowlpox virus antigen in 100 µl of coating buffer (15 mM Na2CO3, 35 mM NaHCO3, pH 9.6) and incubated overnight at 4°C (Buscaglia et al., 1985; Tripathy et al., 1973). Each well is then rinsed once with wash solution (0.29 M NaCl, 0.05% Tween 20) and then blocked with phosphate buffered saline (PBS, pH 7.4) containing 3% bovine serum albumin (BSA) for 1 hour at 37°C. After one wash, serial dilutions of the test sera in PBS containing 1% BSA are added to the wells. After rocking for 2 hours at 37°C, the wells are washed three times prior to the addition of 100 µl/well horseradish-peroxidase-conjugated goat anti-chicken IgG (H+L) antibodies at a recommended dilution in PBS. After 2 hours' incubation at 37°C and three subsequent washes, 100 µl of the peroxidase substrate TMB3 is added to each well. Reactions are terminated by the addition of 1 M phosphoric acid and absorbance at 450 nm is recorded using an ELISA plate reader.

g) Immunoblotting

Antigenic variations between strains of fowlpox virus can be evaluated by means of immunoblotting or Western Blotting. In this method, viral antigens separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) are reacted either with polyclonal or monoclonal antibodies against fowlpox virus (Ghildyal et al., 1989; Singh et al., 2003a; Singh & Tripathy, 2000). This method is not convenient for routine diagnosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Early studies indicated the feasibility of protecting chickens from fowlpox by the use of pigeon pox or fowlpox viruses (Tripathy & Reed, 2003; Winterfield & Hitchner, 1965). Vaccination is indicated in areas where fowlpox is endemic or on premises where infection has been diagnosed previously. Live fowl and pigeon pox virus vaccines, and also fowlpox vectored vaccines that protect against pox, are available commercially. These vaccines are derived from chicken embryos or avian cell cultures.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

Passively acquired immunity should be taken into consideration during vaccination of progeny from flocks that have either had a recent natural infection or been recently vaccinated. As passive immunity (for 2–3 weeks) may interfere with vaccine virus multiplication, such progeny should be vaccinated only after the decline of passively acquired antibody. Fowlpox vaccine is applied by a wing stab method.

1. Seed management

a) Characteristics of the seed

A master seed virus (MSV) must be established and used according to a seed-lot system. A record must be kept of its origin, passage history and characteristics. Viruses used may be either fowlpox or pigeon pox.

3 Kirkegaard and Perry Laboratories, Gaithersberg, Maryland, United States of America.
4 Dynatech, Chantilly, Virginia, United States of America.
viruses. The MSV must be propagated in suitable premises with materials that meet approved standards, and must be tested for freedom from contamination as well as for identity and purity.

b) Method of culture
The MSV may be propagated in specific pathogen free (SPF) chicken embryos, using the CAMs, or in avian cell cultures, such as primary chicken embryo fibroblasts, chicken embryo kidney or chicken embryo dermis.

c) Validation as a vaccine
i) Purity
The MSV may be neutralised with a specific hyperimmune serum before testing for purity. Because of difficulty in neutralising avian pox virus, it is acceptable to centrifuge the MSV at 1000 \( g \) for 20 minutes, followed by filtration through a 0.2 µm filter. The neutralised or filtered MSV is then used in tests to demonstrate freedom from extraneous agents. These tests should be done in embryonating eggs or avian cell cultures, to demonstrate absence of extraneous virus replication, and in SPF chickens, to demonstrate freedom from antibodies to extraneous agents.

ii) Safety
Vaccines should be prepared only from virus that is a stable attenuated strain or a naturally occurring isolate of low virulence.

The vaccine must be shown to be safe by the recommended route of administration, which is wing web stab, in all ages of susceptible birds. A suitable test is to take ten SPF chickens and inoculate each by piercing the wing web with a needle dipped in the vaccine. The birds are observed for 7–10 days for evidence of ‘takes’ and for the absence of adverse effects attributable to the vaccine. A ‘take’ consists of swelling of the skin or a scab at the site where the vaccine was applied and is evidence of successful vaccination. The safety test should be repeated after at least six serial passages of the virus in SPF chickens to show that there has been no reversion to virulence.

iii) Efficacy
Data should be obtained using the highest passage level (fifth passage from the master seed) and the lowest titre of virus to be used in the final product: 20 SPF chickens of the minimum age indicated for vaccination should receive one dose of vaccine by the recommended method. The chickens, together with 20 unvaccinated chickens of the same age and source, should be challenged 3 weeks later by scarification with a virulent strain of fowlpox virus. The birds should be observed for 3 weeks. Ninety per cent of the control birds should develop lesions due to the challenge virus and at least 90% of the vaccinated birds should remain free from such lesions.

2. Method of manufacture
Vaccine is manufactured on a seed-lot system from the validated MSV. This must be done in approved premises designed to avoid the risk of contamination. All media and cell cultures must be tested to ensure freedom from contamination.

3. In-process control
During the process of validation as a vaccine, the efficacy data must be compared to the virus content of the vaccine. A suitable potency can thus be established. The vaccine should be filled into final containers to ensure that each container has sufficient virus to achieve the specified potency.

4. Batch control
a) Sterility
Tests for sterility and freedom from contamination of biological materials may be found in the chapter 1.1.7.

b) Safety
The safety test described in Section C.1.c.ii above, except the requirement for six passages in SPF chickens, should be used on each batch of vaccine.
c) **Potency**

Virus content tests should be carried out using each of at least three containers. The dilutions should span 0–100% infection range, using five-fold dilution steps and at least seven replicates per dilution. Tests should be done in parallel with a standard vaccine, if available. Each lot of vaccine should be titrated in the diluent provided for its use. The virus titre should not normally be higher than 1/10 of the dose at which the vaccine has been shown to be safe and must not be lower than the release titre determined in the test for efficacy. A suitable potency for an attenuated live fowlpox vaccine is likely to be in the region of $10^5$ EID$_{50}$ (50% embryo infective dose) per ml.

d) **Duration of immunity**

The efficacy test given in Section C.1.c.iii may be used to determine the duration of immunity (approximately 6–12 months) by testing at intervals after vaccination, using separate groups of birds for each test.

e) **Stability**

Evidence of stability must be presented to justify the shelf life. This should be based on virus titrations carried out at intervals until 3 months beyond the requested shelf life on at least six batches of vaccine kept under recommended storage conditions.

f) **Preservatives**

Preservatives are not used in live vaccines.

g) **Precautions (hazards)**

It is usually recommended not to vaccinate birds that are in lay. Avoid human contact with the live vaccine. Standard fowlpox vaccine is not to be used in pigeons, though they can be vaccinated with pigeon pox vaccine. In many countries, pigeon pox vaccine has been superseded by attenuated live fowlpox vaccine designed for use in day-old chicks. These products have been safely used in pigeons in the absence of an available pigeon pox vaccine.

5. **Tests on the final product**

a) **Safety**

The safety test described in Section C.1.c.ii above is used on each batch.

b) **Potency**

The potency test described in Section C.4.c above is used on each batch.

**ACKNOWLEDGMENT**

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**REFERENCES**


Chapter 2.3.10. – Fowlpox


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CHAPTER 2.3.11.

FOWL TYPHOID AND PULLORUM DISEASE

SUMMARY

Pullorum disease of chickens is a bacterial infection caused by Salmonella enterica subspecies enterica serovar Gallinarum biovar Pullorum (Salmonella Pullorum)\(^1\). At this time the serovar is referred to as Gallinarum in some parts of the world and Pullorum in others; in this chapter the serovar will be referred to as Gallinarum or Pullorum according to the biovar under discussion as this is more meaningful from a clinical and epidemiological perspective.

In its acute form, Pullorum disease is almost exclusively a septicaemic disease of young chickens. However, the organism may also be associated with disease in turkey poults and may be carried subclinically or lead to reduced egg production and hatchability plus a range of atypical signs in older birds. Ovarian transmission is a major route by which the organism can spread. Game birds and ‘backyard’ poultry flocks may act as reservoirs of infection, and wild birds may act as vectors for the organism and as such are important in the epidemiology of the disease.

Fowl typhoid in chickens and turkeys is caused by S. Gallinarum biovar Gallinarum and is more often observed in the later growing period and in mature stock. Disease is often characterised by rapid spread with high morbidity and acute or subacute mortality. Red mites may be involved in the transmission of disease and persistence in poultry houses.

Clinical signs in chicks and poults include anorexia, diarrhoea, dehydration, weakness and death. In mature birds Pullorum disease is less severe but decreased egg production, poor hatchability and some increased mortality may occur. Fowl typhoid is a more acute septicaemic condition which mainly affects mature birds and may be particularly severe in commercial laying flocks.

Identification of the agent: Samples should not be taken from birds or eggs that have recently been treated with antimicrobial drugs. Swabs or aseptically collected samples from infected tissues, or intestinal and cloacal contents should be used for diagnostic testing. Other materials that may be sampled include eggs, embryos, faecal droppings and hatcher debris, especially fluff, dust and broken eggshells and chick box linings. Samples of tissues such as caecal tonsils and spleen from infected birds are preferable to faecal and environmental samples. Tissue samples should be inoculated into non-selective and selective enrichment broths and on selective agar medium, such as brilliant green agar, as soon as possible after collection. In case of delay, samples should be stored at 4°C. Typical colonies can be identified by serological and biochemical tests. Molecular approaches can also be used to identify and differentiate S. Gallinarum and S. Pullorum. Final serological confirmation of suspect isolates can normally only be completed in a Salmonella Reference Typing Laboratory.

Serological tests: These are satisfactory for identifying the presence and estimating the prevalence of infection within a flock. The test used in the field is the rapid whole blood plate agglutination test. This test is unreliable in turkeys and ducks as many uninfected birds may give positive reactions. In the laboratory a serum agglutination test is used, either as a rapid plate test or as a tube test. These can be applied as macro- or microagglutination tests though the latter may be more likely to give false-positive results with turkey sera. Any positive reactors should be confirmed as being infected by culture at post-mortem examination. Enzyme-linked immunosorbent assays have been reported but no commercial test is available.

The use of vaccines to control S. Enteritidis or S. Gallinarum infections in chickens may cause problems in the interpretation of serological results.

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\(^1\) See the note in Chapter 2.9.9 Salmonellosis for the principles followed concerning the nomenclature of Salmonella.
Chapter 2.3.11. – Fowl typhoid and Pullorum disease

Requirements for vaccines: Live and inactivated vaccines are available for fowl typhoid in some countries. The most commonly used vaccine is a commercial live vaccine derived from the stable rough strain of S. Gallinarum known as ‘9R’.

A. INTRODUCTION

Fowl typhoid and pullorum disease, caused by Salmonella enterica subspecies enterica serovars Gallinarum biovars Gallinarum and Pullorum, respectively, are widely distributed throughout the world but they have been eradicated from commercial poultry in many developed countries of Western Europe, the United States of America (USA), Canada, Australia and Japan. In the United States and the United Kingdom the serovar is referred to as Pullorum (Hitchner, 2004), even though the strains are now considered to be the same serovar that is derived from S.Enteritidis by gene deletion events (Thomson et al., 2008); in this chapter the terms serovar Gallinarum or Pullorum will be used, as this more usefully distinguishes the two biovars that cause clearly distinct clinical syndromes and are therefore epidemiologically different. S. Gallinarum has recently recurred in some European countries (Ivanics et al., 2008).

Salmonella Pullorum remains as a constant reservoir in wild and game birds. Salmonellosis caused by Salmonella bongori or subspecies of Salmonella enterica is covered in chapter 2.9.9 of this Terrestrial Manual.

Clinical signs of fowl typhoid are typical of a septicaemic condition in poultry and include increased mortality and poor quality in chicks hatched from infected eggs. Older birds show signs of anaemia, depression, laboured breathing and diarrhoea causing adherence of faeces to the vent. The highest mortality in pullorum disease occurs in birds of 2–3 weeks of age. In older birds disease may be mild or inapparent. In breeding and laying flocks susceptibility is increased at the point of lay (Wigley et al., 2005), but reduced egg production and hatchability may be the only signs of S.Pullorum. Trans-ovarian infection resulting in infection of the egg and hatched chicks or poults is one of the most important transmission routes for both diseases.

Post-mortem signs of pullorum disease in newly hatched chicks are those of peritonitis with generalised congestion of tissues and an inflamed unabsorbed yolk sac. Longer standing infections commonly lead to typhlitis with development of necrotic caecal casts and small necrotic foci in the liver, lungs and other viscera. Small lesions in the liver and spleen of Pullorum-infected birds may show a ‘white spot’ appearance that is not seen with Gallinarum; however, this lesion is not pathognomic. These Salmonella are very poor at colonisation and survival in the gastrointestinal tract is often indicative of later stages of clinical disease. Adult birds may develop misshapen or shrunken ovaries with follicles attached by pedunculated fibrous stalks. Variant strains of S. Pullorum do not normally cause clinical disease or may result in mild, nonspecific signs but may lead to seroconversion.

In fowl typhoid, as well as generalised signs of septicaemia, the liver is usually enlarged, dark and friable with a distinctive coppery bronze sheen that may only develop after exposure to air. The bone marrow is also often dark brown. Although clinical signs and post-mortem findings of pullorum disease and fowl typhoid may be highly suggestive of the conditions, they are not sufficiently distinct from other causes of septicaemia to be pathognomic. It is therefore necessary to confirm disease by isolation of the organisms. Serological tests can be used to establish the presence of the disease in a flock.

• Zoonotic risk and biosafety requirements

Salmonella Gallinarum and S. Pullorum are host adapted to avian species (Eswarappa et al., 2009) and are considered to pose a minimal zoonotic risk (Shivaprasad, 2000), although the genome is continually evolving, which could theoretically widen the host range in future (Liu et al., 2002). Non-typhoidal Salmonella serovars are normally handled in the laboratory at containment level (CL) 2.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

• Bacteriological culture methods

In the acute stages of disease, the agent of both diseases can be recovered from almost all organs, tissues and faeces. In older birds that have become carriers, S. Pullorum is most commonly recovered from the ova and oviduct; and it is recovered only occasionally from other organs and tissues, including the alimentary tract. In the
acute phase of fowl typhoid the organism is also widely distributed, but in carrier birds, the organism is found most often in the liver, spleen and reproductive tract, and occasionally in the caecal tonsils.

Salmonella Pullorum and S. Gallinarum belong to the Kauffmann–White scheme serogroup D, along with S. Enteritidis, which is closely related (Grimon & Weill, 2007). The organisms are Gram negative non-sporogenic rods 1.0–2.5 µm in length and 0.3–1.5 µm in width. They are considered to be non-motile under normal conditions but inducement of flagellar proteins and motility has been shown in some strains of S. Pullorum when grown in special media (Holt & Chaubal, 1997).

For optimal recovery of the organisms, the birds being sampled should not have been treated with antimicrobial drugs for approximately 2–3 weeks previously.

 Samples may be obtained from live birds, preferably after identifying them as highly sero-positive birds. Fresh or freshly chilled carcasses, egg materials, fresh faeces, or any contaminated materials from housing, incubators or transport boxes may also be taken, but faecal and environmental samples often fail to reveal the presence of the organism because of inconsistent shedding and the poor sensitivity of bacteriological detection methods. Swabs may be taken from the cloaca of sick live birds but post-mortem tissues are preferable. Samples from visibly abnormal tissues are preferred, but aseptically gathered samples can also be taken from the spleen, liver, gall-bladder, kidneys, lungs, heart, ova, testes, alimentary tract or joint lesions. The preferred tissues for routine investigation are liver, ileo-caecal junction and ovaries/oviduct. The surface is seared with a hot spatula and a sample is obtained by inserting a sterile cotton swab or sterile loop through the heat-sterilised surface. The demonstration of infection in serological reactor birds that are apparently normal may, in some cases, require the culture of large volumes of homogenised tissues as well as direct swabbing. Tissue pools may be made from tissues collected from a number of birds.

When floor litter, faecal material or the hatchery is sampled, it should be remembered that S. Pullorum and S. Gallinarum in low numbers associated with subclinically infected carrier birds are more difficult to isolate from faecal and environmental samples than other salmonellae and it is always preferable to culture sick or recently deceased birds. Red mites associated with poultry which are infected with S. Pullorum often contain the organism after feeding and can be cultured. These samples should be cultured by direct inoculation of a selective enrichment broth such as selenite cysteine or selenite F, followed by plating on selective media such as brilliant green agar (Parmar & Davies, 2007; Proux et al., 2002).

Both S. Pullorum and S. Gallinarum grow well in pure culture on non-selective media, but selective and enrichment media have been described that contain substances to inhibit the growth of extraneous organisms. Salmonella Pullorum may grow slowly and produce very small colonies on selective media so incubation of plates for 48 hours is recommended. The efficiency of recovery of Salmonella varies according to circumstances, and experience in the use of a medium is an important but unquantifiable factor. Some complex media may have an inhibitory effect on these organisms, so that it is advisable to use both non-selective and selective media for isolation from tissues. Both solid media and broths can be employed. As the toxic properties of selective media may vary, it is preferable to monitor these by comparing growth of control cultures on both types of medium. The inhibitory media should grow at least 75% of the colonies of the corresponding non-inhibitory medium (Ellis et al., 1976; Mallinsen & Snoeyenbos, 1989).

All the media mentioned below are examples of commonly used media, but there are many others found to be equally satisfactory.

Non-inhibitory media include nutrient agar and blood agar, on which colonies are seen to be smooth, translucent, slightly raised, and about 1–2 mm in diameter. Salmonella Gallinarum grows more rapidly than S. Pullorum and produces larger colonies with a distinctive smell resembling that of seminal fluid on most media. Broths include buffered peptone water and nutrient and meat infusion broths or universal pre-enrichment broth.

- **Selective media:**

  *MacConkey agar*: the agar is inhibitory to non-enteric organisms; it differentiates lactose fermenters (pink colonies) from non-lactose fermenters (colourless colonies). NaCl is omitted to limit the spread of Proteus colonies. Salmonella colonies are smooth and colourless. Salmonella Pullorum produces smaller colonies than other salmonellae. MacConkey is the agar of choice for direct plating from tissues.

  *Xylose lysine deoxycholate agar*: the agar is inhibitory to non-enteric organisms. Salmonella Pullorum grows sparsely as small red translucent colonies. S. Gallinarum colonies are small, dome-shaped, and may have a central black spot due to H2S production, but this reaction may be delayed or variable.

  *Brilliant green agar (BGA)*: the agar is inhibitory to coliforms and most Proteus strains; useful for distinguishing enteric organism colonies. Salmonellae form low, convex, pale red, translucent colonies of 1–3 mm in diameter, similar to *Citrobacter*. Proteus forms pin-point colonies, *Pseudomonas aeruginosa* instrument.
appears as small red colonies, and lactose fermenters are green. *Salmonella* Pullorum produces smaller more pale colonies than other salmonellae. BGA is the agar of choice following enrichment.

**Brilliant green sulphapyridine agar:** the agar is inhibitory to coliforms and *Proteus* strains. The sulphapyridine is added to stabilise selectivity in the presence of egg materials. *Salmonella* Pullorum produces small colonies.

*Salmonella* Pullorum and Gallinarum grow poorly and do not produce typical colonies on newer chromogenic agars such as Rambach agar.

- **Liquid enrichment and selective media:**
  
  **Selenite cysteine and F broths:** inhibitory to coliforms but not *Proteus*, improved by addition of brilliant green. Loss of activity after 24 hours limits its use. Selenite cysteine broth is more stable and less inhibitory than selenite F broth, so is normally preferable (e.g. for hatchery-based monitoring of hatcher basket liners or meconium) except in the case of fresh faecal samples from mature birds where highly competitive flora may be present. Although selenite-based broths are considered to be preferable for isolation of *S. Pullorum* and *S. Gallinarum* from faeces by direct enrichment (Wray & Wray, 2000), if there are difficulties with issues of toxicity or shelf life in particular laboratories the other enrichment broths mentioned below may be used. Most of these other broths are however designed to be used following a non-selective enrichment stage and *S. Gallinarum* and *S. Pullorum* are readily overgrown by competitor organisms in non-selective faecal culture resulting in false-negative tests. Direct selective enrichment is therefore recommended for faeces and intestinal or environmental samples. Non-selective enrichment may give better results for tissues obtained by aseptic post-mortem where there should be no competing organisms (Mallinson & Snoeyenbos, 1989).

  **Tetrathionate/brilliant green broth:** inhibitory to coliforms and *Proteus*, but may also inhibit some strains of *S. Pullorum/Gallinarum*.

  **Rappaport–Vassiliadis soya (RVS) peptone broth:** for selective enrichment following pre-enrichment, use 1 part inoculum to 100 parts medium. *Salmonella* Pullorum and Gallinarum are more likely to be overgrown by other organisms during pre-enrichment of faeces or intestinal contents than salmonellae that are not host-adapted so direct enrichment with RVS may also be attempted.

- **Recovery of salmonellae**

  The methods for recovering *S. Pullorum* and *S. Gallinarum* vary according to the origin of the samples. Although their isolation from cloacal swabs and faeces may be unrewarding, examination of tissues taken at post-mortem is usually more successful. The methods are as follows:

  **Cloacal swabs and fresh faeces from live birds:** swabs dipped in nutrient broth are suitable, small swabs being used for young chickens. The swabs are streaked on selective media, and placed in enrichment broth. The plates and the broth are incubated at 37°C. Higher temperatures may be used with some broths, e.g. 41.5°C for Rappaport–Vassiliadis (RV). Subcultures are made on to selective agar after 24 and 48 hours.

  **Gall-bladder contents:** swabs of gall-bladder contents are streaked on to non-selective and selective agars and placed in inhibitory and non-inhibitory broths, followed by incubation at 37°C and subculture on to selective agar after 24 and 48 hours.

  **Organs and tissues:** swabs or segments of tissues are taken in an aseptic manner from individual tissues and lesions and cultured on to non-selective and selective media, and into similar non-selective and selective broths. These are incubated at 37°C and subcultured on to selective agar after 24 and 48 hours. Parallel incubation at a higher temperature, e.g. 40°C, can also be used to enhance the overall isolation rate. **Carrier birds:** larger amounts of material may be required to identify the carrier birds. The ovary and oviduct are the tissues of choice for *S. Pullorum*, and the liver, gall-bladder and caecal tonsils as well as ovary and oviduct should be tested for *S. Gallinarum*. In practice it is usually best to pool samples from a variety of tissues including the spleen, but intestinal tissues should not be pooled with other tissues. Tissues are homogenised in a small volume of broth and directly plated out. Approximately 10 ml of homogenate is also added to 100 ml of non-selective enrichment broth (e.g. buffered peptone water) and selective enrichment broth (e.g. selenite cysteine broth or brilliant green broth), and incubated at 37°C. These broths are subcultured on to non-selective and selective agar after 24 hours.

  **Alimentary canal, including the caecal tonsils and intestinal contents:** after grinding or homogenisation in a small volume of broth, 10 ml of the homogenate is incubated in 100 ml of selective enrichment broth at 37°C. In general better isolation is achieved with selenite cysteine broth.
**Eggshells:** the broken eggshells are placed in a tenfold volume of enrichment broth (e.g. selenite cysteine broth). The broth is incubated at 37°C and subcultured on to selective agar after 24 and 48 hours.

**Egg contents:** Aseptically taken contents of fresh eggs are homogenised and mixed with 200 ml of buffered peptone water or nutrient broth, incubated at 37°C, and subcultured on to non-selective and selective agar after 24 and 48 hours. Incubated eggs, whether infertile or containing small embryos, can be similarly treated.

**Embryos:** Homogenised viscera and swabs from the yolk sacs of well developed embryos may be streaked on to non-selective and selective agar, one swab being placed in 10 ml of both non-selective and enrichment broth (e.g. selenite cysteine broth or brilliant green broth). Incubation is carried out at 37°C, and subcultures are made on to non-selective and selective agars after 24 and 48 hours.

**Environmental samples:** These include hatcher fluff, debris and macerated egg/chick waste samples and chick box liners or floor faecal or litter samples; 25 g is mixed with 225 ml of enrichment broth (e.g. selenite cysteine broth, brilliant green broth), incubated at 37°C, and subcultured on to selective agar after 24 and 48 hours.

Polymerase chain reaction (PCR) based tests may also be used, but have not been fully validated internationally (Cha et al., 2008; Oliviera et al., 2003).

- **Confirmatory procedures**

  Typical *S. Gallinarum* colonies on non-selective media are round, translucent, glistening, domed, smooth, and 1–2 mm in diameter after 24–48 hours’ incubation. *Salmonella* Pullorum colonies are slightly smaller and translucent. On selective media their appearance varies with the medium, but suspect colonies can be investigated serologically by testing for ‘O’9 somatic antigens, observing for motility and testing biochemically. After incubation for 20–24 hours, the plates should be examined carefully for the presence of typical *S. Pullorum* and *S. Gallinarum* colonies. If growth is slight after 24 hours’ incubation, the plates should be re-incubated for a further 24 hours and examined again. For biochemical and serological confirmation from each plate, five typical or suspect colonies should be chosen for further examination. If there are fewer than five typical or suspect colonies, all of them should be taken for further examination. Selected colonies should be streaked on to the surface of nutrient agar, in a manner that allows the growth of separate colonies. For biochemical confirmation, only pure cultures taken from non-selective media should be used. The following media should be streaked using an inoculating loop: triple sugar iron (TSI) agar; lysine iron agar (or L-lysine decarboxylation medium); urea agar according to Christensen; tryptone/tryptophan medium for indole reaction; glucose with an inverted Durham tube for acid and gas production; dulcitol, maltose, ornithine decarboxylation medium and semisolid agar, for motility. The reactions shown in Table 1 occur.

Identification kits are commercially available, for example Analytical Profile Index (API) system for Enterobacteriaceae. However, care must be taken when using API because *S. Pullorum* may be misidentified as *Hafnia* spp. Molecular tests using ribotyping techniques and PCR have been developed in research laboratories (Kang et al., 2011), and can be used for confirmation and differentiation between *S. Gallinarum* and *S. Pullorum*.

For serological confirmation to serogroup level, colonies from non-selective media (nutrient or blood agar) are used. The first stage is elimination of autoagglutinatable strains. For this, material taken from a single colony of pure culture is transferred to a glass slide and mixed with a drop of sterile saline. The slide is rocked gently or the drop stirred with a loop for 30–60 seconds and observed for agglutination against a dark background, preferably with the aid of a magnifying glass or dissecting microscope. If the bacteria have clumped into more or less distinctive units, the strain is considered to be autoagglutinable and must not be submitted to the following tests. If the bacterial sample is recognised as non-autoagglutinable, it is tested with a polyvalent ‘O’ (A–G) antiserum. For this purpose, the material from a single colony is dispersed in the drop of polyvalent ‘O’ antiserum on the glass slide to produce a light suspension to check for autoagglutination (‘rough strains’). If no agglutination takes place, one or two loops of antisera are added, the drop stirred with a loop and observed for agglutination. *Salmonella* Pullorum and *S. Gallinarum* should agglutinate with polyvalent ‘O’ antisera but not with polyvalent flagella (poly ‘H’ phase 1 and phase 2) antisera. If the reaction is positive, the single colony is tested further in the same manner using group-specific sera for *S. Pullorum* and *S. Gallinarum* serotypes (‘O’9 antiserum). After serogrouping, isolates may be sent to a reference laboratory for serotyping.
Table 1. Biochemical investigation of Salmonella Pullorum and S. Gallinarum

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<th><em>Salmonella Pullorum</em></th>
<th><em>Salmonella Gallinarum</em></th>
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<tbody>
<tr>
<td>TSI glucose (acid formation)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TSI glucose (gas formation)</td>
<td>v</td>
<td>–</td>
</tr>
<tr>
<td>TSI lactose</td>
<td>–</td>
<td>–</td>
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<tr>
<td>TSI saccharose</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TSI hydrogen sulphide</td>
<td>v</td>
<td>v</td>
</tr>
<tr>
<td>Gas from glucose (medium with Durham tube)</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Urea hydrolysis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lysine decarboxylation</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ornithine decarboxylation</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Maltose fermentation</td>
<td>– or late +</td>
<td>+</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

+ = 90% or more positive reaction within 1 or 2 days; – = No reaction (90% or more); v = Variable reactions.

It is also possible to confirm and differentiate *S. Gallinarum* by specific PCR (Shah *et al.*, 2005).

- **Test procedure for culture of visceral, faecal, intestinal and environmental samples for *S. Pullorum* and *S. Gallinarum***
  
i) Where possible, begin laboratory procedures on the same day as samples are collected.
  
ii) Homogenise the material as much as possible by manual mixing, gentle macerating or stomaching with a small volume of sterile saline if the material is dry.
  
iii) Stir the mixture with a small rectal swab or loop and streak thickly on to one-quarter of a brilliant green agar plate. (Swabs from uncontaminated tissues sampled in an aseptic manner can also be streaked on to blood agar.)
  
iv) From this deposit of material on the plate, streak the rest of the plate to obtain individual colonies.
  
v) Add 5–25 g of the homogenised sample to freshly made selenite cysteine broth (see note on liquid enrichment and selective media above) to make a 1:10 sample to broth ratio. Shake or stir to disperse the sample in the broth.
  
vi) Incubate the brilliant green agar plates and selenite cysteine broth at 37°C for 24 hours.
  
vii) Examine the plate after 24 hours’ culture. Carry out agglutination tests on up to five suspect colonies with polyvalent ‘O’ (A–G) antisera and polyvalent H (phase 1 and phase 2) antisera. If agglutination is unclear subculture suspect colonies on to nutrient agar or blood agar and repeat tests after 24 hours’ incubation of those media.
  
viii) If poly ‘O’ is positive then check with ‘O’9 antiserum. If ‘O’9 is positive and poly ‘H’ is negative, this is indicative of the possible presence of *S. Pullorum* or *S. Gallinarum*.
  
ix) If there are no positive colonies on the brilliant green agar plate, streak out a 10 µl loop of incubated selenite cysteine broth onto brilliant green agar as in step iv above.
  
x) Incubate the brilliant green agar plates at 37°C for 24 hours and re-incubate the previous (negative) brilliant green agar plates and the selenite cysteine broths for a further 24 hours.
  
xi) Repeat examination of plates as in step vii above.
  
xii) If plates are still negative, re-plate from selenite cysteine broth and incubate brilliant green agar plate, that was inoculated in step ix, for a further 24 hours and examine as in step vii above.
  
xiii) Confirm *S. Pullorum* and *S. Gallinarum* using biochemical tests as shown in Table 1. Isolates can be sent to a *Salmonella* reference laboratory for confirmation of serotype and for phage typing of *S. Pullorum*.

- **Molecular epidemiology**
  
Standard molecular ‘fingerprinting’ techniques used for *Salmonella*, such as plasmid profile analysis, pulsed field gel electrophoresis PCR-restriction fragment length polymorphism (RFLP) or ribotyping can be used for investigating outbreaks of *S. Pullorum* or *S. Gallinarum*. Random amplified polymorphic DNA (RAPD) has proved particular useful in one study (Habtamu-Taddele *et al.*, 2011). It is often necessary to use
combinations of such methods and different restriction enzyme combinations to obtain maximum discrimination because of a high level of clonality. The most effective techniques may also vary by country because of the nature of circulating clones in that region. High throughput sequencing has also been applied to *S. Gallinarum*, but is not yet economically viable for outbreak investigation (Richardson et al., 2011).

2. Serological tests

Serological tests are best applied as a flock test as results for individual birds will vary according to the stage of infection. It is therefore necessary to take sufficient individual samples to determine infection in the flock. The number of samples will depend on the expected prevalence and level of confidence desired (see Chapter 1.1.1 Collection and shipment of diagnostic specimens). If the test is to be used for detecting individual infected birds for culling, it should be repeated at least twice and preferably until the whole flock has given at least two negative tests.

The tests that are most readily applied include rapid whole blood agglutination, rapid serum agglutination (RST), tube agglutination and micro-agglutination (USDA, 1996). Other invasive *Salmonella* such as *S. Enteritidis* and *S. Typhimurium* or use of vaccination may lead to false-positive results in serological tests for *S. Pullorum*.

Both *S. Pullorum* and *S. Gallinarum* possess ‘O’ antigens 9 and 12 and may also possess O antigen 1 (Brooks et al., 2008). However, in the case of *S. Pullorum*, there is a variation in the ratio of $12_1$, $12_2$ and $12_3$; the standard strain contains more $12_3$ than $12_2$, while the reverse is true of the variant form. Intermediate forms also exist.

(There appears to be no such form variation in the case of *S. Gallinarum.*) As this variation occurs, it is necessary to use a polyvalent antigen in immunodiagnostic tests. The same antigen is used to detect both *S. Pullorum* and *S. Gallinarum*, but detection of the latter may be relatively poor (Proux et al., 2002).

a) Rapid whole blood agglutination test

The rapid whole blood agglutination test can be used under field conditions for detecting both *S. Pullorum* and *S. Gallinarum*, and the reactors can be identified immediately. However, it is not reliable in turkeys as the test results in a significant proportion of false-positive results. Sera can be screened by rapid slide agglutination test and positive reactions confirmed by the more specific tube agglutination test. Chickens can be tested at any age, although some authorities specify a minimum age of 4 months (USDA, 1996; Wray & Wray 2000) and positive results from chicks less than 4 weeks of age may be due to maternal antibodies.

- **Preparation of stained antigen for the rapid whole blood or rapid serum agglutination test**

Incubate one standard form strain of *S. Pullorum* (antigenic structure 9, $12_1$, $12_2$) and one variant form (antigenic structure 9, $12_1$, $12_3$) at 37°C and harvest separately until final mixing for the complete antigen.

Sow strains on to separate agar slopes, incubate at 37°C for 24 hours, emulsify growth with sterile normal saline and spread an inoculum over an agar plate to produce easily selected discrete colonies. For this the plates are incubated for 48 hours, as the colonies are marked out and each is tested for agglutination on a slide with 1/500 acriflavine in saline. Smooth-phase colonies do not produce agglutination. Pick off typical colonies that do not produce any agglutination, seed on to agar slopes, and incubate for 24 hours. Emulsify the growth in saline and evenly distribute 2 ml over the surface of the medium (200 ml) in a Roux or similar flask. Incubate the flasks for 60 hours.

For harvesting the bacterial growth, flood the surface of each flask with enough sterile buffered formol saline, pH 6.5 (8.5 g/litre sodium chloride, 10 ml/litre neutral formalin, 4 ml/litre 0.5 M sodium phosphate: made up to 1 litre with distilled water, pH adjusted to 6.5 using 1 M orthophosphoric acid or 1 M sodium hydroxide), to give dense cell suspensions (about 10 ml per flask). Add 12–15 sterile glass beads of 3–5 mm diameter and shake the mixture and allow to stand for 36 hours or until precipitation is complete. Check the morphology and purity of the suspensions by preparing and examining Gram-stained films. Bulk the suspension from each flask containing the same strains. To each 100 ml of suspension, add 200 ml of absolute alcohol. Shake the mixture and allow to stand for 36 hours or until precipitation is complete. Check the agglutinability of the standard and variant precipitate by first centrifuging a sample to separate the alcohol, which is removed, dilute with normal saline and test with a known positive and negative serum. If satisfactory, remove the clear supernatant alcohol (centrifugation at 2000 g for 10 minutes may be helpful in precipitation), and add sufficient phosphate buffered saline (PBS) containing 10% (v/v) glycerol to standardise the density to 75 × No. 1 Wellcome opacity tube (or 50 × tube No. 1.0 on the McFarland scale). Add equal volumes of standard and variant strains, and add 1% (v/v) of 3% (w/v) alcoholic crystal violet solution to the final mixture, and allow to stand for 48 hours at room temperature. Store in a tightly closed container at 0–4°C for up to 6 months. To assess safety, carry out a culture test on blood agar for non-viability of the unwashed antigen before standardisation. Each bottle of antigen must be tested after alcoholic precipitation and before standardisation against standard titre antisera for *S. Pullorum* and *S. Gallinarum*, and against a negative serum. If possible, also test with known positive and negative serum and blood from positive and negative chickens.
Stained antigen products for the whole blood plate agglutination test are available commercially, and although there seems to be some slight differences in their sensitivity (Gast, 1997), it is unlikely that poultry flocks infected with the different variants of S. Pullorum would be missed.

- **Test procedure**
  i) Use a clean white tile marked into squares of about 3 × 3 cm. If a tile with 3 × 4 squares is used, up to 12 blood samples can be tested at the same time.
  ii) Place 1 drop (about 0.02 ml) of crystal-violet-stained antigen in the centre of each square.
  iii) Obtain a sample of fresh whole blood. This is conveniently done by making a stab of a wing vein using a needle with a triangular point.
  iv) Place an equal size drop of fresh whole blood next to a drop of antigen.
  v) Mix the drops of antigen and blood using a fine glass rod, which is wiped clean between samples.
  vi) Use a gentle rocking motion to keep the drops agitated for up to 2 minutes. Several tests may be carried out simultaneously on the same tile, but the drops should not be allowed to dry out during this time. In very warm conditions, larger drops may be required to avoid drying out.
  vii) A positive reaction is indicated by easily visible clumping of the antigen within 2 minutes.
  viii) A negative reaction is indicated by absence of clumping of the antigen within 2 minutes.
  ix) Include known positive and negative control sera on each testing occasion, using them in the same way as the blood.
  x) On completion of a set of tests, the tile is washed and dried, ready for further use.

In the absence of positive reactions, any doubtful reactions can only be interpreted in the light of the previous Salmonella testing history of the flock. Where there are positive reactors, any doubtful reactor should be regarded as positive. Also, recently infected birds may not show a typical positive reaction until they are retested after 3–4 weeks.

b) Rapid serum agglutination test

The RST is performed in the same manner, except that serum is substituted for whole blood. For export test purposes an initial screening of sera by RST followed by confirmation of positives by the tube agglutination test is the optimal approach. Ideally serum samples tested by any method should be tested within 72 hours of collection as nonspecific reactions may increase in older samples. Fresh samples can be frozen for later testing if a delay is unavoidable.

c) Tube agglutination test

Fresh serum from chickens, turkeys or other birds is used at an initial dilution of 1/25, obtained by mixing 0.04 ml of serum with 1.0 ml of antigen. Positive and negative control sera are included in each test. The antigen is prepared from unstained S. Pullorum or S. Gallinarum cultures diluted to a concentration of No. 1 on the McFarland scale (as described above). The mixture is incubated at 37 or 50°C for 18–24 hours before reading. A positive reaction consists of a granular white deposit with a clear supernatant fluid; a negative reaction shows uniform turbidity. Samples positive at a dilution of 1/25 are retested at a higher range of dilutions and a titre of 1/50 is usually considered to be positive, although this figure seems to vary in the literature. In many cases a single dilution of 1/50 is used but this may fail to detect some flock infections if only small numbers of samples are taken.

d) Micro-agglutination test

This resembles the tube agglutination test, but requires much smaller volumes of reagents. The test is performed in microtest plates. Sera are first diluted by adding 10 µl of serum to 90 µl of normal saline, and then adding 100 µl of previously standardised stained microtest antigen to give a final dilution of 1/20. By titrating the serum in doubling dilutions and adding an equal volume of standardised stained antigen, an endpoint (titre) can be obtained. The plates are sealed and incubated at 37°C for 18–24 or 48 hours. A positive reaction consists of a fine diffuse precipitation, whereas a negative reaction shows a button-like precipitate. Titres of 1/40 are usually considered to be positive but this test is more liable to produce false-positive results with turkey sera.

Other serological tests include micro-antiglobulin (Coombs), immunodiffusion, haemagglutination and enzyme-linked immunosorbent assay (ELISA).

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2 For preparation of small volumes of somatic antigens see chapter 2.9.9.
ELISA techniques have been described for detecting antibodies to S. Pullorum and S. Gallinarum (Oliveira et al., 2004). The indirect ELISA using lipopolysaccharide antigen is likely to be the most sensitive and specific serological flock test for Salmonella, including S. Gallinarum and S. Pullorum. It is relatively easy to perform with serum or yolk, and can be used for quantifying the titre of antibody (Barrow, 1992; 1994; Wray & Wray, 2000). No commercial ELISA kits for S. Pullorum and S. Gallinarum are currently available.

C. REQUIREMENTS FOR VACCINES

1. Background

a) Rationale and intended use of the product

Although both live and inactivated vaccines have been prepared for use against S. Gallinarum (Paiva et al., 2009), the vaccine most widely used is made from the rough 9R strain (Harbourne et al., 1963). It is normally only employed in chickens. The number of viable organisms per dose is important; these organisms can survive in vaccinated birds for many months and may be transmitted through the egg (and perhaps from bird to bird). Vaccination may reduce flock losses, but will not prevent infection with field strains. In addition, vaccination with 9R may sometimes precipitate high mortality in infected birds (Silva et al., 1981), and may stimulate the production of transient antibodies. It is usual to vaccinate at 8 weeks and again at 16 weeks of age. Antimicrobials should be avoided before and after vaccination.

Currently available vaccines, however, have only a minor role to play in the control of fowl typhoid as they offer short-lived protection against clinical disease and limited or variable protection against infection. Autogenous or locally produced vaccines can also be used to control clinical disease, but care must be taken to avoid strain instability leading to reversion to virulence (Okamoto et al., 2010). Control can best be achieved by biosecurity, hygiene, good management, monitoring and removal of infected flocks. Commercially available 9R vaccines have been used for reduction of S. Enteritidis in laying flocks in some countries but may be prohibited or are not commercially available in some countries where fowl typhoid is not present (Lee et al., 2005). Even in countries with fowl typhoid, use of vaccine may complicate control as it does not prevent infection, only reduce clinical disease and allow production to continue from infected flocks. It is therefore preferable to aim for eradication of the organism rather than acceptance of on-going disease, but this is often not economically viable in large multi-age holdings as eradication of red mites is necessary to ensure continued freedom from infection (Wales et al., 2010).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements. Most vaccines are produced in highly industrial commercial processes and are regulated by national veterinary medicines licensing authorities. Smaller quantities of emergency herd vaccines or autogenous vaccines are produced by private laboratories, but each production has to be specifically licensed. It is recommended that a validated commercial vaccine is used unless there is no alternative because of the need to maintain quality and avoid risk associated with reversion to virulence. Live vaccines must also be bacteriologically distinguishable from field strains or surveillance and control programmes may be compromised. Observations from some countries suggest that it is not always straightforward to distinguish between S. Gallinarum vaccines and field strains. There will inevitably be some interference with serological monitoring for S. Gallinarum and potential interference with serological monitoring for S. Enteritidis, unless a stepwise approach is used in which a sensitive LPS-based ELISA is used to test for antibodies to O9 antigens and positive sera are further tested with a flagella antigen ELISA, which will give a negative reaction in cases of S. Gallinarum infection (Wray & Wray, 2000). Recent work on the molecular mechanisms of infection should lead to the development of improved vaccines in future (Barrow & Freitas Neto, 2011).

2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed

i) Biological characteristics

For killed or live vaccines, the bacterial strain should be an organism as closely related to currently circulating field strains as possible. It should be carefully chosen from cases of severe clinical disease, and virulence and antigen production should be assessed. It is best to evaluate a panel of potential strains in this way before testing the final selection. The final vaccinal strain should be identified by historical records and characterised by stable phenotypic and/or genetic markers. Living vaccinal strains should be marked by stable characters allowing distinction from wild strains. Markers such as resistance to antimicrobials, for example rifampcin, or auxotrophism may be used. Attenuation of virulence should be stable and preferably
Chapter 2.3.11. – Fowl typhoid and Pullorum disease

obtained by two independent defined mutations. The stability of live vaccine strains can be verified by
regular checks using sensitive molecular fingerprinting and micro-array techniques.

Live fowl typhoid vaccine is a suspension of suitably attenuated living organisms of a rough strain of
*S. Gallinarum*, e.g. 9R. The organisms in the vaccine give the biochemical reactions characteristic of
*S. Gallinarum*. Colonies of a 24-hour culture prepared from the vaccine on nutrient agar plates are rough
when examined by the acriflavine slide test. The culture should not contain the somatic antigens
characteristic of the smooth forms of *S. Gallinarum*

ii) Quality criteria (sterility, purity, freedom from extraneous agents)

*Sterility and purity*

The vaccine strain must be checked as follows:

- Staining of a smear of bacterial suspension on a glass slide using Gram stain.
- Homogeneity of culture on non-selective media.
- Metabolic requirements as indicated by biochemical tests.
- Detection of markers, and phage type.
- Agglutination with specific antiserum.
- The vaccine culture and any adjuvants, preservatives or other materials must be microbiologically
sterile and non-toxic at the concentrations used.

*Safety*

The LD$_{50}$ (50% lethal dose) or ID$_{50}$ (50% infectious dose) may be determined in chickens or,
preferably, signs of more mild adverse reactions should be checked in the target species. Ten times
the field dose of live vaccine or twice the dose for killed vaccines must be given to the target species at
the recommended age and by the recommended route. The animals are observed for absence of
adverse reactions. Stability and non-reversion to virulence after serial passages in susceptible species
should be shown for live vaccines. It is also necessary to consider repeat vaccination. Live vaccine
should be shown not to persist for long in vaccinated animals or be transmitted to milk or eggs that may
be consumed, and the method of application should not present a hazard to operators. In the case of
*S. Gallinarum* vaccine at least six healthy, susceptible (preferably specific pathogen free [SPF])
chickens, 8–16 weeks of age, are each injected subcutaneously with ten doses of vaccine, and are
observed for at least 7 days; no local or systemic reaction should develop.

*Efficacy*

Laboratory experiments and field trials should be used to show that the vaccine is effective. The
laboratory experiments consist of vaccination–challenge tests in the target species at the
recommended dose and age. The efficacy data can also be used as the basis for a batch potency test.
Field trials are more difficult to undertake with respect to testing efficacy because of difficulties with
standardising the challenge and providing appropriate controls. In the case of *S. Gallinarum* 9R vaccine
or similar vaccines at least fifteen healthy chickens, 8–16 weeks of age, of a brown layer hybrid breed,
and taken from a stock that is free from *S. Pullorum* infection, are each injected subcutaneously with a
quantity of vaccine corresponding to one field dose, i.e. $5 \times 10^7$ viable organisms. After an interval of
21–28 days, the vaccinated chickens and an equal number of similar unvaccinated chickens are
deprived of food for approximately 18 hours. The chickens are then challenged by oral administration
of 1 ml of a broth suspension containing $5 \times 10^7$ organisms of a virulent strain of *S. Gallinarum* mixed
with 300 mg of a powder consisting of chalk (40%), light kaolin (43%) and magnesium trisilicate (17%).
All the chickens are observed for 14–21 days. The vaccine passes the test if at the end of this period
the number of surviving vaccinated chickens that show no macroscopic lesions of fowl typhoid at post-
mortem exceeds by eight or more the number of similarly defined control chickens.

*Environmental aspects*

Live vaccine strains should be tested for their ability to persist in the environment and infect non-target
species such as rodents and wild birds that are likely to be exposed. Prolonged survival of some live
vaccines in faeces and litter may present an unacceptable environmental hazard when the material is
removed from the animal houses. Live vaccines should not be used in commercial laying flocks during lay.

b) Method of manufacture

i) Procedure

The seed culture is propagated and maintained using suitable media, of which many have been
described (in textbooks) for growth of *Salmonella*. The media used must not contain serum or animal
tissues. Culture may be on solid medium, in Roux flasks, or in liquid medium, in which case large-scale fermentation equipment may be used. Iron limitation or low temperature incubation on a minimal media may enhance lipopolysaccharide (LPS) antigen production by the vaccine strain. In the case of S. Gallinarum (9R), the vaccine may be prepared by inoculation of a suitable medium, such as peptone broth, with a fresh culture of S. Gallinarum (9R) and incubation at 37°C for 24 hours, with agitation. The organisms are harvested by sedimentation or centrifugation.

Alternatively the organisms may be grown on and harvested from a solid medium, such as nutrient agar. In either case, the suspension is diluted in PBS solution, pH 7.0, and may be freeze-dried. The dose used per bird is between $5 \times 10^6$ and $5 \times 10^7$ organisms.

Vaccine must be produced in suitable clean rooms to which only approved personnel have access. Care must be taken to avoid cross-contamination between areas where live organisms are processed and other areas. Contamination from operators and/or the environment must be avoided and vaccine preparation should take place in a separate area from diagnostic culture work. Operators must not work with vaccine whilst ill and must not be subject to immunosuppressive conditions or medications. Personnel must be provided with protective clothing in production areas and in animal rooms.

Seed-lot cultures are prepared from the primary seed-lot, and the number of passages is dependent on the validation of the process. The vaccine may be prepared by inoculation of a suitable medium, such as nutrient broth, with a fresh culture and incubation on a shaker at 37°C for 24 hours, with or without aeration. The organisms are harvested by sedimentation or centrifugation. Alternatively, the organisms may be grown on and harvested from a solid medium, such as nutrient agar. In the case of live vaccines, the suspension is diluted in PBS, pH 7.0, and may be freeze-dried.

The time of inactivation of dead vaccines should be at least 33% more than that taken to reduce the viable number to an undetectable level. The inactivation process must be applied to the whole volume of the vaccine cell harvest.

Preservatives, excipient for lyophilisation, stabiliser for multi-dose containers or other substances added to or combined with a vaccinal preparation must have no deleterious effect on the immunising potency of the product.

ii) Requirements for substrates and media

All chemicals and growth media used should be guaranteed to be fit for purpose and checked by the use of suitable controls.

iii) In-process controls

The following points require attention:

• Visual control of the suspension, homogeneity by Gram stain, culture on non-selective medium.

• Slide agglutination with specific antisera.

• Titration of bacteria by turbidimetry and/or plate count.

• Test of effective inactivation (killed vaccine) by plating on non-selective medium or use of a medium that gives optimum chance of recovery, e.g. production medium with neutralisation of the inactivating compound.

• Titration of viable bacteria (living vaccine) before and after lyophilisation.

iv) Final product batch tests

Sterility/purity

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

Safety

A laboratory test that has previously shown a correlation with safety in the target species may be used to determine the absence of deleterious effects on vaccinated animals. Each batch should be tested in the target species at the recommended age and route, using at least twice the field dose for killed vaccines and ten times the dose for live vaccines. Observations are made on any adverse effects on the demeanour and health of the vaccinated animals and an assessment may be made of tissue reactions at the injection site.

Batch potency

Potency is tested using vaccination–challenge assay in chickens and/or other species, including (if practicable) any other target species and immunological response in target species.
c) Requirements for authorisation

i) Safety requirements

Certain killed vaccines may occasionally cause reactions in vaccinated animals because of their LPS content or the adjuvant used, and likewise live vaccines should be used with caution in animals that are not completely healthy at the time of vaccination. It is often necessary, however, to vaccinate flocks in the face of clinical fowl typhoid. Vaccines may also cause swelling at the site of injection, particularly if an oil-emulsion adjuvant is used.

Target and non-target animal safety

Killed vaccines are assessed in a double-dose test, and live vaccines are assessed in a test using ten times the dose, ideally in the target species. Live vaccines should be proven to be harmless in relevant non-target species that could be exposed to vaccine excreted by vaccinated animals. As S. Gallinarum and S. Pullorum are host specific, non-target species are less of a concern.

Reversion to virulence for attenuated/live vaccines

Live vaccines shall be shown in replication tests in target species to not revert to virulent strains during a suitably large number of replications. Mutations, especially undefined mutations, should be shown to be stable, and checks on stability can be made by molecular fingerprinting methods or sequencing. Although the risk is small it is wise not to use live vaccines in a country where the organism in the vaccine has been eradicated. Special care should also be taken to ensure that attenuated vaccines are not incompletely attenuated or contaminated with seed organisms.

Environmental consideration

Live vaccines should not be able to replicate in the environment or persist for more than a short period.

ii) Efficacy requirements

For animal production

The duration of immunity is likely to vary considerably between products, vaccination regimes and individual vaccinated animals. The vaccine should provide protection throughout the laying period, and this can be measured by potency (efficacy) tests at stages during lay. A booster dose during lay may be required, but should not be used during lay in flocks providing eggs for human consumption.

Immunity to Salmonella is normally serovar or serogroup specific. Consultation among colleagues suggests that some killed vaccines will provide some protection for 6 months, while some live vaccines given by injection may elicit stronger immunity, which may persist for 1 year or more. It should be remembered, however, that a strong challenge such as that associated with continuously occupied farms or infected wild birds and mite populations may overwhelm vaccinal immunity and commercial live vaccines may be attenuated to reduce environmental survival in a way that reduces the immune response. There may also be problems with ensuring effective oral administration with live vaccines that are delivered orally or accuracy of injection with killed and live injectable vaccines. The Salmonella vaccines are intended to limit the extent of clinical disease in poultry, and also to reduce the risk of introduction of infection to flocks. If possible, the potency test should relate to the efficacy of the vaccine in the target species, and suitable criteria should be applied for passing batches. It may be possible to assess killed and injected vaccines by the antibody response produced, although it should be remembered that serum antibodies are only part of the host's protective mechanism against Salmonella. Alternatively, the potency of the vaccine may be assessed by its effect on challenged vaccinated animals compared quantitatively and statistically with unvaccinated controls.

For control and eradication

Vaccines for Salmonella are not capable of eradicating infection from flocks but can increase the threshold for infection, reduce the level of excretion of the organism and reduce vertical transmission in poultry that results in contamination of hatching or table eggs. Vaccination is therefore an aid to other eradication and control measures such as culling, all in-all out production, biosecurity and farm hygiene.

iii) Stability

Information is lacking on the stability of killed vaccines. Stability is affected by storage conditions and by the presence of contaminating microorganisms growing in the product. Chemicals with antimicrobial activity, such as thiomersal, phenol or crystal violet, are often included as preservatives in killed bacterial vaccines. The stability is assessed by potency tests repeated at appropriate time intervals. The stability of live vaccines can be assessed by performing counts of the number of viable organisms repeated at appropriate time intervals, and genotyping tests to identify genetic changes during fermentation production.
3. Vaccines based on biotechnology

a) Vaccines available and their advantages

None commercially available.

b) Special requirements for biotechnological vaccines, if any

Not applicable.

REFERENCES


Chapter 2.3.11. – Fowl typhoid and Pullorum disease


* * *
CHAPTER 2.3.12.

INFECTIOUS BURSAL DISEASE
(Gumboro disease)

SUMMARY

Infectious bursal disease (IBD) is caused by a virus that is a member of the genus Avibirnavirus of the family Birnaviridae. Although turkeys, ducks, guinea fowl and ostriches may be infected, clinical disease occurs solely in chickens. Only young birds are clinically affected. Severe acute disease of 3–6-week-old birds is associated with high mortality, but a less acute or subclinical disease is common in 0–3-week-old birds. This can cause secondary problems due to the effect of the virus on the bursa of Fabricius. IBD virus (IBDV) causes lymphoid depletion of the bursa, and if this occurs in the first 2 weeks of life, significant depression of the humoral antibody response may result. Two serotypes of IBDV are recognised. These are designated serotypes 1 and 2. Both serotypes can be differentiated by cross-neutralisation assays. Clinical disease has been associated with only serotype 1 and all commercial vaccines are prepared against this serotype. Antigenic variants of IBD serotype 1 have been described and these may require special vaccines for maximum protection. Very virulent strains of classical serotype 1 are now common and are causing serious disease in many countries.

Clinical disease due to infection with the IBDV, also known as Gumboro disease, can usually be diagnosed by a combination of characteristic signs and post-mortem lesions. Laboratory confirmation of disease, or detection of subclinical infection, can be carried out by demonstration of a humoral immune response in unvaccinated chickens or by detecting the presence of viral antigen or viral genome in tissues. In the absence of such tests, histological examination of bursae may be helpful.

Identification of the agent: Isolation of IBDV is not usually carried out as a routine diagnostic procedure. Specific antibody-negative chickens may be used for this purpose, as may cell cultures or embryonating eggs from specific antibody-negative sources. However, some difficulty may be experienced if using the latter two systems as the virus does not readily adapt to them. If successful, the identity of the virus can be confirmed by the virus neutralisation (VN) test.

The agar gel immunodiffusion (AGID) test can be used to detect viral antigen in the bursa of Fabricius. A portion of the bursa is removed, homogenised, and used as antigen in a test against known positive antiserum. This is particularly useful in the early stages of the infection, before the development of an antibody response. An immunofluorescence test using IBDV-specific chicken antiserum can also be used to detect antigen in bursal tissue. Antigen-capture enzyme-linked immunosorbent assays (ELISAs) based on plates coated with IBDV-specific antibodies have also been described for the demonstration of IBDV antigens in bursal homogenates. The reverse-transcription polymerase chain reaction (RT-PCR) with specific primers may be used to detect viral genomic RNA in the bursa of Fabricius.

Strain characterisation: IBDV strains can be further identified by testing their pathogenicity in specific antibody-negative chickens, by investigating their antigenic reactivity in cross VN assays or in tests based on monoclonal antibodies, by determining the nucleotide sequence of RT-PCR amplification products derived from IBDV genome, or by studying the number and size of the restriction fragments obtained following digestion of such RT-PCR products with restriction endonucleases. Several protocols have been described for each of these different approaches. Tests should be performed by specialised laboratories and should include a panel of reference strains as controls. Although the molecular basis for antigenic variation is now better understood, no validated virulence marker has been described yet.
Serological tests: An AGID, VN or ELISA may be carried out on serum samples. The infection usually spreads rapidly within a flock of birds. Because of this, only a small percentage of the flock needs to be tested to detect the presence of antibodies. If positive reactions are found in unvaccinated birds then the whole flock must be regarded as infected.

Requirements for vaccines and diagnostic biologicals: Both live attenuated and inactivated (killed) vaccines are available to control the disease. A live recombinant vaccine expressing the VP2 antigen of IBDV has also been licensed recently. It is important that live vaccines be stable, with no tendency to revert to virulence on passage. To be effective, the inactivated vaccines need to have a high antigen content.

Live vaccines are used to produce an active immunity in young chickens. A complementary approach to this is to provide chickens with passive protection by vaccinating the parents using a combination of live and killed vaccines. Effective vaccination of breeding stock is therefore of great importance.

Live vaccines: Attenuated strains of IBD viruses are used. These are referred to as either mild, intermediate, or ‘intermediate plus’ (‘hot’) vaccines. The mild vaccines cause limited bursal damage, while the intermediate and intermediate plus vaccines cause some lymphocytic depletion in the bursa of Fabricius. Usually none of the vaccine types causes immunosuppression when used in birds over 14 days old that have been hatched from IBD immune parents.

Mild vaccines are rarely used in broilers, but are used widely to prime broiler parents prior to inoculation with inactivated vaccine. Intermediate and ‘hot’ vaccines are more capable of overcoming low levels of maternally derived antibodies (MDA). Live vaccines are usually administered by spray or in drinking water. In the absence of MDA, mild vaccines are given at 1-day old. When MDA are present at 1 day of age, vaccination should be delayed until MDA in most of the flock has waned. The best schedule can be determined by serological testing of the birds to detect the time at which MDA has fallen to a low level. More recently, vaccines have been developed that can be administered in ovo at 18 days of incubation.

Killed vaccines: These are usually used to stimulate high and uniform levels of antibody in parent chickens so that the progeny will have high and uniform levels of MDA. The killed vaccines may occasionally be used in young valuable birds with MDA. The killed vaccines are manufactured in oil emulsion adjuvant and given by injection. They must be used in birds already sensitised by primary exposure, either to live vaccine or to field virus. This can be checked serologically. High levels of MDA can be obtained in breeder birds by giving, for example, live vaccine at approximately 8 weeks of age, followed by inactivated vaccine at approximately 18 weeks of age.

A. INTRODUCTION

Infectious bursal disease (IBD) is caused by a virus that is a member of the genus Avibirnavirus of the family Birnaviridae. Although turkeys, ducks, guinea fowl and ostriches may be infected, clinical disease occurs solely in chickens. Only young birds are clinically affected. Severe acute disease of 3–6-week-old birds is associated with high mortality, but a less acute or subclinical disease is common in 0–3-week-old birds. This can cause secondary problems due to the effect of the virus on the bursa of Fabricius. IBD virus (IBDV) causes lymphoid depletion of the bursa, and if this occurs in the first 2 weeks of life, significant depression of the humoral antibody response may result. Two distinct serotypes of infectious bursal disease virus (IBDV) are known to exist. Serotype 1 virus causes clinical disease in chickens younger than 10 weeks. Older chickens usually show no clinical signs. Antibodies are sometimes found in other avian species, but no signs of infection are seen. Serotype 2 antibodies are very widespread in turkeys and are sometimes found in chickens and ducks. There are no reports of clinical disease caused by infection with Serotype 2 virus (Lasher & Shane, 1994).

B. DIAGNOSTIC TECHNIQUES

Isolation and identification of the agent provide the most certain diagnosis of IBD, but are not usually attempted for routine diagnostic purposes as the virus may prove difficult to isolate (Lukert & Saif, 1997). In practice, laboratory diagnosis of IBD depends on detection of specific antibodies to the virus, or on detection of the virus in tissues, using immunological or molecular methods.
1. Identification of the agent

Clinical IBD has clearly characteristic signs and post-mortem lesions. A flock will show very high morbidity with severe depression in most birds lasting for 5–7 days. Mortality rises sharply for 2 days then declines rapidly over the next 2–3 days. Usually between 5% and 10% of birds die, but mortality can reach 30–40%. The main clinical signs are watery diarrhoea, ruffled feathers, reluctance to move, anorexia, trembling and prostration. Post-mortem lesions include dehydration of the muscles with numerous ecchymotic haemorrhages, enlargement and discoloration of the kidneys, with urates in the tubules. The bursa of Fabricius shows the main diagnostic lesions. In birds that die at the peak of the disease outbreak, the bursa is enlarged and turgid with a pale yellow discoloration. Intrafollicular haemorrhages may be present and, in some cases, the bursa may be completely haemorrhagic giving the appearance of a black cherry. Peribursal straw-coloured oedema will be present in many bursae. Confirmation of clinical disease or detection of subclinical disease is best done by using immunological methods as the IBDV is difficult to isolate. For virus isolation, the methods described below should be followed. Differentiation between serotypes 1 and 2 or between serotype 1 subtypes or pathotypes should be undertaken by a specialised laboratory (e.g. the OIE Reference Laboratories for infectious bursal disease [see Table given in Part 4 of this Terrestrial Manual]).

a) Sample preparation

Remove the bursae of Fabricius aseptically from approximately five affected chickens in the early stages of the disease. Chop the bursae using two scalpels, add a small amount of peptone broth containing penicillin and streptomycin (1000 µg/ml each), and homogenise in a tissue blender. Centrifuge the homogenate at 3000 g for 10 minutes. Harvest the supernatant fluid for use in the investigations described below. Filtration through a 0.22 µ filter may prove necessary to further control bacterial contamination, although this may cause a reduction in virus titre.

b) Identification by the agar gel immunodiffusion test

A protocol for the AGID test is described in Section B.2.a. For detection of antigen in the bursa of Fabricius by AGID, the bursae should be removed aseptically from about ten chickens at the acute stage of infection. The bursae are minced using two scalpels in scissor movement, then small pieces are placed in the wells of the AGID plate against known positive serum. Freeze–thaw cycles of the minced tissue may improve the release of IBDV antigens from the infected bursal tissue, and the freeze–thaw exudate may be used to fill the wells.

c) Identification by immunofluorescence

Sections of bursa are prepared using a microtome cryostat, dried at room temperature and then fixed in cold acetone. Fluorescent-labelled IBDV-specific antisera are applied to the sections, which are then incubated at 37°C for 1 hour in a humid atmosphere. At the end of the incubation period, they are washed for 30 minutes using phosphate buffered saline (PBS), pH 7.2, then rinsed in distilled water. The sections are mounted using buffered glycerol, pH 7.6, and examined by UV microscopy for IBDV-specific fluorescence (Meulemans et al., 1977).

d) Identification by antigen-capture enzyme-linked immunosorbent assay (AC-ELISA)

Different protocols have been described for the detection of serotype 1 IBDV using an antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) (Eterradossi et al., 1997; Kwang et al., 1987; Snyder et al., 1988). Briefly, ELISA plates are coated with IBDV-specific antibodies. Depending on the chosen AC-ELISA protocol, the capture antibody may be a mouse anti-IBDV monoclonal antibody (MAb), or a mix of such MAbs, or a chicken post-infectious anti-IBDV polyclonal serum. It has been suggested that AC-ELISAs using polyclonal antibodies may have a higher sensitivity. Samples of bursal homogenates (see above) diluted 1/10 to 1/25 (w/v) in a suitable dilution buffer are incubated in the coated wells. Unbound antigens are discarded at the end of the incubation period by washing with a suitable washing buffer (e.g. PBS, pH 7.2 + 0.2% Tween 20). The captured antigens are then revealed, as in an indirect ELISA, with a detection antibody (which must have been developed from a different animal species than the capture antibody), followed by an enzyme conjugate that binds to the detection antibody only (in some protocols the detection antibody may be directly conjugated to the enzyme), followed by the enzyme substrate. Finally, optical densities, which parallel the amount of captured IBDV antigens, are read with an ELISA reader.

The AC-ELISA is based on the use of samples possibly containing live virus and should be performed only in suitable containment facilities such as a class II safety cabinet. All liquid (washing buffers) and solid wastes should be considered to be contaminated by IBDV and decontaminated accordingly before disposal.

Critical steps in the implementation or assessment of AC-ELISA are i) the need to perform extensive washings between each step of the reaction to keep background reactions low, ii) the requirement for known positive and negative samples to be included in each assay as controls, and iii) the need for both the capture
and detection antibodies to positively react with all serotype 1 IBDV strains (i.e. neither capture nor detection should critically depend on IBDV antigenic variation that occurs among serotype 1 strains).

e) Identification by molecular techniques

Molecular virological techniques have been developed that allow IBDV to be identified more quickly than by virus isolation (Davis & Boyle, 1990; Jackwood, 1990; Wu et al., 1992). The most frequently used molecular method is the detection of IBDV genome by the reverse-transcription polymerase chain reaction (RT-PCR) (Lin et al., 1993; Wu et al., 1992). This method can detect the genome of IBDV, which is unable to grow in cell culture, because it is not necessary to grow the virus before amplification.

RT-PCR is performed in three steps: extraction of nucleic acids from the studied sample, reverse transcription (RT) of IBDV RNA into cDNA, and amplification of the resulting cDNA by PCR. The two latter steps require that the user selects oligonucleotidic primers that are short sequences complementary to the virus-specific nucleotidic sequence. Different areas of the genome will be amplified depending on the location from which the primers have been selected. The example below allows the amplification of the middle third of the gene encoding the outer capsid protein VP2 (Eterradossi et al., 1998; 1999).

- **Extraction of nucleic acids**

  Single-stranded RNA is extremely susceptible to degradation by RNases. IBDV double-stranded RNA (dsRNA) genome resists degradation by RNases. However, infected cells also contain IBDV-derived positive-sense single-stranded RNA species that can be used as a template at the RT step and may contribute to improving the sensitivity of the assay. It is thus important that RNA extraction be performed using gloves and RNase-free reagents and labware.

  IBDV RNA can be extracted from infected tissues using some kits available from commercial suppliers of molecular biology reagents. Alternatively, IBDV RNA can be extracted by adding 1% (weight/volume final concentration) sodium dodecyl sulphate and 1 mg/ml proteinase K to 700 µl of virus suspension (e.g. bursal homogenate). Incubate for 60 minutes at 37°C. Nucleic acids are obtained using a standard protocol for phenol/chloroform extraction (caution: phenol is toxic and should be handled and disposed accordingly). Nucleic acids are harvested from the final aqueous phase by ethanol precipitation and are resuspended in RNase-free distilled water or a suitable buffer. Water-diluted RNA should be kept frozen at a temperature below –20°C until use.

- **Reverse transcription**

  A variety of reverse transcriptases are commercially available. Follow the supplier’s instructions to prepare the RT reaction mix. Use the ‘lower’ PCR primer (complementary to the positive strand of IBDV genome, see below) for reverse transcription, as this allows the synthesis of cDNA both from the positive strand of IBDV dsRNA genome and from IBDV-derived positive-sense single-stranded RNAs previously contained in infected cells. Alternatively, random primers (hexanucleotides) can be used to prime cDNA synthesis.

  The IBDV RNA matrix must be denatured before transfer to the RT reaction mix. Add one part (by volume) molecular biology grade dimethylsulphoxide to four parts the unfrozen solution of IBDV RNA. Heat for 3 minutes at 92°C and chill on ice; an alternative method is to heat for 5 minutes and immediately incubate the mixture in liquid nitrogen. Transfer the relevant volume of denatured matrix to the reaction mix. Incubate according to the instructions of the enzyme supplier.

  The cDNA solution obtained after the RT step should be kept frozen at a temperature below –20°C. Delaying the PCR step for several weeks after the cDNA synthesis may cause false-negative PCR results.

- **Polymerase chain reaction**

  A variety of DNA polymerases suitable for PCR are commercially available. Follow the manufacturer’s instructions to prepare the PCR reaction mix. Protocols for the amplification and molecular typing of IBDV have been reviewed recently (Wu et al., 2007). As an example, the U3/L3 and +290/–861 pairs of PCR primers shown below can be suggested and have been found useful for amplifying the middle third of the VP2 gene in segment A of serotype 1 IBDV strains (Eterradossi et al., 1998; 1999), and a region at the 5’ extremity of IBDV segment B (Le Nouen et al., 2006), respectively. Both regions have been shown to be suitable for molecular epidemiology studies (Le Nouen et al., 2005). Although a significant number of IBDV strains have two nucleotide changes at position 35 (G–A) and 38 (T–C) of the U3 primer (including isolates from Japan [OKYM], Hong Kong [HK46], UK [UK661], Nigeria [N4]), it has been shown that the U3-L3 primer pair successfully amplifies some of these viruses that exhibit both mutations. This is probably because the 3’ extremity of U3 is highly conserved. However, as with most PCR assays, IBDV strains may exist with nucleotide changes at the annealing positions of the primers, thus requiring the use of other primers for optimised RT-PCR detection.
The combination of segment A- and segment B-targeted RT-PCR protocols enhances the probability that, if present, serotype 1 IBDV will indeed be detected; it also allows a thorough genetic characterisation of the IBDV strains detected.

Nucleotide sequence of the U3 and L3 IBDV-specific PCR primers (specific for Segment A, VP2 gene):

Upper U3:  5' -TGT-AAA-ACG-AGC-GCC-AGT-GCA-TGC-GGT-ATG-TGA-GGC-TTG-GTG-AC-3'

Nucleotide sequence of the +226 and −793 IBDV-specific PCR primers (specific for Segment B, VP1 gene):

Upper +290:  5' -TGT-AAA-ACG-AGC-GGC-AGT-GAA-TTC-AGA-TTC-TGC-AGC-CAC-GGT-CTC-T-3'
Lower −861:  5' -CAG-GAA-ACA-GCT-ATG-ACC-CTG-CAG-TTG-ATG-ACC-GGG-TGA-GTT-TTT-TC-3'

The U3 and L3 primers are both 44 nucleotides long, whereas primers +290 and −861 are 46 and 47 nucleotides long, respectively. The four primers include an IBDV-specific 3' extremity (in italics in the sequence shown above) corresponding to nucleotide positions 657–676 and 1193–1212 of IBDV segment A in primers U3 and L3, respectively (numbering as in segment A of strain P2, Acc No X84034), and to nucleotide positions 290–311 and 861–883 of IBDV segment B in primers +290 and −861, respectively (numbering as in segment B of strain D6948, Acc No AF240687). The IBDV-specific extremity is coupled to a non-IBDV 5' extremity (bold type in the sequence above) corresponding to the M13 and RM13 universal primers in the upper and lower primers, respectively. The M13 and RM13 universal primers are commonly used as primers in DNA sequencing reactions, so that purified PCR products resulting from amplification with the U3/L3 and +290/−861 primer pairs can be easily sequenced in both directions. Finally, restriction sites (underlined in the above sequence) are included for the following restriction endonucleases: SphI (in primer U3), EcoRI (in primers L3 and +290), and PstI (in primer −861). These restriction sites are positioned so that the PCR products resulting from amplification with the U3/L3 or +290/−861 primer pairs can be cloned if required. The U3/L3 pair generates a 604 base pair (bp) product, 516 bp of which are specific of the amplified IBDV sequence and encompass the region encoding the hyper-variable region of the VP2 protein. The +290/−861 pair generates a 642 bp product, 549 bp of which are specific of the amplified IBDV sequence. Both products are derived from genomic regions that are suitable for phylogenetic analysis (Errardossi et al., 1998; 1999; Le Nouen et al., 2005; 2006).

Perform an initial denaturation step as recommended by the DNA polymerase supplier, followed by 35 cycles, each including one denaturation, one annealing and one elongation step. In such cycles, denaturation at 95°C for 30 seconds and annealing at 64°C for 45 seconds may be used with both the U3/L3 and +290/−861 primer pairs (the annealing temperature should be adapted if other primers are used). The parameters for the elongation step should be set according to the supplier’s recommendations.

Revelation may be performed by electrophoresis with the PCR products and DNA molecular weight markers in a 1% agarose gel stained with ethidium bromide (caution: ethidium bromide is toxic and carcinogenic. It should be handled and disposed accordingly).

Three PCR reactions should be performed for each cDNA sample (pure, 10- and 100-fold diluted cDNA) to avoid false-negative results due to PCR inhibition in mixes containing high amounts of the cDNA preparation.

Each PCR should include negative and positive control reactions. Protocols that include an internal control avoid false-negative results due to PCR inhibition in mixes containing high amounts of the cDNA preparation.

Delaying the PCR for several weeks after the RT step may cause false negative PCR results.

f) **Isolation of virus in cell culture**

Inoculate 0.5 ml of sample into each of four freshly confluent chicken embryo fibroblast (CEF) cultures (from a specific pathogen free [SPF] source) in 25 cm² flasks. Adsorb at 37°C for 30–60 minutes, wash twice with Earle’s balanced salt solution and add maintenance medium to each flask. Incubate the cultures at 37°C, observing daily for evidence of cytopathic effect (CPE). This is characterised by small round refractive cells. If no CPE is observed after 6 days, discard the medium, then freeze and thaw the cultures and inoculate the resulting lysate into fresh cultures. This procedure may need to be repeated at least three times. If CPE is observed, the virus should be tested against IBDV antiserum in a tissue culture virus neutralisation (VN) test (see below). The more pathogenic IBDV strains usually cannot be adapted to grow in CEF unless the virus has first been submitted to extensive serial passage in embryos (see below).

g) **Isolation of virus in embryos**

Inoculate 0.2 ml of sample into the yolk sac of five 6–8-day-old specific antibody negative (SAN) chicken embryos and on to the chorioallantoic membrane (American Association of Avian Pathologists, 1998) of five 9- to 11-day-old SAN chicken embryos. SAN embryos are derived from flocks shown to be serologically
negative to IBDV. Candle daily and discard dead embryos up to 48 hours post-inoculation. Embryos that die after this time are examined for lesions. Serotype 1 IBD produces dwarfing of the embryo, subcutaneous oedema, congestion and subcutaneous or intracranial haemorrhages. The liver is usually swollen, with patchy congestion producing a mottled effect. In later deaths, the liver may be swollen and greenish, with areas of necrosis. The spleen is enlarged and the kidneys are swollen and congested, with a mottled effect. If lesions are observed, the virus should then be tested against a monospecific anti-IBDV serum in an embryo-revealed virus neutralisation assay.

Serotype 1 IBDV usually causes death in at least some of the embryos on primary isolation.

Serotype 2 IBDV does not induce subcutaneous oedema or haemorrhages in the infected embryos, but embryos are of a smaller size with a pale yellowish discolouration.

For the preparation of embryo-propagated stock virus or for subsequent passaging, embryos with lesions or embryos suspected to be infected, respectively, are harvested aseptically. Their head and limbs are discarded and the main body is minced as described in Section B.1.a for the preparation a virus suspension.

h) Isolation of virus in chickens

This method has been used in the past but is no longer recommended due to animal welfare concerns. Five susceptible and five IBD-immune chickens (3–7 weeks of age) are inoculated by the eye-drop route with 0.05 ml of sample. Kill the chickens 72–80 hours after inoculation, and examine their bursae of Fabricius. The bursae of chickens infected with virulent serotype 1 IBDV appear yellowish (sometimes haemorrhagic) and turgid, with prominent striations. Peribursal oedema is sometimes present, and plugs of caseous material are occasionally found. The plicae are petechiated.

The presence of lesions in the bursae of susceptible chickens along with the absence of lesions in immune chickens is diagnostic of IBD. The bursae from both groups may be used as antigen in an agar gel immunodiffusion (AGID) test against known positive IBD antiserum (see Section B.1.b).

The extent of bursal damage may vary considerably with the pathogenicity of the studied IBDV strain. However, as the samples submitted for virus isolation may vary in virus content, the extent of bursal damage observed in susceptible chickens at the isolation stage gives a limited indication on strain pathogenicity.

The bursae of chickens infected with serotype 2 IBDV do not exhibit any gross lesions.

i) Strain differentiation

IBDV strains can be further identified by testing their pathogenicity in SAN chickens, by investigating their antigenic reactivity in cross VN tests or using MAbs, by determining the nucleotide sequence of RT-PCR amplification products derived from IBDV genome, or by studying the number and size of the restriction fragments obtained following digestion of such RT-PCR products with restriction endonucleases. Several protocols have been described for each of these approaches. Tests should be performed by specialised laboratories and should include a panel of reference strains as controls. Although the molecular basis for antigenic variation is now better understood, no validated virulence marker has been described yet.

• Pathogenicity testing

Studies to compare the pathogenicity of IBDV strains must be carried out in secure biocontainment facilities to avoid the dissemination of the studied virus (see Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities). SAN birds with a known microbial status (ideally SPF chickens) must be used to avoid interference by contaminating agents.

The main variables when comparing the results of pathogenicity trials are the breed, age and immune status of the challenged chickens, the dose and route of inoculation of the challenge virus, and the possible presence of contaminating agents in the inoculum. Light layer breeds have been reported to be more susceptible than heavy broilers (Van den Berg & Meulemans, 1991). Differences in susceptibility may also occur between different SPF chicken lines. The highest susceptibility to acute IBD occurs in chickens between 3 and 6 weeks of age (Lukert & Saif, 1997). (The influence of the immune status is described in Section C.) A high dose of challenge virus such as that recommended in Section C.1.c is necessary so that all inoculated chickens become infected at once without requiring bird-to-bird transmission of the inoculated virus. Finally, the presence in the inoculum of contaminating agents, such as adenovirus or chicken infectious anaemia virus, may modify the severity of IBD and signs observed after challenge (Rosenberger et al., 1975).

The terms ‘variant’, ‘classical’ and ‘very virulent’ have been used to describe IBDV strains that exhibit a different pathogenicity. Based on the signs and lesions observed in two lines of White Leghorn SPF chickens during acute experimental IBD following a $10^5$ 50% embryo infective dose (EID$_{50}$) challenge, North American ‘Variant’ IBDVs induce little if any clinical signs and no mortality but marked bursal lesions, ‘Classical’ IBDVs induce
approximately 10–50% mortality with typical signs and lesions whereas 'very virulent' IBDVs induce approximately 50–100% mortality with typical signs and lesions (Eterradossi et al., personal observation).

• Antigenicity testing

Antigenic relatedness among IBDV strains may be assayed in cross VN tests, which correlate best with cross protection. Such tests have to be performed in SAN embryonated eggs when the studied viruses do not grow in CEF (e.g. very virulent IBDV [vvIBDV]). Differences in cross VN results among serotype 1 IBDV strains have led to the definition of serotype 1 ‘subtypes’, some of which include the antigenically ‘variant’ North American IBDV isolates (Jackwood & Saif, 1987).

Another approach to the study of genetic relatedness is the use of mouse MAbs that bind to IBDV neutralising epitopes. Several panels of MAbs exist world-wide (Eterradossi et al., 1997; Fahey et al., 1991; Snyder et al., 1992). Some of the MAbs have been included in commercially available kits, but no unified MAb panel as yet been proposed. All neutralising epitopes of IBDV characterised to date have been mapped into a major immunogenic domain in the middle third (amino acid positions 200 to 340) of the VP2 outer capsid protein (Eterradossi et al., 1998; Schnitzler et al., 1993; Vakharia et al., 1994). This region is termed 'VP2 variable domain' because most amino acid changes observed among IBDV strains are clustered in it. Within vVP2, four amino acid stretches are of critical importance to antigenicity and are referred to as vVP2 hydrophilic peaks. These are amino acid positions 210 to 225 (major peak A), 249 to 252 (minor peak 1), 281 to 292 (minor peak 2) and 313 to 324 (major peak B) (Azad et al., 1987; Van den Berg et al., 1996). Both North American 'variants' and 'very virulent' IBDV exhibit in these areas amino acid changes that correlate with epitope variation (Eterradossi et al., 1999; Vakharia et al., 1994). To date, no antigenic marker has been shown to correlate strictly with IBDV pathogenicity.

• Molecular identification

Most efforts at molecular identification have focused on the characterisation of the larger segment of IBDV (segment A) and especially of the vVP2 encoding region. Several protocols have been published on characterisation using restriction endonucleases of RT-PCR products. These approaches are known as RT-PCR/RE or RT-PCR-RFLP (restriction fragment length polymorphism) (Jackwood & Jackwood, 1997; Lin et al., 1993; Zierenberg et al., 2001). The usefulness of the information they provide depends on the identification of enzymes that cut in restriction sites that are phenotypically relevant. Some sites involved in antigenicity have already been identified (see above), however, restriction sites reliably related to virulence still need to be defined and validated. Nucleotide sequencing of RT-PCR products, although more expensive than restriction analysis, provides an approach to assessing more precisely the genetic relatedness among IBDV strains. Markers have been demonstrated experimentally, using a reverse genetics approach, for cell culture-adapted strains, which exhibit amino acid pairs 279 N–284 T (Lim et al., 1999) or 253 H–284 T (Mundt, 1999). In most very virulent viruses, four typical amino acids are present (222 A, 256 I, 294 I and 299 S) (Brown et al., 1994; Eterradossi et al., 1999; Lie et al., 1993). However, it is not yet known whether these amino acids play a role in virulence or if they are merely an indication of the clonal origin of most vvIBDV isolates. Several recent studies indicate that although VP2 is an important virulence determinant, it may not be the only one (Boot et al., 2000). It has been reported that segment A and B of IBDV mostly co-evolve (i.e. most significant IBDV clusters, such as vvIBDV-related strains, may be identified by analysis of both genome segments), however some potentially reassortant viruses have been identified (Le Nouen et al., 2006).

2. Serological tests

a) Agar gel immunodiffusion test

The AGID test is the most useful of the serological tests for the detection of specific antibodies in serum, or for detecting viral antigen or antibodies in bursal tissue.

Blood samples should be taken early in the course of the disease, and repeat samples should be taken 3 weeks later. As the virus spreads rapidly, only a small proportion of the flock needs to be sampled. Usually 20 blood samples are enough. For detection of antigen in the bursa of Fabricius, the bursae should be removed aseptically from about ten chickens at the acute stage of infection. The bursae are minced using two scalpels in a scissor movement, then small pieces are placed in the wells of the AGID plate against known positive serum. Freeze–thaw cycles of the minced tissue may improve the release of IBDV antigens from the infected bursal tissue.

• Preparation of positive control antigen

Inoculate 3–5-week-old susceptible chickens, by eye-drop, with a clarified 10% (w/v) bursal homogenate known to contain viable IBDV1. Kill the birds 3 days post-inoculation, and harvest the bursae aseptically.

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1 A suitable classical strain of IBDV (serotype 1, classical pathotype) is strain 52/70, obtainable from one of the OIE Reference Laboratories (see Table given in Part 4 of this Terrestrial Manual).
Chapter 2.3.12. — Infectious bursal disease (Gumboro disease)

Discard haemorrhagic bursae and pool the remainder, weigh and add an equivalent volume of cold distilled water (or of a suitable buffer such as PBS or tryptose phosphate broth) and an equivalent volume of undiluted methylene chloride. (Caution: methylene chloride is toxic and possibly carcinogenic. It should be handled and disposed accordingly. A possible alternative to avoid health hazards caused by methylene chloride is to use trichlorotrifluoroethane). Thoroughly homogenise the mixture in a tissue blender and centrifugate at 2000 g for 30 minutes. Harvest the supernatant fluid and dispense into aliquots for storage at −40°C. The antigen contains live virus and should be handled only in suitable containment facilities such as a class II safety cabinet. If required, the antigen can be inactivated prior to dispensing: add 0.3% (v/v) β-propiolactone to the harvested supernatant, then further incubate at 37°C for 2 hours. It is important that incubation takes place on an orbital shaker or a mechanical rocker, so that any inner part of the vial that has been in contact with live virus indeed gets into contact with β-propiolactone. Dispense and store as above. Check the efficacy of the inactivation process by attempting IBDV isolation from the inactivated antigen, with three serial passages on SAN embryonated eggs (see Section B.1.g).

- **Preparation of positive control antiserum**

Inoculate 4–5-week-old susceptible chickens, by eye-drop, with 0.05 ml of a clarified 10% (w/v) bursal homogenate known to contain viable IBDV. Exsanguinate 28 days post-inoculation. Pool and store serum in aliquots at −20°C.

- **Preparation of agar**

Dissolve sodium chloride (80 g) and phenol (5 g) in distilled water (1 litre) (caution: phenol is toxic and should be handled and disposed of accordingly). Add agar (12.5 g) and steam until the agar has dissolved. To avoid the health and environmental hazards caused by the use of phenol, another suitable recipe for the preparation of agar is as follows: sodium chloride (80 g), kalium dihydrogenophosphate (0.45 g), sodium hydrogenophosphate dihydrate (1.19 g), agar (10 g) and distilled water to a final volume of 1 litre (final pH 7.1 at 20–25°C). This second recipe can be homogenised by heating up to 90°C under agitation. While the mixture is still very hot, filter it through a pad of cellulose wadding covered with a few layers of muslin and dispense the medium in 20 ml volumes into glass bottles. The medium without phenol can further be sterilised by autoclaving at (at most) 115°C for 15 minutes. Store the bottles at 4°C until required for use.

- **Test procedure**

  i) Prepare plates from 24 hours to 7 days before use. Dissolve the agar by placing in a steamer or boiling water bath. Take care to prevent water entering the bottles.

  ii) Pour the contents of one bottle into each of the required number of 9 cm plastic Petri dishes laid on a level surface. (Some laboratories prefer to pour the gel on 25 × 75 mm glass slides, 3 mm deep.)

  iii) Cover the plates and allow the agar to set, and then store the plates at 4°C. (If the plates are to be used the same day that they are poured, dry them by placing them opened but inverted at 37°C for from 30 minutes to 1 hour.)

iv) Cut three vertical rows of wells 6 mm in diameter and 3 mm apart, using a template and tubular cutter.

![Fig. 1. Protocol for tests for antibody.](image1.png)

**AG** = Positive antigen
**AB** = Positive antiserum

![Fig. 2. Protocol for tests for antigen.](image2.png)

**T** = test sera

Notes:

1. The linear pattern of wells is preferred although a hexagonal pattern may be used. Each test serum or bursa should be placed adjacent to a positive control antibody (AB) or antigen (AG), respectively.

2. Wells, 3 mm deep, 6 mm in diameter, and 3 mm apart (or wells of any other size previously shown to be effective), are used.

iv) Cut three vertical rows of wells 6 mm in diameter and 3 mm apart, using a template and tubular cutter.
v) Remove the agar from the wells by aspiration or remove using a pen and nib, taking care not to damage the walls of the wells.

vi) Using a pipette, dispense 50 µl of the test sera into the wells as shown in Figure 1.

Or, for the detection of IBDV antigens in bursae:

Dispense small pieces of finely minced test bursae by means of curved fine-pointed forceps into the wells, as shown in Figure 2, to just fill the wells. Alternatively, the freeze–thaw exudate of minced tissues can be used to fill the wells.

vii) Dispense 50 µl of the positive and negative control reagents into the relevant wells.

viii) Incubate the plates at between 22°C and 37°C for up to 48 hours in a humid chamber to avoid drying the agar.

ix) Examine the plates against a dark background with an oblique light source after 24 and 48 hours.

**Quantitative agar gel immunodiffusion tests**

The AGID test can also be used to measure antibody levels by using dilutions of serum in the test wells and taking the titre as the highest dilution to produce a precipitin line (Cullen & Wyeth, 1975). This can be very useful for measuring maternal or vaccinal antibodies and for deciding on the best time for vaccination; however, this AGID quantitative determination has now been largely replaced by the ELISA.

b) Virus neutralisation tests

VN tests are carried out in cell culture. The test is more laborious and expensive than the AGID test, but is more sensitive for detecting antibody. This sensitivity is not required for routine diagnostic purposes, but may be useful for evaluating vaccine responses or for differentiating between IBDV 1 and 2 serotypes.

First, 0.05 ml of virus diluted in tissue culture medium to contain 100 TCID_{50} (50% tissue culture infective doses) per 0.05 ml is placed in each well of a tissue-culture grade microtitre plate (Spearman–Kärber [American Association of Avian Pathologists, 1998] or the Reed & Muench [1938]). The test sera are heat-inactivated at 56°C for 30 minutes. Serial doubling dilutions of the sera are made in the diluted virus. After 30 minutes at room temperature, 0.2 ml of SPF chicken embryo fibroblast cell suspension, with a cell density allowing confluent layers to be obtained after 24 hours of incubation, is dispensed into each well. Plates are sealed and incubated at 37°C for 4–5 days, after which the monolayers are observed microscopically for typical CPE. The end-point (serum titre) is expressed as the reciprocal of the highest serum dilution that did not show CPE. To reduce test-to-test and operator-to-operator variation, a standard reference antiserum may be included with each batch of tests and the titre of the virus suspension must be reassessed in each new experiment using a sufficient number of repeats (wells) per virus dilution.

c) Enzyme-linked immunosorbent assay

ELISAs are in use for the detection of antibodies to IBD. Coating the plates requires a purified, or at least semipurified, preparation of virus, necessitating special skills and techniques. Methods for preparation of reagents and application of the assay were described by Marquardt et al. (1980). Commercial kits are available.

The test sera are diluted according to the established protocol or kit instructions and each is dispensed into the requisite number of wells. After incubation under the appropriate conditions, the sera are discarded from the plates, and the wells are washed thoroughly. Anti-chicken immunoglobulins conjugated to an enzyme are dispensed into the wells, and the plates are again incubated as appropriate. The plates are emptied and rewashed before substrate containing a chromogen that gives a colour change in the presence of the enzyme used is added to the plate. After a final incubation step, the substrate/chromogen reaction is stopped by addition of a suitable stopping solution and the colour reactions are quantified by measuring the optical density of each well. The Sample to Positive (S/P) ratio for each test sample is calculated.

d) Interpretation of results

The AGID test is surprisingly sensitive, though not as sensitive as the VN test; the latter will often give a titre when the AGID test is negative. Positive reactions indicate infection in unvaccinated birds without maternal antibodies. As a guide, a positive AGID reaction in a vaccinated bird or young bird with maternal antibody indicates a protective level of antibody. ELISA gives more rapid results than VN or AGID and is less costly in terms of labour, although the reagents are more expensive. VN and AGID titres correlate well, but as VN is more sensitive, AGID titres are proportionally lower. Correlation between ELISA and VN and between ELISA

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2 A suitable reference antiserum may be obtained from the OIE Reference Laboratories (see Table given in Part 4 of this Terrestrial Manual).
and AGID is more variable depending on the source of the ELISA reagents. When testing for the decay of maternally derived antibodies (MDA), it is not uncommon to find residual VN antibodies at an age when ELISA results are already negative. A formula has been devised that allows ELISA titres to be used to calculate the optimal age for vaccination (Kouwenhoven & Van Der Bos, 1993), which will vary depending on the vaccine used. Nonspecific positive reactions may occur with most ELISAs because they are usually designed for monitoring vaccine responses, in which case sensitivity is regarded as more important than specificity. This should be taken into account when the ELISA is used for diagnosis. In commercial chicken flocks, the possibility that a serotype 1 ELISA antigen also detects antibodies induced by a natural infection with serotype 2 IBDV cannot be ruled out, however this possible cross-reactivity has not yet been demonstrated to interfere with serological monitoring programmes of IBD based on the ELISA.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Two types of vaccine are mostly available for the control of IBD. These are live attenuated vaccines, or inactivated oil-emulsion adjuvanted vaccines (Thornton & Pattison, 1975). A live recombinant vaccine expressing IBDV antigens has also been licensed (Darteil et al., 1995).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

To date, IBD vaccines have been made with serotype 1 IBDV only, although a serotype 2 virus has been detected in poultry. The serotype 2 virus has not been associated with disease, but its presence will stimulate antibodies. Serotype 2 antibodies do not confer protection against serotype 1 infection, neither do they interfere with the response to type 1 vaccine. There have been numerous descriptions of antigenic variants of serotype 1 virus (Rosenberger & Cloud, 1986). Cross-protection studies have shown that inactivated vaccines prepared from ‘classical’ serotype 1 virus require a high antigenic content to provide good protection against some of these variants. IBD vaccines that contain both classical and variant IBD serotype 1 viruses are now licensed. vvIBDV strains with limited antigenic changes as compared with ‘classical’ serotype 1 viruses have emerged since 1986. Active immunisation with a ‘classical’ serotype 1 virus or vaccine provides a good protection against the vvIBDVs (Jackwood, 1990), however the latter viruses are less susceptible to neutralisation by maternally derived antibodies than ‘classical’ pathogenic viruses (Van den Berg & Meulemans, 1991).

- Live vaccines: methods of use

Live IBD vaccines are produced from fully or partially attenuated strains of virus, known as ‘mild’, ‘intermediate’, or ‘intermediate plus’ (‘hot’), respectively.

Mild or intermediate vaccines are used in parent chickens to produce a primary response prior to vaccination near to point-of-lay using inactivated vaccine. They are susceptible to the effect of MDA so should be administered only after all MDA has waned. Application is by means of intramuscular injection, spray or in the drinking water, usually at 8 weeks of age (Skeeles et al., 1979).

Intermediate or intermediate plus vaccines are used to protect broiler chickens and commercial layer replacements. Some of these vaccines are also used in young parent chickens if there is a high risk of natural infection with virulent IBD. Although intermediate vaccines are susceptible to the presence of MDA, they are sometimes administered at 1-day old, as a coarse spray, to protect any chickens in the flock that may have no or only minimal levels of MDA. This also establishes a reservoir of vaccine virus within the flock that allows lateral transmission to other chickens when their MDA decay. Second and third applications are usually administered, especially when there is a high risk of exposure to virulent forms of the disease or when the vaccinated chicks exhibit uneven MDA levels. The timing of additional applications will depend on the antibody titres of the parent birds at the time the eggs were laid. As a guide, the second dose is usually given at 10–14 days of age when about 10% of the flock is susceptible to IBD, and the third dose 7–10 days later. The route of administration is by means of spray or in the drinking water. Intramuscular injection or eye-drop is used rarely. If the vaccine is given in the drinking water, clean water with a neutral pH must be used that is free from smell or taste of chlorine or metals. Skimmed milk powder may be added at a rate of 2 g per litre. Care must be taken to ensure that all birds receive their dose of vaccine. To this end, all water should be removed (cut off) for 2–3 hours before the medicated water is made available and care must be taken that no residual water remains in the water adduction pipes or in the drinkers. It is possible to divide the medicated water into two parts, giving the second part 30 minutes after the first.

Recently, technology has been developed to deliver live vaccine into eggs during the incubation period. Live vaccine virus is blended with IBD antibody and the complex is injected in ovo at 18 days of incubation. The eggs go on to hatch and the vaccine virus is released when the chicks are about 7 days of age. In this way, the problem of maternally derived IBD antibody is overcome and the chicks are effectively immunised (Haddad et al., 1997).
A live recombinant vaccine that uses a viral vector (herpes virus of turkeys) to express the VP2 antigen of IBDV in chickens has been licensed recently in Europe. There is limited information available on the use of this vaccine.

Live IBD vaccines are generally regarded as compatible with other avian vaccines. However, it is possible that IBD vaccines that cause bursal damage could interfere with the response to other vaccines. Only healthy birds should be vaccinated. The vials of lyophilised vaccine should be kept at temperatures between 2°C and 8°C up to the time of use.

- **Inactivated vaccines: method of use**

Inactivated IBD vaccines are mostly used to produce high, long-lasting and uniform levels of antibodies in breeding hens that have previously been primed by live vaccine or by natural exposure to field virus during rearing (Cullen & Wyeth, 1976). The usual programme is to administer the live vaccine at about 8 weeks of age. This is followed by the inactivated vaccine at 16–20 weeks of age. Occasionally, inactivated vaccines may be used in programmes combining inactivated and live vaccines, in young valuable birds with high MDA levels reared in areas with high risk of exposure to virulent IBDV. The inactivated vaccine is manufactured as a water-in-oil emulsion, and has to be injected into each bird. The preferred routes are intramuscular into the leg muscle, avoiding proximity to joints, tendons or major blood vessels or the subcutaneous route. A multidose syringe may be used. All equipment should be cleaned and sterilised between flocks, and vaccination teams should exercise strict hygiene when going from one flock to another. Vaccine should be stored at between 4°C and 8°C. It should not be frozen or exposed to bright light or high temperature.

Only healthy birds, known to be sensitised by previous exposure to IBDV, should be vaccinated. Used in this way the vaccine should produce such a good antibody response that chickens hatched from those parents will have passive protection against IBD for up to about 30 days of age (Wyeth & Cullen, 1979). This covers the period of greatest susceptibility to the disease and prevents bursal damage at the time when this could cause immunosuppression. It has been shown that bursal damage occurring after about 15 days of age has little effect on immunocompetence as by that time the immunocompetent cells have migrated into the peripheral lymphoid tissues. However, if there is a threat of exposure to infection with very virulent IBDV, live vaccines should be applied as described above. The precise level and duration of immunity conferred by inactivated IBD vaccines will depend mainly on the concentration of antigen present per dose. The manufacturing objective should be to obtain a high antigen concentration and hence a highly potent vaccine.

1. **Seed management**

a) **Characteristics of the seed**

- **Live vaccine**

  The seed virus must be shown to be free from extraneous viruses, bacteria, mycoplasma and fungi, particularly avian pathogens. This includes freedom from contamination with other strains of IBDV. For vaccine strains that claim to be attenuated and nonimmunosuppressive, the seed virus must be shown to be stable, with no tendency to revert to virulence. This can be confirmed by carrying out at least five consecutive chicken-to-chicken passages at 3–4-day intervals using bursal suspension as inoculum in SPF chickens of the minimum age recommended for vaccination. It must be shown that the virus was transmitted. A histological comparison is then made to show that there is no difference between bursae from birds inoculated with the initial and the final passage material. Bursal scoring (Muskett et al., 1979) and imaging techniques have been developed.

  **Test for immunosuppression:** An important characteristic is that the virus should not produce such damage to the bursa of Fabricius that it causes immunosuppression in susceptible birds. Live vaccines of the ‘intermediate’ or ‘intermediate plus’ type may be licensed even though they may be capable of causing immunosuppression. A possible protocol for the experimental assessment of immunosuppression is the following: The vaccine is administered by injection or eye-drop, one field dose per bird, to each of 20 SPF chickens, at 1-day old. A further group of birds of the same age and source are housed separately as controls. At 2 weeks of age, each bird in both groups is given one field dose of live Newcastle disease vaccine by eye-drop. Alternatively, the IBDV vaccine may be administered at the minimum age recommended for vaccination, and the Newcastle disease vaccine at the time when bursal lesions induced by the IBDV vaccine are maximal. The haemagglutination inhibition (HI) response of each bird to Newcastle disease vaccine is measured 2 weeks after the administration of the Newcastle Disease vaccine, and the protection is measured against challenge with $10^{5.0}$ to $10^{6.5}$ ELD$_{50}$ (50% embryo lethal doses) Herts 33/56 strain (or similar) of Newcastle disease virus. The IBD vaccine fails the test if the HI response and protection afforded by Newcastle disease vaccine is significantly less (<0.01) in the group given IBD vaccine than in the control group. In countries where Newcastle disease virus is exotic, an alternative is to use sheep erythrocytes or *Brucella abortus*-killed antigen as the test antigen, measuring the response using the haemagglutination or serum agglutination test, respectively. However, another live vaccine is a preferable test system because it also evaluates cell-mediated immunity.
• Killed vaccine
For killed vaccines, the most important characteristics are high yield and good antigenicity. Both virulent and attenuated strains have been used. The seed virus must be shown to be free from extraneous viruses, bacteria, mycoplasma and fungi, particularly avian pathogens (Thornton & Muskett, 1982).

b) Method of culture
Seed virus may be propagated in various culture systems, such as SPF chicken embryo fibroblasts, or chicken embryos. In some cases, propagation in the bursa may be used. The bulk is distributed in aliquots and freeze-dried in sealed containers. There have been claims that bursal origin vaccines are better immunogens than tissue culture vaccines. In controlled studies, it was concluded that both vaccines, when containing similar antigenic mass, elicited similar immune responses.

c) Validation as a vaccine
Data on efficacy should be obtained before bulk manufacture of vaccine begins. The vaccine should be administered to birds in the way in which it will be used in the field. Live vaccine can be given to young birds and the response measured serologically and by resistance to experimental challenge. In the case of killed vaccines, a test must be carried out in older birds that go on to lay, using the recommended vaccination schedule, so that their progeny can be challenged to determine resistance due to MDA at the beginning and end of lay.

• Live vaccine
**Efficacy test:** Administer one vaccine dose of the minimum recommended titre to each of 20 SPF chickens of the minimum age of vaccination. Inoculate separate groups for each of the recommended routes of application. Leave 20 chickens from the same hatch as uninoculated controls. After 14 days, challenge each of the chickens by eye-drop with approximately 100 CID_{50} (50% chicken infective dose) of a virulent strain of IBDV as recommended by one of the OIE Reference Laboratories for IBD (see Table given in Part 4 of this Terrestrial Manual). Observe the chickens daily for 10 days. Register the number of birds that die or exhibit IBD signs. Perform a histological examination of the bursa in chickens that survive at day 10. The vaccine fails the test unless at least 90% of the vaccinated chickens survive without showing either clinical signs or severe lesions in the bursae of Fabricius at the end of the observation period. If more than half the controls do not show IBD signs, or one or more control chicken does not exhibit severe lesions of the bursa of Fabricius, or control or inoculated birds die from causes not attributable to the test, the test is invalid. Lesions are considered to be severe if at least 90% of follicles show greater than 75% depletion of lymphocytes. Providing results are satisfactory, this test need be carried out on only one batch of all those batches prepared from the same seed lot.

• Killed vaccine
**Efficacy test:** At least 20 unprimed SPF birds are given one dose of vaccine at the recommended age (near to point-of-lay) at least one of the recommended routes; an alternative recommended procedure is to test one dose of vaccine in the recommended routes listed on the label, using 20 unprimed SPF birds for each route. The antibody response is measured between 4 and 6 weeks after vaccination by serum neutralisation with reference to a standard antiserum³.

Eggs are collected for hatching 5–7 weeks after vaccination, and 25 progeny chickens are then challenged at 3 weeks of age by eye-drop with approximately 100 CID_{50} of a recognised virulent strain of IBDV. Ten control chickens of the same breed but from unvaccinated parents are also challenged. Protection is assessed 3–4 days after challenge by removing the bursa of Fabricius from each bird; each bursa is then subjected to histological examination or tested for the presence of IBD antigen by the agar gel precipitin test. Not more than three of the chickens from vaccinated parents should show evidence of IBD infection, whereas all those from unvaccinated parents should be affected.

These procedures should be repeated towards the end of the period of lay when the vaccinated birds are at least 60 weeks of age, but, on this occasion challenge of the progeny should be undertaken when they are 15 days old.

The efficacy test should be repeated on primed birds vaccinated by the recommended schedule. The final dose of killed vaccine is given at the earliest recommended age. Chickens hatched from fertile eggs collected at the beginning and the end of lay are tested for protection against challenge as described above.

These tests need to be performed once only using a typical batch of vaccine.

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³ See footnote 2
2. Method of manufacture

The vaccine must be manufactured in suitable clean and secure accommodation, well separated from diagnostic facilities or commercial poultry.

Production of the vaccine should be on a seed-lot system using a suitable strain of virus of known origin and passage history. SPF eggs must be used for all materials employed in propagation and testing of the vaccine. Live vaccines are made by growth in eggs or cell cultures. Inactivated IBD vaccines may be made using virulent virus grown in the bursae of young birds, or using attenuated, laboratory-adapted strains of IBDV grown in cell culture or embryonated eggs. A high virus concentration is required. These vaccines are made as water-in-oil emulsions. A typical formulation is to use 80% mineral oil to 20% suspension of bursal material in water, with suitable emulsifying agents.

3. In-process control

Antigen content: Having grown the virus to high concentration, its titre should be assayed by use of cell cultures, embryos or chickens as appropriate to the strain of virus being used. The antigen content required to produce satisfactory batches of vaccine should be based on determinations made on test vaccine that has been shown to be effective in laboratory and field trials.

Inactivation of killed vaccines: This is often done with either ß-propiolactone or formalin. The inactivating agent and the inactivation procedure must be shown under the conditions of vaccine manufacture to inactivate the vaccine virus and any potential contaminants, e.g. bacteria, that may arise from the starting materials.

Prior to inactivation, care should be taken to ensure a homogeneous suspension free from particles that may not be penetrated by the inactivating agent. A test for inactivation of the vaccine should be carried out on each batch of both the bulk harvest after inactivation and the final product. An alternative approach is to test inactivation of the final or bulk harvest, but not both. The test selected should be appropriate to the vaccine virus being used and should consist of at least two passages in susceptible cell cultures, embryos or chickens, with ten replicates per passage. No evidence of the presence of any live virus or microorganism should be observed.

Sterility of killed vaccines: Oil used in the vaccine must be sterilised by heating at 160°C for 1 hour, or by filtration, and the procedure must be shown to be effective. Tests appropriate to oil-emulsion vaccines are carried out on each batch of final vaccine as described, for example, in the European Pharmacopoeia.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

b) Safety

• Live vaccine safety test

Ten field doses of vaccine are administered by eye-drop to each of 15 SPF chickens of the minimum age recommended for vaccination and not older than 2 weeks. The chickens are observed for 21 days. If more than two chickens die due to causes not related to the vaccine, the test must be repeated. The vaccine fails the test if any chickens die or show signs of disease attributable to the vaccine. This test is performed on each batch of final vaccine.

• Killed vaccine safety test

Ten SPF birds, 14–28 days of age, are inoculated by the recommended routes with the recommended dose or twice the field dose. The birds are observed for 3 weeks. No abnormal local or systemic reaction should develop. The test is performed on each batch of final vaccine.

c) Potency

• Live vaccine potency test

A potency test (virus titration) in eggs or cell cultures must be carried out on each serial (batch) of vaccine produced. In addition, the method described in Section C.1.c ‘Live vaccine (efficacy test)’ must be used on one batch representative of all the batches prepared from the same seed lot.
Chapter 2.3.12. — Infectious bursal disease (Gumboro disease)

- Killed vaccine potency test

Ten SPF chickens, approximately 4 weeks of age, are each vaccinated with one dose of vaccine given by the recommended route. An additional ten control birds of the same source and age are housed together with the vaccinated. The antibody response of each bird is determined 4–6 weeks after vaccination in a VN test with reference to a standard antiserum. The mean antibody level of the vaccinated birds should not be significantly less than the level recorded in the test for protection. No antibody should be detected in the control birds. This test must be carried out on each batch of final vaccine.

d) Stability

Evidence should be provided on three batches of vaccine to show that the vaccine passes the batch potency test at the requested shelf life or as an alternative at 3 months beyond.

e) Preservatives

A preservative is normally required for vaccine in multidose containers. The concentration of the preservative in the final vaccine and its persistence throughout shelf life should be checked. A suitable preservative already established for such purposes should be used.

f) Precautions (hazards)

Oil-emulsion vaccines cause serious injury to the vaccinator if accidentally injected into the hand or other tissues. In the event of such an accident the person should go at once to a hospital, taking the vaccine package with them. Each vaccine bottle and package should be clearly marked with a warning of the serious consequences of accidental self-injury. Such wounds should be treated by the casualty doctor as a ‘grease gun injury’.

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

REFERENCES


Chapter 2.3.12. — Infectious bursal disease (Gumboro disease)


Chapter 2.3.12. — Infectious bursal disease (Gumboro disease)

REVIEWs


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NB: There are OIE Reference Laboratories for Infectious bursal disease (Gumboro disease) (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for infectious bursal disease (Gumboro disease).
CHAPTER 2.3.13.

MAREK’S DISEASE

SUMMARY

Marek’s disease (MD) is a lymphomatous and neuropathic disease of domestic fowl caused by an alphaherpesvirus.

Diagnosis is made on clinical signs and gross or microscopic lesions. Chickens may become persistently infected with MD virus (MDV) without developing clinical disease. Infection by MDV is detected by virus isolation and the demonstration of viral antigen or antibodies.

MD is prevented by vaccination with monovalent or multivalent live virus vaccines of various types. The vaccine is injected in ovo or at hatch.

In chickens, MD occurs at 3–4 weeks of age or older and is most common between 12 and 30 weeks of age. Clinical signs observed are paralysis of the legs and wings, with enlargement of peripheral nerves, but nerve involvement is sometimes not seen, especially in adult birds. MDV strains of higher virulence may also cause increased mortality in young birds of 1–2 weeks of age, especially if they lack maternal antibodies. Depending on the strain of MDV, lymphomatosis can occur, especially in the ovary, liver, spleen, kidneys, lungs, heart, proventriculus and skin. As opposed to the uniform cell population that comprises the tumours caused by lymphoid leukosis, the nerve infiltration and lymphomas caused by MDV consist of lymphoid cells of various types. Tumours that resemble those produced by MDV can also be induced by avian retroviruses such as avian leukosis virus (ALV) and reticuloendotheliosis virus (REV) and differentiation of MD from these tumours is important.

Identification of the agent: Under field conditions, most chickens become infected with MDV during the first few weeks of life and then carry the infection throughout their lives, often without developing overt disease. The infection is usually detected by inoculating live buffy coat cells on to monolayer cultures of chicken kidney cells or duck embryo fibroblasts, in which characteristic viral plaques develop within a few days. Two serotypes of MDV are recognised – 1 and 2 – and a third serotype is represented by the related herpesvirus of turkeys (HVT). Serotype 1 includes all the virulent strains and some attenuated vaccine strains. Serotype 2 includes the naturally avirulent strains, some of which are used as vaccines. MDV genomic DNA and viral antigens can be detected in the feather tips of infected birds using polymerase chain reaction (PCR) and radial immunoprecipitation test, respectively (see below).

Serological tests: Antibodies to MDV develop within 1–2 weeks of infection and are commonly recognised by the agar gel immunodiffusion test, the indirect fluorescent antibody test, and sometimes by other serological tests such as enzyme-linked immunosorbent assay.

Requirements for vaccines: MD is prevented by vaccinating chickens in ovo or at 1 day of age. Live viral vaccines are used. HVT (serotype 3), in either a cell-free (lyophilised) form, or a cell-associated (‘wet’) form, is one of the widely used vaccines. Attenuated variants of serotype 1 strains of MDV are the most commonly used vaccine. Serotype 2 strains may also be used, particularly in bivalent vaccines, together with HVT. Serotype 1 and 2 vaccines are only available in the cell-associated form. Bivalent vaccines consisting of serotypes 1 and 3 or trivalent vaccines consisting of serotypes 1, 2, and 3 are also used. The bivalent and trivalent vaccines have been introduced to combat the very virulent strains of MDV that are not well controlled by the monovalent vaccines.

Vaccination greatly reduces clinical disease, but does not prevent the persistent infection by MDV. The vaccine viruses are also carried throughout the life of the fowl and are continued to be shed, which results in the ubiquitous presence of MDV.
**A. INTRODUCTION**

Marek’s disease (MD) (Davison & Nair, eds., 2004; Schat & Nair, 2008; Sharma, 1998) is a disease of domestic fowl (chickens) caused by a herpesvirus. Birds get infected by inhalation of infected dust from the poultry houses, and following a complex life cycle, the virus is shed from the feather follicle of infected birds (Baigent & Davison, 2004). MD occurs at 3–4 weeks of age or older and is most common between 12 and 30 weeks of age. MD is associated with several distinct pathological syndromes, of which the lymphoproliferative syndromes are the most frequent and are of most practical significance. In the classical form of the disease, characterised mainly by the involvement of nerves, mortality rarely exceeds 10–15% and can occur over a few weeks or many months. In the acute form, in which there is usually lymphoma formation in the viscera, a disease incidence of 10–30% in the flock is not uncommon and outbreaks involving up to 70% can occur. Mortality may increase rapidly over a few weeks and then cease, or can continue at a steady or slowly falling rate for several months. Currently, the acute form of the disease with extensive visceral lymphomas is most prevalent. In its classical form, the most common clinical sign of MD is partial or complete paralysis of the legs and wings. In the acute form, birds are often severely depressed and some may die without showing signs of clinical disease. Non-neoplastic disease involving brain pathology with vasogenic oedema resulting in transient paralysis is increasingly recognised with MD induced by the more virulent strains.

In the classical form, the characteristic finding is enlargement of one or more peripheral nerves. Those most commonly affected and easily seen at post-mortem are the brachial and sciatic plexuses, coeliac plexus, abdominal vagus and intercostal nerves. Affected nerves are often two or three times their normal thickness, the normal cross-striated and glistening appearance is absent, and the nerve may appear greyish or yellowish, and sometimes oedematous. Lymphomas are sometimes present in the classical form of MD, most frequently as small, soft, grey tumours in the ovary, and sometimes also in the lungs, kidneys, heart, liver and other tissues. ‘Grey eye’ caused by an iridocyclitis that renders the bird unable to accommodate the iris in response to light and causes a distorted pupil is common in older (16–18 week) birds, and may be the only presenting sign.

In the acute form, the typical finding is widespread, diffuse lymphomatous involvement of the liver, gonads, spleen, kidneys, lungs, proventriculus and heart. Sometimes lymphomas also arise in the skin around the feather follicles and in the skeletal muscles. Affected birds usually have enlarged peripheral nerves, as in the classical form. In younger birds, liver enlargement is usually moderate in extent, but in adult birds the liver may be greatly enlarged and the gross appearance identical to that seen in lymphoid leukosis, from which the disease must be differentiated. Nerve lesions are often absent in adult birds with MD.

In both the classical and acute forms of MD, the disease starts as a proliferation of lymphoid cells, which is progressive in some cases and regressive in others. The peripheral nerves may be affected by proliferative, inflammatory or minor infiltrative changes, which are termed type A, B, and C lesions, respectively. The A-type lesions consist of infiltration by proliferating lymphoblasts, large, medium and small lymphocytes, and macrophages, and appear to be neoplastic in nature. The B-type lesion is characterised by interneuritic oedema, infiltration by mainly small lymphocytes and plasma cells, and Schwann cell proliferation, and appears to be inflammatory. The C-type lesion consists of a light scattering of mainly small lymphocytes, and is often seen in birds that show no gross lesions or clinical signs. It is thought to be a regressive, inflammatory lesion. Demyelination frequently occurs in nerves affected by the A- and B-type lesions, and is responsible for the clinical paralysis.

Lymphomas in the visceral organs and other tissues are similar cytologically to the lymphoproliferations in the A-type lesions in nerves. Usually the lymphoid cells are of mixed types, often with a preponderance of small and medium lymphocytes, but sometimes, particularly in acute MD in adult birds, large lymphocytes and lymphoblasts may predominate.

The heterogeneous population of lymphoid cells in MD lymphomas, as seen in haematoxylin-and-eosin-stained sections, or in impression smears of lymphomas stained by May–Grünwald–Giemsa, is an important feature in differentiating the disease from lymphoid leukosis, in which the lymphomatous infiltrations are composed of uniform lymphoblasts. Another important difference is that, in lymphoid leukosis, gross lymphomas occur in the bursa of Fabricius, and the tumour has an intrafollicular origin and pattern of proliferation. In MD, although the bursa is sometimes involved in the lymphoproliferation, the tumour is less apparent, diffuse and interfollicular in location. Peripheral nerve lesions are not a feature of lymphoid leukosis as they are in MD. The greatest difficulty comes in distinguishing between lymphoid leukosis and forms of MD sometimes seen in adult birds in which the tumour is lymphoblastic with marked liver enlargement and absence of nerve lesions. If post-mortems are conducted on several affected birds, a diagnosis can usually be made based on gross lesions and histopathology. However there are other specialised techniques described. The expression of a Meq biochemical marker has been used to differentiate between MD tumours, latent MDV infections and retrovirus-induced tumours (Schat & Nair, 2008). The procedure may require specialised reagents and equipment and it may not be possible to carry out these tests in laboratories without these facilities. Other techniques, such as detection by immuno-fluorescence of activated T cell antigens present on the surface of MD tumour cells (MD tumour-associated surface antigen or MATSA), or of B-cell antigens or IgM on the tumour cells of lymphoid leukosis can give a presumptive diagnosis, but these are not specific to MD tumour cells.
Nerve lesions and lymphomatous proliferations induced by certain strains of reticuloendotheliosis virus are similar, both grossly and microscopically, to those present in MD. Although reticuloendotheliosis virus is not common in chicken flocks, it should be borne in mind as a possible cause of lymphoid tumours; its recognition depends on virological and serological tests on the flock. Reticuloendotheliosis virus can also cause neoplastic disease in turkeys, ducks, quail and other species. Another retrovirus, designated lymphoproliferative disease virus (LPDV), also causes lymphoproliferative disease in turkeys. Although chicken flocks may be seropositive for reticuloendotheliosis virus, neoplastic disease is rare. The main features in the differential diagnosis of MD, lymphoid leukosis and reticuloendotheliosis are shown in Table 1. Peripheral neuropathy is a syndrome that can easily be confused with the neurological lesions caused by MD virus (MDV). This is not very common but its incidence may be increasing in some European flocks (Bacon et al., 2001). There are no recognised health risks to humans working with MDV or the related herpesvirus of turkeys (HVT).

**Table 1. Features useful in differentiating Marek’s disease, lymphoid leukosis and reticuloendotheliosis**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Marek’s disease</th>
<th>Lymphoid leukosis</th>
<th>Reticuloendotheliosis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Any age. Usually 6 weeks or older</td>
<td>Not under 16 weeks</td>
<td>Not under 16 weeks</td>
</tr>
<tr>
<td>Signs</td>
<td>Frequently paralysis</td>
<td>Non-specific</td>
<td>Non-specific</td>
</tr>
<tr>
<td>Incidence</td>
<td>Frequently above 5% in unvaccinated flocks. Rare in vaccinated flocks</td>
<td>Rarely above 5%</td>
<td>Rare</td>
</tr>
</tbody>
</table>

**Macroscopic lesions**

| Neuropathy | Frequent | Absent | Infrequent |
| Bursa of Fabricius | Diffuse enlargement or atrophy | Nodular tumours | Nodular tumours |
| Tumours in skin, muscle and proventriculus, ‘grey eye’ | May be present | Usually absent | Usually absent |

**Microscopic lesions**

| Neuropathy | Yes | No | Infrequent |
| Liver tumours | Often perivascular | Focal or diffuse | Focal |
| Spleen | Diffuse | Often focal | Focal or diffuse |
| Bursa of Fabricius | Interfollicular tumour and/or atrophy of follicles | Intrafollicular tumour | Intrafollicular tumour |
| Central nervous system | Yes | No | No |
| Lymphoid proliferation in skin and feather follicles | Yes | No | No |
| Cytology of tumours | Pleomorphic lymphoid cells, including lymphoblasts, small, medium and large lymphocytes and reticulum cells. Rarely can be only lymphoblasts | Lymphoblasts | Lymphoblasts |
| Category of neoplastic lymphoid cell | T cell | B cell | B cell |

*Reticuloendotheliosis virus may cause several different syndromes. The bursal lymphoma syndrome is most likely to occur in the field and is described here.
B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Virus isolation

Infection by MDV in a flock may be detected by isolating the virus from the tissues of infected chickens. However, the ubiquitous nature of MDV must be taken into consideration and the diagnosis of MD should be based on a combination of MDV isolation or detection of the genome by polymerase chain reaction (PCR) and clinical disease. Commonly used sources are buffy coat cells from heparinised blood samples, or suspensions of lymphoma cells or spleen cells. When these samples are collected in the field, it is suggested that they be transported to the laboratory under chilled conditions. As MDV is highly cell associated, it is essential that these cell suspensions contain viable cells. The cell suspensions are inoculated into monolayer cultures of chicken kidney cells or duck embryo fibroblasts (CEF) are less sensitive for primary virus isolation). Serotype 2 and 3 viruses (see Section C.1.a) are more easily isolated in CEF than in chicken kidney cells. Usually a 0.2 ml suspension containing from $10^6$ to $10^7$ live cells is inoculated into duplicate monolayers grown in plastic cell culture dishes (60 mm in diameter). Inoculated and uninoculated control cultures are incubated at 38.5°C in a humid incubator containing 5% CO$_2$. Alternatively, closed culture vessels may be used. Culture medium is replaced at 2-day intervals. Areas of cytopathic effects, termed plaques, appear within 3–5 days and can be enumerated at about 7–10 days.

Another, less commonly used source of MDV for diagnostic purposes is feather tips, from which cell-free MDV can be extracted. Tips about 5 mm long, or minced tracts of skin containing feather tips, are suspended in an SPGA/EDTA (sucrose, phosphate, glutamate and albumin/ethylenediamine tetra-acetic acid) buffer for extraction and titration of cell-free MDV (Calnek et al., 1970). The buffer is made as follows: 0.2180 M sucrose (7.462 g); 0.0038 M monopotassium phosphate (0.052 g); 0.0072 M dipotassium phosphate (0.125 g); 0.0049 M L-monosodium glutamate (0.083 g); 1.0% bovine albumin powder (1.000 g); 0.2% EDTA (0.200 g); and distilled water (100 ml). The buffer is sterilised by filtration and should be at approximately pH 6.5.

This suspension is sonicated and then filtered through a 0.45 µm membrane filter for inoculation on to 24-hour-old drained chicken kidney cell monolayers. After absorption for 40 minutes, the medium is added, and cultures are incubated as above for 7–10 days.

Using these methods, MDV of serotypes 1 and 2 may be isolated, together with the HVT (serotype 3), if it is present as a result of vaccination. With experience, plaques caused by the different virus serotypes can be differentiated fairly accurately on the basis of time of appearance, rate of development, and plaque morphology. HVT plaques appear earlier and are larger than serotype 1 plaques, whereas serotype 2 plaques appear later and are smaller than serotype 1 plaques.

MDV and HVT plaques may be identified as such using specific antibodies raised in chickens. Monoclonal antibodies may be used to differentiate serotypes (Lee et al., 1983).

b) Antigen detection

A variation of the AGID test used for serology (see below) may be used to detect MDV antigen in feather tips as an indication of infection by MDV. Glass slides are prepared with a coating of 0.7% agarose (e.g. A37) in 8% sodium chloride, containing MDV antiserum. Tips of small feathers are taken from the birds to be examined and are inserted vertically into the agar, and the slides are maintained as described below. The development of radial zones of precipitation around the feather tips denotes the presence in the feather of MDV antigen and hence of infection in the bird.

c) Polymerase chain reaction (PCR)

Genomes of all three serotypes have been completely sequenced (Afonso et al., 2001; Lee et al., 2000). PCR tests have been developed for the diagnosis MD. Real-time quantitative PCR (qPCR) to quantify MDV genome copies has also been described (Abdul-Careem et al., 2006; Baigent et al., 2005; Islam et al., 2004). In addition, PCR tests that enable differentiation of oncogenic and non-oncogenic strains of serotype 1 MDV, and of MDV vaccine strains of serotypes 2 and 3 (Becker et al., 1992; Bumstead et al., 1997; Handberg et al., 2001; Silva, 1992; Zhu et al., 1992) have been described. PCR may also be used to quantitate virus load in tissues (Baigent et al., 2005; Bumstead et al., 1997; Burgess & Davison, 1999; Reddy et al., 2000) or differentially detect MDV and HVT in the blood or feather tips (Baigent et al., 2005; Davidson & Borenshtain, 2002).
2. Serological tests

The presence of antibodies to MDV in non-vaccinated chickens from about 4 weeks of age is an indication of infection. Before that age, such antibodies may represent maternal transmission of antibody via the yolk and are not evidence of active infection.

Viruses, antigens and antisera can be obtained from commercial suppliers or from OIE Reference Laboratories for Marek’s Disease (see Table in Part 4 of this Terrestrial Manual), but international standard reagents have not yet been produced.

a) Agar gel immunodiffusion

There is no prescribed test for trade, but the agar gel immunodiffusion (AGID) test is employed most commonly to detect antibody. The test is conducted using glass slides coated with 1% agar in phosphate buffered saline containing 8% sodium chloride. Adjacent wells are filled with antigen or serum and these are incubated in a humid atmosphere at 37°C for 24 hours for diffusion to take place; positive sera show reactions of identity with known positive serum and antigen. The antigen used in this test is either disrupted MDV-infected tissue culture cells or an extract of feather tips, or skin containing feather tracts obtained from MDV-infected chickens. The cell culture antigen is prepared by propagating MDV in chicken kidney cells or chicken embryo fibroblast cells. When cytopathic effect is confluent, the cells are detached from the culture vessel and suspended in culture medium or phosphate buffered saline without tryptose phosphate broth (presence of tryptose phosphate broth may produce non-specific precipitin lines) at a concentration of about $1 \times 10^7$ cells/ml. This suspension is then freeze–thawed three times and used as antigen.

- Test procedure
  i) Make a 1% solution of Difco Bactoagar in 8% sodium chloride by standing the mixture in a boiling water bath.
  ii) Either a microscope slide or a Petri dish can be used and the agar is poured to a thickness of 2–3 mm.
  iii) Cut holes in the agar using a template with a centre well and 6 wells spaced at equal distance around the centre well. The diameter of wells should be approximately 5.3 mm, and the wells should be about 2.4 mm apart. A template with cutters is commercially available.
  iv) The antigen is placed in the centre well and the standard antiserum is placed in alternate exterior wells. Serum samples to be tested are placed in the remaining three wells so that a continuous line of identity is formed between an unknown sample that is positive and the known positive control sera.
  v) Incubate the slide for 24 hours at 37°C in a humid container and read the results over a lamp in a darkened room.

b) Other tests

Other tests for MDV antibody include the direct and indirect fluorescent antibody test. These demonstrate the ability of a test serum to stain MDV plaques in cell cultures (Silva et al., 1997; Spencer & Calnek, 1970). These tests are group specific and more sensitive than the AGID test. A virus neutralisation test for the ability of a serum to neutralise the plaque-forming property of cell-free MDV can also be employed. However, this test is more suitable for research purposes than for routine diagnostic use. Enzyme-linked immunosorbent assays (ELISA) for detecting MDV antibodies are available (Cheng et al., 1984; Sharma, 1998; Zelnik et al., 2004). To prepare antigen for the ELISA, wells of a 96-well microtitre plate are coated with MDV-infected cells.

C. REQUIREMENTS FOR VACCINES

1. Background

a) Rationale and intended use of the product

Control of MD is essentially achieved by the widespread use of live attenuated vaccines (Nair, 2004). Commercial biological products used in the control of MD are the cell-associated or cell-free (lyophilised) live virus or HVT, respectively (see below). Marek’s disease vaccines are injected in ovo at the 17th or 18th day of embryonation (Sharma, 1999) or subcutaneously at hatch.
2. Outline of production and minimum requirements for conventional vaccines

The requirements for producing vaccines are outlined below, and in Chapter 1.1.6 Principles of veterinary vaccine production, but other sources should be consulted for further information on the procedures (Code of Federal Regulations [CFR], 2000; European Pharmacopoeia, 1997a and 1997b; Merieux et al., 1974; Ministry of Agriculture, Fisheries and Food, UK, 1990;); Thornton, 1985). Protocols are given in the British Pharmacopoeia Monograph 589, and the CFR, Title 9, Part 113 (CFR, 2000). The guidelines in this Terrestrial Manual are intended to be general in nature and may be supplemented by national and regional requirements.

a) Characteristics of the seed

i) Biological characteristics

Viruses of the MDV group are classified under three serotypes – 1, 2, and 3 – on the basis of their antigenic relatedness.

Serotype 1: This includes all the pathogenic strains of the virus, ranging from strains that are very virulent plus (e.g. 648A), very virulent (e.g. Md/5, Md/11, Ala-8, RB-1B), virulent (e.g. HPRS-16, JM GA), mildly virulent (e.g. HPRS-B14, Conn A) and finally to weakly virulent (e.g. CU-2, CVI-988). These strains may be attenuated by passage in tissue culture, with loss of pathogenic properties but retention of immunogenicity, to provide strains that have been used as vaccines. Those that have been used commercially include attenuated HPRS-16 and CVI-988 (Rispens) strains. Attenuated variants of the very virulent stains have been used in experimental vaccines to protect against the variant form of acute MD caused by the very virulent strains. Md11/75C/R2/23 is one such strain (Witter, 2001) licensed for use in the United States of America. Serotype 1 vaccines are prepared in a cell-associated ('wet') form that must be stored in liquid nitrogen.

Serotype 2: This includes naturally avirulent strains of MDV (e.g. SB-1, HPRS-24, 301B/1, HN-1), and several of these have been shown to provide protection against virulent strains. The SB-1 and 301B/1 strains have been developed commercially and used, particularly with HVT, in bivalent vaccines for protection against the very virulent strains. Serotype 2 vaccines exist only in the cell-associated form.

Serotype 3: This contains the strains of naturally avirulent HVT (e.g. FC126, PB1), which are widely used as a monovalent vaccine, and also in combination with serotype 1 and 2 strains in bivalent or trivalent vaccines against the very virulent strains of MDV. HVT may be prepared in a cell-free form as a freeze-dried (lyophilised) vaccine or in a cell-associated ('wet') form.

ii) Quality criteria (sterility, purity, freedom from extraneous agents)

The substrates used for commercial vaccine production are primary chicken embryo fibroblasts (CEF) derived from specific pathogen free (SPF) flocks or duck embryo fibroblasts. CEF from SPF flocks are preferred to duck cells because more is known about chicken-embryo-transmitted pathogens and methods for their detection.

Methods for testing SPF flocks for freedom from infection are available (Ministry of Agriculture, Fisheries and Food, UK, 1990; Thornton, 1985). SPF chicken flocks should be free from avian adeno viruses, including egg-drop syndrome 76 virus, avian leukosis virus (subgroups A, B and J), avian nephritis virus, avian reoviruses, avian rotaviruses, chicken anaemia virus, fowl pox virus, infectious bronchitis virus, infectious bursal disease virus, infectious laryngotracheitis virus, influenza type A virus, MDV, Mycoplasma gallisepticum, Mycoplasma synoviae, Newcastle disease virus, reticuloendotheliosis virus, Salmonella spp., and turkey rhinotracheitis virus.

SPF duck flocks should be free from avian adeno viruses, avian reoviruses, Chlamydia, duck virus enteritis, duck virus hepatitis types I and II, influenza type A virus, Newcastle disease virus, Pasteurella (now Riemerella) anatipestifer, REV, and Salmonella infections. Freedom from other infections may also be required as they become recognised.

Seed virus must be free from the agents listed for SPF flocks and from other contaminants that may be acquired in the laboratory. A vaccine strain derived from turkeys must also be free from LPDV and haemorrhagic enteritis virus.

The ability of the master seed virus – and derived vaccines at the limit of the passage range used to produce vaccinal virus (usually not more than five tissue culture passages) – to protect against MD must be determined. Standardised protection tests are published. They involve vaccination of MD-susceptible SPF chickens at 1 day of age and challenge with sufficient virulent MDV 8 days later to cause at least a 70% incidence of MD in unvaccinated chickens. Two types of tests are used. In the protection index test, a single field dose (1000 PFU, plaque-forming units) of vaccine is given and the incidence of MD in vaccinated birds is compared with that in unvaccinated birds. Protective indices should be greater than 80, i.e. vaccinated birds should show at least 80% reduction in the incidence of gross MD, compared with unvaccinated controls.
A PD\textsubscript{50} (50% protective dose) test is also used, involving the inoculation of five four-fold serial dilutions of vaccine virus selected to provide protection above and below the 50% level, followed by challenge 8 days later to determine the PD\textsubscript{50} value. The assays are conducted using a standard reference vaccine for comparison. The PD\textsubscript{50} may be as low as 4 PFU, but higher values can be obtained depending on the vaccine strain, whether cell-free or cell-associated and the presence or absence of maternal antibodies in the test chickens. On the basis of the PD\textsubscript{50} test, it has been suggested that the minimum vaccine field dose should be the greater of two values: 10\textsuperscript{3} PFU or 100 PD\textsubscript{50}.

Extensive field trials of a new vaccine strain in the presence of field challenge should be conducted, using different breeds of birds of varying MDV maternal antibody status, to ensure efficacy and persistence of immunity. Experience suggests that vaccinal immunity, once acquired, is lifelong.

b) Method of manufacture

i) Procedure

Vaccines against MD are prepared from live attenuated strains belonging to the 3 serotypes using CEF as the substrates.

ii) Requirements for substrates and media

Substrate cells are seeded into flat-bottomed vessels for stationary incubation, or into cylindrical vessels for rolled incubation. Media commonly used are Eagle’s minimal essential medium, or 199 medium, buffered with sodium bicarbonate and supplemented with 5% calf serum. Incubation is at 38–39°C for 48 hours.

For cell-associated vaccine, cultures are infected with production HVT or MDV seed-virus stock, in cell-associated form, which is usually two passages beyond the master seed stock. Cultures are incubated for 48 hours then the infected cells are harvested by treating the washed cell sheet with an EDTA/trypsin solution to allow the cells to begin to detach. The flasks are then returned to the incubator (38.5°C) to allow complete detachment. The cells are subjected to low-speed centrifugation, and then resuspended in the freezing mixture consisting of cell growth medium containing 7.5–15% dimethylsulphoxide (DMSO), and held at 4°C or dispensed immediately into the final vaccine containers, usually glass ampoules, which are flame sealed and frozen in liquid nitrogen.

Cell-free lyophilised vaccine may be prepared from HVT, but not from MDV strains. For the production of this form of vaccine, HVT-infected cultures are incubated for 72 hours, infected cells are detached from the vessel as described above, or scraped from the walls of the vessel. The cells are suspended in a small volume of growth medium, centrifuged, and resuspended in a buffered stabiliser solution containing 8% sucrose, but free from protein to prevent frothing. The cell suspension is sonicated to release virus, the cell debris is removed, the suspension is diluted with a complete stabiliser—such as SPGA—filled into the final containers, and lyophilised.

The dilution rate for both cell-associated and cell-free vaccines is based on previous experience, as is the number of doses required per container, because the virus content of the harvested material cannot be assayed prior to filling the final containers. The virus content of the finished product can subsequently be added to the label.

iii) In-process controls

For optimal results in preparing cell-associated vaccine, a slow rate of freezing (1–5°C per minute) and rapid thawing are essential. The infectivity titre of the infected cells, and hence the number of doses per ampoule, are determined after filling the ampoules. Similarly for cell-free vaccine, the virus content of the final suspension, and hence the number of doses per container, is determined after filling.

iv) Final product batch tests

Using immunofluorescence assay (IFA) with monospecific serum, checks should be carried out to show that the product is of the same specificity as the seed virus. This is best done using monoclonal antibodies.

Sterility/purity

Extensive testing is required of the materials used to produce the vaccine, and of the final product. Substrate cells should come from an SPF flock, in particular, free from vertically transmitted agents. Substances of animal origin used in the preparation of vaccines such as serum, trypsin, and bovine serum albumin, must be free from extraneous agents.

Batches of the final vaccine produced should be tested for freedom from contaminating bacteria, fungi, mycoplasma and the viruses listed for SPF flocks; tests for purity of the diluent should also be conducted. Suitable tests for the detection of extraneous agents at all stages of vaccine production are recommended by several official bodies (Ministry of Agriculture, Fisheries and Food, UK, 1990; CFR,
2000; Thornton, 1985) and in Chapter 1.1.7 Tests for sterility and freedom from contamination of biological materials.

Safety
Ten doses of vaccine or a quantity of diluent equivalent to two doses of vaccine should be inoculated into separate groups of ten 1-day-old SPF chickens. No adverse reactions should occur during a 21-day observation period.

With cell-associated vaccine, care is necessary to avoid injury from ampoules that may explode when they are removed from liquid nitrogen. Eye protection must be worn.

Batch potency
The standard dose of each type of vaccine is 1000 PFU per chicken or egg. Virus content assays are conducted on batches of vaccine to ensure that the correct dose per bird will be achieved.

c) Requirements for authorisation
i) Safety requirements
Target animal safety
The master seed virus should be shown to be non-pathogenic for chickens by inoculating ten times the field dose into 1-day-old SPF chickens of a strain susceptible to MD, to ensure that it does not cause gross lesions or significant microscopic lesions of MD by 120 days of age. It should be noted that some vaccine strains of MDV and HVT may produce minor and transient microscopic nerve lesions.

Reversion-to-virulence for attenuated/live vaccines
No increase in virulence should occur during six serial passages of the vaccine strain in 1-day-old SPF MD-susceptible chickens. Ten times the field dose of vaccine is inoculated initially and then passaged by inoculation of heparinised blood at 5–7-day intervals, and tests for viraemia are run to check that virus is transferred at each passage. The birds receiving the final passage are kept for 120 days and should be free from MD lesions. However, some strains such as Rispens, may cause some mild MD lesions. The important observation is that the virulence should not change. This is a difficult test because the genetic resistance of the chickens fundamentally affects the apparent virulence of the virus, so does the type of inoculum. After successful completion of laboratory safety tests, the safety of the strain should be confirmed in extensive field trials.

ii) Efficacy requirements
A test for duration of immunity is carried out on the seed virus only. Such immunity is apparently lifelong. Preservatives are not included in the vaccine or diluent. During use, reconstituted vaccine must be kept cool and cell-associated vaccine should be agitated to keep cells in suspension.

iii) Stability
Tests for stability are carried out on six representative batches of vaccine to show that titre is maintained during the stated shelf life of the vaccine. These tests should be conducted under the conditions of storage of the vaccine. The lyophilised product should have a shelf life of 12 months when stored at 2–8°C. Manufacturers may double the virus content of the vaccine to compensate for some loss of titre during storage. Appropriate diluting fluids are provided for use with cell-associated and freeze-dried vaccines. The stability of reconstituted vaccine over a 2-hour period should be tested.

3. Vaccines based on biotechnology

a) Vaccines available and their advantages
Although genetically engineered recombinant vaccines have been developed (Reddy et al., 1996) and tested in laboratory and field trials (Lee et al., 2010), they are currently not in commercial use.

b) Special requirements for biotechnological vaccines, if any

None.
REFERENCES


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**NB:** There are OIE Reference Laboratories for Marek’s disease (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Marek’s disease.
CHAPTER 2.3.14.
NEWCASTLE DISEASE

SUMMARY

Newcastle disease (ND) is caused by virulent strains of avian paramyxovirus type 1 (APMV-1) of the genus Avulavirus belonging to the family Paramyxoviridae. There are ten serotypes of avian paramyxoviruses designated APMV-1 to APMV-10.

ND virus (NDV) has been shown to be able to infect over 200 species of birds, but the severity of disease produced varies with both host and strain of virus. Even APMV-1 strains of low virulence may induce severe respiratory disease when exacerbated by the presence of other organisms or by adverse environmental conditions. The preferred method of diagnosis is virus isolation and subsequent characterisation.

Identification of the agent: Suspensions in an antibiotic solution prepared from tracheal or oropharyngeal and cloacal swabs (or faeces) obtained from live birds, or of faeces and pooled organ samples taken from dead birds, are inoculated into the allantoic cavity of 9–11-day-old embryonating fowl eggs. The eggs are incubated at 37°C for 4–7 days. The allantoic fluid of any egg containing dead or dying embryos, as they arise, and all eggs at the end of the incubation period are tested for haemagglutinating activity and/or by use of validated specific molecular methods.

Any haemagglutinating agents should be tested for specific inhibition with a monospecific antiserum to APMV-1. APMV-1 may show some antigenic cross-relationship with some of the other avian paramyxovirus serotypes, particularly APMV-3 and APMV-7.

The intracerebral pathogenicity index (ICPI) can be used to determine the virulence of any newly isolated APMV-1. Alternatively, virulence can also be evaluated using molecular techniques, i.e. reverse-transcription polymerase chain reaction and sequencing. ND is subject to official control in most countries and the virus has a high risk of spread from the laboratory; consequently, appropriate laboratory biosafety and biosecurity must be maintained; a risk assessment should be carried out to determine the level needed.

Serological tests: The haemagglutination inhibition (HI) test is used most widely in ND serology, its usefulness in diagnosis depends on the vaccinal immune status of the birds to be tested and on prevailing disease conditions.

Requirements for vaccines: Live viruses of low virulence (lentogenic) or of moderate virulence (mesogenic) are used for the vaccination of poultry depending on the disease situation and national requirements. Inactivated vaccines are also used.

Live vaccines may be administered to poultry by various routes. They are usually produced by harvesting the infective allantoic/amniotic fluids from inoculated embryonated fowl eggs; some are prepared from infective cell cultures. The final product should be derived from the expansion of master and working seeds.

Inactivated vaccines are given intramuscularly or subcutaneously. They are usually produced by the addition of formaldehyde to infective virus preparations, or by treatment with beta-propiolactone. Most inactivated vaccines are prepared for use by emulsification with a mineral or vegetable oil.

Recombinant Newcastle disease vaccines using viral vectors such as turkey herpesvirus or fowl poxvirus in which the HN gene, F gene or both are expressed have recently been developed and licensed.
Chapter 2.3.14. – Newcastle disease

If virulent forms of NDV are used in the production of vaccines or in challenge studies, the facility should meet the OIE requirements for Containment Group 4 pathogens, which is generally equivalent to the United States Department of Agriculture’s Biosafety Level 3-Agriculture or Enhanced (BSL3-Ag or BSL3-E). Additional regulatory oversight may be required in some countries.

A. INTRODUCTION

Newcastle disease (ND) is caused by virulent strains of avian paramyxovirus type 1 (APMV-1) serotype of the genus Avulavirus belonging to the subfamily Paramyxovirinae, family Paramyxoviridae. The paramyxoviruses isolated from avian species have been classified by serological testing and phylogenetic analysis into ten subtypes designated APMV-1 to APMV-10 (Miller et al., 2010a); ND virus (NDV) has been designated APMV-1 (Alexander & Senne, 2008b).

Since its recognition in 1926, ND is regarded as being endemic in many countries. Prophylactic vaccination is practised in all but a few of the countries that produce poultry on a commercial scale.

One of the most characteristic properties of different strains of NDV has been their great variation in pathogenicity for chickens. Strains of NDV have been grouped into five pathotypes on the basis of the clinical signs seen in infected chickens (Alexander & Senne, 2008b). These are:

1. Viscerotropic velogenic: a highly pathogenic form in which haemorrhagic intestinal lesions are frequently seen;
2. Neurotropic velogenic: a form that presents with high mortality, usually following respiratory and nervous signs;
3. Mesogenic: a form that presents with respiratory signs, occasional nervous signs, but low mortality;
4. Lentogenic or respiratory: a form that presents with mild or subclinical respiratory infection;
5. Asymptomatic: a form that usually consists of a subclinical enteric infection.

Pathotype groupings are rarely clear-cut (Alexander & Allan, 1974) and even in infections of specific pathogen free (SPF) birds, considerable overlapping may be seen. In addition, exacerbation of the clinical signs induced by the milder strains may occur when infections by other organisms are superimposed or when adverse environmental conditions are present. As signs of clinical disease in chickens vary widely and diagnosis may be complicated further by the different responses to infection by different hosts, clinical signs alone do not present a reliable basis for diagnosis of ND. However, the characteristic signs and lesions associated with the virulent pathotypes will give rise to strong suspicion of the disease.

NDV is a human pathogen and the most common sign of infection in humans is conjunctivitis that develops within 24 hours of NDV exposure to the eye (Swayne & King, 2003). Reported infections have been non-life threatening and usually not debilitating for more than a day or two (Chang, 1981). The most frequently reported and best-substantiated clinical signs in human infections have been eye infections, usually consisting of unilateral or bilateral reddening, excessive lachrymation, oedema of the eyelids, conjunctivitis and sub-conjunctival haemorrhage. Although the effect on the eye may be quite severe, infections are usually transient and the cornea is not affected. There is no evidence of human-to-human spread. There is one report of the isolation of a pigeon-like APMV-1 from lung tissue, urine and faeces of an immunocompromised patient who died of pneumonia (Goebel et al., 2007).

ND, as defined in Section B.1.f of this chapter, is subject to official control in most countries and the virus has a high risk of spread from the laboratory; consequently, a risk assessment should be carried out to determine the level of biosafety and biosecurity needed for the diagnosis and characterisation of the virus. The facility should meet the requirements for the appropriate Containment Group as determined by the risk assessment and as outlined in Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities. Within the facility, work should be carried out at biosafety level 2 or above. Countries lacking access to such a specialised national or regional laboratory should send specimens to an OIE Reference Laboratory.
Chapter 2.3.14. – Newcastle disease

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Samples for virus isolation

When investigations of ND are the result of severe disease and high mortality in poultry flocks, it is usual to attempt virus isolation from recently dead birds or moribund birds that have been killed humanely. Samples from dead birds should consist of oro-nasal swabs, as well as samples collected from lung, kidneys, intestine (including contents), caecal tonsils, spleen, brain, liver and heart tissues. These may be collected separately or as a pool, although brain and intestinal samples are usually processed separately from other samples.

Samples from live birds should include both tracheal or oropharyngeal and cloacal swabs, the latter should be visibly coated with faecal material. Swabbing may harm small, delicate birds, but the collection of fresh faeces may serve as an adequate alternative.

Where opportunities for obtaining samples are limited, it is important that cloacal swabs (or faeces), tracheal (or oropharyngeal) swabs or tracheal tissue be examined as well as organs or tissues that are grossly affected or associated with the clinical disease. Samples should be taken in the early stages of the disease.

The samples should be placed in isotonic phosphate buffered saline (PBS), pH 7.0–7.4, containing antibiotics. Protein-based media, e.g. brain–heart infusion (BHI) or tris-buffered tryptose broth (TBTB), have also been used and may give added stability to the virus, especially during shipping. The antibiotics can be varied according to local conditions, but could be, for example, penicillin (2000 units/ml); streptomycin (2 mg/ml); gentamycin (50 µg/ml); and mycostatin (1000 units/ml) for tissues and tracheal swabs, but at five-fold higher concentrations for faeces and cloacal swabs. It is important to readjust the concentrated stock solution to pH 7.0–7.4 before adding it to the sample. If control of Chlamydophila is desired, 0.05–0.1 mg/ml oxytetracycline should be included. Faeces and finely minced tissues should be prepared as 10–20% (w/v) suspensions in the antibiotic solution. Suspensions should be processed as soon as possible after incubation for 1–2 hours at room temperature. When immediate processing is impracticable, samples may be stored at 4°C for up to 4 days.

b) Virus isolation (the prescribed test for international trade)

The supernatant fluids of faeces or tissue suspensions and swabs, obtained through clarification by centrifugation at 1000 g for about 10 minutes at a temperature not exceeding 25°C, are inoculated in 0.2 ml volumes into the allantoic cavity of each of at least five embryonated SPF fowl eggs of 9–11 days incubation. If SPF eggs are not available, at least NDV antibody negative eggs are required. After inoculation, these are incubated at 35–37°C for 4–7 days. To accelerate the final isolation, it is possible to carry out two passages at a 3-day interval, obtaining results comparable to two passages at 4–7-day intervals (Alexander & Senne, 2008a). Eggs containing dead or dying embryos as they arise, and all eggs remaining at the end of the incubation period, should first be chilled to 4°C for 4 hours or overnight and the allantoic fluids tested for haemagglutination (HA) activity. Fluids that give a negative reaction should be passed into at least one further batch of eggs. Routine checks for contamination should be conducted by streaking samples in Luria Broth agar plates and reading these at 24 and 48 hours of incubation against a light source. Contaminated samples can be treated by incubation with increased antibiotic concentrations for 2–4 hours (gentamicin, penicillin g, and amphotericin b solutions at final concentrations to a maximum of 1 mg/ml, 10,000 U/ml, and 20 µg/ml, respectively). Samples heavily contaminated by bacteria that cannot be removed by centrifugation or controlled by antibiotics can be filtrated through 0.45 and 0.2 micron sterile filters. Filtration should be used only when other methods fail because aggregation may significantly reduce virus titre.

Suspension of homogenated organs, faeces or swabs prepared as for isolation in eggs may also be used for attempted isolation in cell cultures. APMV-1 strains can replicate in a variety of cell cultures of avian and non-avian origin, among which the most widely used are: chicken embryo liver (CEL) cells, chicken embryo kidney (CEK) cells, chicken embryo fibroblasts (CEF), African green monkey kidney (Vero) cells, avian myogenic (QM5) and chicken-embryo-related (CER) cells (Terregino & Capua, 2009). Primary cell cultures of avian origin are the most susceptible. In order to optimise the chances of viral recovery for isolates of low virulence, trypsin should be added to the culture medium. The concentration of trypsin will vary depending on the type of trypsin and the type of cells used. One example is to add 0.5 µg/ml of porcine trypsin to CEFs. Viral growth is usually accompanied by cytopathic effects typically represented by disruption of the monolayer and formation of syncytia.

The optimal culture system for the virus is to some extent strain-dependent. Some strains of APMV-1 grow poorly in cell culture and replicate to higher titre in embryonated eggs, whereas some strains of Pigeon
PMV-1 (PPMV-1) and of APMV-1, such as the apathogenic Ulster strain, can be isolated in chicken liver or chicken kidney cells but not in embryonated eggs (Kouwenhoven, 1993). If possible, mainly when dealing with samples suspected of being infected with PPMV-1, virus isolation should be attempted using both substrates (embryonated eggs and primary chicken embryo cells). As the viral titre obtained in cell culture is usually very low, additional replication steps in embryonated eggs should be performed prior to characterisation of the isolate by HI or other phenotypic methods.

c) Virus identification

HA activity detected in bacteriologically sterile fluids harvested from inoculated eggs may be due to the presence of any of the ten subtypes of APMV (including NDV) or 16 haemagglutinin subtypes of influenza A viruses, or. Nonsterile fluid could contain bacterial HA. NDV can be confirmed by the use of specific antiserum in a haemagglutination inhibition (HI) test. Usually chicken antiserum that has been prepared against one of the strains of NDV is used.

In the HI test, some level of cross-reactivity may be observed among the various avian paramyxovirus serotypes. Cross-reactivity can be observed between APMV-1 and APMV-3 viruses (particularly with the psittacine variant of APMV-3, commonly isolated from pet or exotic birds) or APMV-7. The risk of mistyping an isolate can be greatly reduced by using a panel of reference sera or monoclonal antibodies (MAbs) specific for APMV-1, APMV-3 and APMV-7.

At present, RT-PCR-based techniques for the detection and typing (pathotyping and genotyping) of APMV-1 RNA in allantoic fluid of inoculated fowl eggs is becoming increasingly common in diagnostic laboratories. However, the genetic variability of APMV-1 isolates should be considered carefully as potential cause for false negative results of genetic-based laboratory tests. See Sections 1e; 1h and 1i of the present chapter.

d) Pathogenicity index

The extreme variation in virulence of different NDV isolates and the widespread use of live vaccines means that the identification of an isolate as APMV-1 from birds showing clinical signs does not confirm a diagnosis of ND, so that an assessment of the virulence of the isolate is also required (see Section B.1.f below ‘Definition of Newcastle disease’). In the past, such tests as the mean death time in eggs, the intravenous pathogenicity test and variations of these tests have been used (Alexander & Senne, 2008b), but by international agreement, a definitive assessment of virus virulence is based on the intracerebral pathogenicity test (ICPI). The current OIE definition (Section B.1.f below) also recognises the advances made in understanding the molecular basis of pathogenicity and allows confirmation of virus virulence, but not lack of virulence, by in-vitro tests that determine the amino acid sequence at the F0 protein cleavage site. Because of the severity of the procedure, ICPI should only be used where there is strong justification based on the epidemiological circumstances, for example in the first isolate from an outbreak. It would not be appropriate to use ICPI for isolates detected in the course of routine surveillance of healthy birds.

The in vivo tests on strains isolated from species other than chickens (pigeons or doves for instance) can cause some problems and may not produce accurate readings until passaged in chickens or embryonated chicken eggs (Alexander & Parsons, 1986.) A more accurate indication of the true pathogenicity of ND viruses for a susceptible species could come from experimental infection of a statistically significant number (≥10) of young and adult birds with a viral standard dose (e.g. 10⁵EID₅₀) administered via natural routes (e.g. oro-nasal route).

• Intracerebral pathogenicity index

i) Fresh infective allantoic fluid with a HA titre >2⁴ (>1/16) is diluted 1/10 in sterile isotonic saline with no additives, such as antibiotics.

ii) 0.05 ml of the diluted virus is injected intracerebrally into each of ten chicks hatched from eggs from an SPF flock. These chicks must be over 24-hours and under 40-hours old at the time of inoculation.

iii) The birds are examined every 24 hours for 8 days.

iv) At each observation, the birds are scored: 0 if normal, 1 if sick, and 2 if dead. (Birds that are alive but unable to eat or drink should be killed humanely and scored as dead at the next observation. Dead individuals must be scored as 2 at each of the remaining daily observations after death.)

v) The intracerebral pathogenicity index (ICPI) is the mean score per bird per observation over the 8-day period.

The most virulent viruses will give indices that approach the maximum score of 2.0, whereas lentogenic and asymptomatic enteric strains will give values close to 0.0.
e) Molecular basis for pathogenicity

During replication, APMV-1 particles are produced with a precursor glycoprotein, F0, which has to be cleaved to F1 and F2 for the virus particles to be infectious. This post-translational cleavage is mediated by host-cell proteases. Trypsin is capable of cleaving F0 for all NDV strains.

It would appear that the F0 molecules of viruses virulent for chickens can be cleaved by a host protease or proteases found in a wide range of cells and tissues, and thus spread throughout the host damaging vital organs, but F0 molecules in viruses of low virulence are restricted in their cleavability to certain host proteases resulting in restriction of these viruses to growth only in certain host-cell types.

Most APMV-1 viruses that are pathogenic for chickens have the sequence \(^{112}\text{R/K-R-Q/K/R-K/R-R}_{116} \) (Choi et al., 2010; Kim et al., 2008a) at the C-terminus of the F2 protein and F (phenylalanine) at residue 117, the N-terminus of the F1 protein, whereas the viruses of low virulence have sequences in the same region of \(^{112}\text{G/E-K/R-Q-G/E-R}_{116} \) and L (leucine) at residue 117. Some of the pigeon variant viruses (PPMV-1) examined have the sequence \(^{112}\text{G-R-Q/K-K-R-F}_{117} \), but give high ICPI values (Meulemans et al., 2002). Thus there appears to be the requirement of at least one pair of basic amino acids at residues 116 and 115 plus a phenylalanine at residue 117 and a basic amino acid (R) at 113 if the virus is to show virulence for chickens. However, some PPMV-1 may have virulent cleavage sites with low ICPI values (Collins et al., 1994). This phenomena has been associated not with the fusion protein (Dortmans et al., 2010), but with the replication complex consisting of the nucleoprotein, phosphoprotein and polymerase (Dortmans et al., 2010).

Several studies have been done using molecular techniques to determine the F0 cleavage site sequence by reverse-transcription polymerase chain reaction (RT-PCR), either on the isolated virus or on tissues and faeces from infected birds, followed by analysis of the product by restriction enzyme analysis, probe hybridisation or nucleotide sequencing with a view to establishing a routine in-vitro test for virulence (Miller et al., 2010b). Determination of the F0 cleavage sequence may give a clear indication of the virulence of the virus, and this has been incorporated into the definition of ND (see Section B.1.f).

In the diagnosis of ND it is important to understand that the demonstration of the presence of virus with multiple basic amino acids at the F0 cleavage site confirms the presence of virulent or potentially virulent virus, but that failure to detect virus or detection of NDV without multiple basic amino acids at the F0 cleavage site using molecular techniques does not confirm the absence of virulent virus. Primer mismatch, or the possibility of a mixed population of virulent and avirulent viruses means that virus isolation and an in-vivo assessment of virulence, such as an ICPI, will still be required.

Analyses of viruses isolated in Ireland in 1990 and during the outbreaks of ND in Australia since 1998 have given strong evidence that virulent viruses may arise from progenitor viruses of low virulence (Alexander & Senne, 2008b). Virulent NDV has also been generated experimentally from low virulence virus by passage in chickens (Shengqing et al., 2002).

f) Definition of Newcastle disease

The vast majority of bird species appear to be susceptible to infection with APMV-1 of both high and low virulence for chickens, although the clinical signs seen in infected birds vary widely and are dependent on factors such as: the virus, host species, age of host, infection with other organisms, environmental stress and immune status. In some circumstances infection with the extremely virulent viruses may result in sudden high mortality with comparatively few clinical signs. Thus, the clinical signs are variable and influenced by other factors so that none can be regarded as pathognomonic.

Even for susceptible hosts, ND viruses produce a considerable range of clinical signs. Generally, variation consists of clusters around the two extremes in the ICPI test, but, for a variety of reasons, some viruses may show intermediate virulence. The broad variation in virulence and clinical signs necessitates the careful definition of what constitutes ND for the purposes of trade, control measures and policies. The definition of ND currently in use in all member states of the European Union is defined in Directive 92/66/EEC of the Commission for European Communities.

The OIE definition for reporting an outbreak of ND is:

*Newcastle disease is defined as an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that meets one of the following criteria for virulence:

a) The virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (Gallus gallus) of 0.7 or greater.

or
b) Multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term ‘multiple basic amino acids’ refers to at least three arginine or lysine residues between residues 113 and 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterisation of the isolated virus by an ICPI test.

In this definition, amino acid residues are numbered from the N-terminus of the amino acid sequence deduced from the nucleotide sequence of the F0 gene, 113–116 corresponds to residues –4 to –1 from the cleavage site.

g) Monoclonal antibodies

Mouse monoclonal antibodies (MAbs) directed against strains of NDV have been used in HI tests to allow rapid identification of NDV without the possible cross-reactions with other APMV serotypes that may occur with polyclonal sera. Many MAbs have been produced that give reactions in HI tests that are specific for particular strains or variant NDV isolates (Alexander et al., 1997).

Panels of MAbs have been used to establish antigenic profiles of NDV isolates based on whether or not they react with the viruses. Typical patterns of reactivity of PPMV-1 strains to MAbs can be used to differentiate these from other APMV-1.

h) Phylogenetic studies

Development of improved techniques for nucleotide sequencing, the availability of sequence data of more APMV-1 viruses in computer databases and the demonstration that even relatively short sequence lengths could give meaningful results in phylogenetic analyses have led to a considerable increase in such studies in recent years. Considerable genetic diversity has been detected, but viruses sharing temporal, geographical, antigenic or epidemiological parameters tend to fall into specific lineages or clades and this has proven valuable in assessing both the global epidemiology and local spread of ND (Aldous et al., 2003; Cattoli et al., 2010; Czegledi et al., 2006; Kim et al., 2007).

Although in the past phylogenetic studies have been impracticable as a routine tool, the greater availability and increased speed of production of results obtained using sophisticated, commercially available kits for RT-PCR and automatic sequencers now means such studies are within the capabilities of many more diagnostic laboratories and can give meaningful results that are contemporaneous rather than retrospective (Miller et al., 2010b). Aldous et al. (2003) proposed that genotyping of NDV isolates should become part of diagnostic virus characterisation for reference laboratories by producing a 375-nucleotide sequence of the F gene, which includes the F0 cleavage site, routinely for all viruses and comparing the sequences obtained with other recent isolates and 18 viruses representative of the recognised lineages and sub-lineages. Such analysis should allow rapid epidemiological assessment of the origins and spread of the viruses responsible for ND outbreaks.

i) Molecular techniques in diagnosis

In addition to the use of RT-PCR and other similar techniques for the determination of the virulence of ND viruses (see Section B.1.e) or for phylogenetic studies (see Section B.1.h), there has been increasing use of molecular techniques to detect NDV in clinical specimens, the advantage being the extremely rapid demonstration of the presence of virus. Care should be taken in the selection of clinical samples as some studies have demonstrated lack of sensitivity in detecting virus in some organs and particularly in faeces (Creelan et al., 2002; Nanthakumar et al., 2000). Tracheal or oropharyngeal swabs are often used as the specimens of choice because they are easy to process and usually contain little extraneous organic material that can interfere with RNA recovery and amplification by PCR. However, tissue and organ samples and even faeces have been used with some success. The system used for RNA extraction will also affect the success of RT-PCR on clinical specimens and even with commercial kits care should be taken in selecting the most appropriate or validated for the samples to be analysed.

Usually RT-PCR systems have been used to amplify a specific portion of the genome that will give added value; for example by amplifying part of the F gene that contains the F0 cleavage site so that the product can be used for assessing virulence (Creelan et al., 2002). Perhaps the most serious problem with the use of RT-PCR in diagnosis is the necessity for post-amplification processing because of the high potential for contamination of the laboratory and cross contamination of samples. Extreme precautions and strict regimens for handling samples are necessary to prevent this (see Chapter 1.1.5 Principles and methods of validation of diagnostic assays for infectious diseases).

One of the strategies used to avoid post-amplification processing is to employ real-time RT-PCR (rRT-PCR) techniques. The advantages of such assays are that rRT-PCR assays based on the fluorogenic hydrolysis
probes or fluorescent dyes eliminate the post-amplification processing step and that results can be obtained in less than 3 hours. At present, the widest application of an rRT-PCR assay for APMV-1 detection was in the United States of America (USA) during the ND outbreaks of 2002–2003, when the assay described by Wise et al. (2004) was employed. The primers and probes in this report were validated on lentogenic, mesogenic and velogenic strains circulating in the USA. At the peak of the outbreak, between 1000 and 1500 samples were tested daily by rRT-PCR. However those protocols do not detect all NDV strains and a more conserved part of the genome should be targeted or a multiple testing approach (i.e. at least two distinct independent laboratory tests for antigen detection) may be needed for detecting the index case.

In fact, one important problem is that APMV-1 isolates have been shown to be genetically distinct. For example, one group of viruses, which were placed in genogroup 6 by Aldous et al. (2003) and subsequently Class I by Czegledi et al. (2006), are so different from all the other APMV-1 isolates, i.e. Class II viruses (Czegledi et al., 2006) that different primers would be necessary for their detection in RT-PCR tests. Furthermore, it has been recently demonstrated that also within class II APMV-1 viruses, the matrix gene is not truly highly conserved and false negatives occurred in case of outbreak investigations or routine surveillance in poultry using the USDA-validated real-time RT-PCR assay targeting this gene (Cattoli et al., 2009, 2010; Khan et al., 2010). In addition the matrix gene-based real-time PCR that is generally used to identify APMV-1 does not discriminate between lentogenic and mesogenic/velogenic strains, therefore it should be used as screening test for the presence of APMV-1 RNA in the samples and not for detection or confirmation of ND outbreaks. This is particularly true in regions or countries that use live vaccines in poultry routinely. A universal fusion gene-specific rRT-PCR test to detect and pathotype determine virulence would be useful as it would allow for quick pathotyping, however because of the variability of the region encoding for the cleavage site, available tests are of limited use and could fail to detect variants. A promising approach that involves the inclusion of class I viruses within one rRT-PCR was done by Kim et al. 2008, combining primers for class I and class II (Kim et al., 2008b). At present, it should be noted that multiplexing RT-PCR or rRT-PCR assays aiming at broadening the range of virus detection frequently result in reduced sensitivity of the test compared with single target assays (Fuller et al., 2010; Liu et al., 2010).

2. Serological tests

NDV may be employed as an antigen in a wide range of serological tests, enabling neutralisation or enzyme-linked immunosorbent assays (ELISA) and HI to be used for assessing antibody levels in birds. At present, the HI test is most widely used for detecting antibodies to APMV-1 in birds while the use of commercial ELISA kits to assess post-vaccination antibody levels is common. In general, virus neutralisation or HI titres and ELISA-derived titres correlate at the flock level rather than at the level of individual birds. Serological assays are also used in diagnostic laboratories to assess antibody response following vaccination, but have limited value in surveillance and diagnosis of ND because of the almost universal use of vaccines in domestic poultry.

a) Haemagglutination and haemagglutination inhibition tests

Chicken sera rarely give nonspecific positive reactions in the HI test and any pretreatment of the sera is unnecessary. Sera from species other than chickens may sometimes cause agglutination of chicken red blood cells (RBCs), so this property should first be determined and then removed by adsorption of the serum with chicken RBCs. This is done by adding 0.025 ml of packed chicken RBCs to each 0.5 ml of antisera, shaking gently and leaving for at least 30 minutes; the RBCs are then pelleted by centrifugation at 800 g for 2–5 minutes and the adsorbed sera are decanted.

Variations in the procedures for HA and HI tests are practised in different laboratories. The following recommended examples apply in the use of V-bottomed microwell plastic plates in which the final volume for both types of test is 0.075 ml. The reagents required for these tests are isotonic PBS (0.01 M), pH 7.0–7.2, and RBC taken from a minimum of three SPF chickens and pooled in an equal volume of Alsever's solution. (If SPF chickens are not available, blood may be taken from unvaccinated birds monitored regularly and shown to be free from antibodies to NDV.) Cells should be washed three times in PBS before use as a 1% (packed cell v/v) suspension. Positive and negative control antigens and antisera should be run with each test, as appropriate.

- **Haemagglutination test**
  i) 0.025 ml of PBS is dispensed into each well of a plastic V-bottomed microtitre plate.
  ii) 0.025 ml of the virus suspension (i.e. infective or inactivated allantoic fluid) is placed in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/3, 1/5, 1/7, etc.
  iii) Twofold dilutions of 0.025 ml volumes of the virus suspension are made across the plate.
  iv) A further 0.025 ml of PBS is dispensed to each well.

OIE Terrestrial Manual 2012 561
**v)** 0.025 ml of 1% (v/v) chicken RBCs is dispensed to each well.

**vi)** The solution is mixed by tapping the plate gently. The RBCs are allowed to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C if ambient temperatures are high, when control RBCs should be settled to a distinct button.

**vii)** HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA (no streaming); this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

- **Haemagglutination inhibition test (an alternative test for international trade)**
  
  **i)** 0.025 ml of PBS is dispensed into each well of a plastic V-bottomed microtitre plate.
  
  **ii)** 0.025 ml of serum is placed into the first well of the plate.
  
  **iii)** Twofold dilutions of 0.025 ml volumes of the serum are made across the plate.
  
  **iv)** 4 HAU virus/antigen in 0.025 ml is added to each well and the plate is left for a minimum of 30 minutes at room temperature, i.e. about 20°C, or 60 minutes at 4°C.
  
  **v)** 0.025 ml of 1% (v/v) chicken RBCs is added to each well and, after gentle mixing, the RBCs are allowed to settle for about 40 minutes at room temperature, i.e. about 20°C, or for about 60 minutes at 4°C if ambient temperatures are high, when control RBCs should be settled to a distinct button.
  
  **vi)** The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (positive serum, virus/antigen and PBS controls) should be considered to show inhibition.
  
  **vii)** The validity of results should be assessed against a negative control serum, which should not give a titre >1/4 (>2² or >log₂ 2 when expressed as the reciprocal), and a positive control serum for which the titre should be within one dilution of the known titre.

The value of serology in diagnosis is clearly related to the expected immune status of the affected birds. HI titres may be regarded as being positive if there is inhibition at a serum dilution of 1/16 (2⁴ or log₂ 4 when expressed as the reciprocal) or more against 4 HAU of antigen. Some laboratories prefer to use 8 HAU in HI tests. While this is permissible, it affects the interpretation of results so that a positive titre is 1/8 (2³ or log₂ 3) or more. Back titration of antigen should be included in all tests to verify the number of HAU used.

In vaccinated flocks that are being monitored serologically, it may be possible to identify anamnestic responses as the result of a challenge infection with field virus (Alexander & Allan, 1974), but great care should be exercised as variations may occur from other causes. For example, it has been demonstrated that APMV-3 virus infections of ND-virus-vaccinated turkeys will result in substantially increased titres to NDV (Alexander et al., 1983).

**b) Enzyme-linked immunosorbent assay**

There are a variety of commercial ELISA kits available and these are based on several different strategies for the detection of NDV antibodies, including indirect, sandwich and blocking or competitive ELISAs using MAbs. At least one kit uses a subunit antigen. Usually such tests have been evaluated and validated by the manufacturer, and it is therefore important that the instructions specified for their use be followed carefully. The HI test and ELISA may measure antibodies to different antigens; depending on the system used ELISAs may detect antibodies to more than one antigen while the HI test is probably restricted to those directed against the HN protein. However, comparative studies have demonstrated that the ELISAs are reproducible and have high sensitivity and specificity; they have been found to correlate well with the HI test (Brown et al., 1990). Conventional ELISAs have the disadvantage that it is necessary to validate the test for each species of bird for which they are used. Competitive ELISAs may not recognise all strains of APMV-1 if they use MAb known for their specificity for single epitopes.

**C. REQUIREMENTS FOR VACCINES**

1. **Background**

   **a) Rationale and intended use of the product**

   A detailed account of all aspects of NDV vaccines, including their production and use, has been published (Allan et al., 1978) and should be referred to for details of the procedures outlined here. Guidelines for the
production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements. If virulent forms of NDV are used in the production of vaccines or in challenge studies, the facility should meet the OIE requirements for Containment Group 4 pathogens as described in chapter 1.1.3.

In this section, conventional live and inactivated vaccines will be considered, as these are still used universally. However, it should be remembered that there has been much recent work on the application of molecular biology techniques to the production of new vaccines, and success has been reported in obtaining protective immunity with recombinant fowlpox virus, vaccinia virus, pigeonpox virus, turkey herpesvirus and avian cells in which the HN gene, the F gene, or both, of NDV are expressed. Several of these recombinant viruses have been licensed for use in certain countries.

NDV strains used in conventional commercial live virus vaccines fall into two groups: lentogenic vaccines, such as Hitchner-B1, LaSota, V4, NDW, I2 and mesogenic vaccines, such as Roakin, Mukteswar and Komarov. Strains from both these groups have been subjected to selection and cloning to fulfil different criteria in their production and application. The mesogenic vaccine viruses all have two pairs of basic amino acids at the F0 cleavage site and ICPI values of around 1.4. This means that infections of birds with these viruses would fall within the intended definition of ND (Section B.1.f), but as these vaccines are used primarily in countries where ND is endemic this may not necessarily preclude their use. In the USA, the 9CFR 121.3b.818 states that NDV strains with ICPI values equal to or greater than 0.7 are virulent and reportable, leaving NDV isolates of low virulence to be used as vaccines. The European Union stated in their Commission Decision 93/152/EEC (European Commission, 1993) that for routine ND vaccination programs the viruses used as live NDV vaccines are to be tested under specific conditions and have an ICPI of less than 0.4 or 0.5, depending on the dose of vaccine given. The OIE Biological Standards Commission similarly recommended in 2000 that in principle vaccines should have an ICPI <0.7. However, in order to account for interassay and interlaboratory variability a safety margin should be allowed so that vaccine master seed virus strains should not have an ICPI exceeding 0.4.

Live virus vaccines may be administered to birds by incorporation in the drinking water, delivered as a coarse spray (aerosol), or by intranasal or conjunctival instillation. A live vaccine formulated from a NDV of low virulence for use in ovo has been licensed for use in the USA. Some mesogenic strains are given by wing-web intradermal inoculation. Vaccines have been constructed to give optimum results through application by specific routes.

Inactivated vaccines are considerably more expensive than live vaccines, and their use entails handling and injecting individual birds. They are prepared from allantoic fluid that has had its infectivity inactivated by the addition of formaldehyde or beta-propiolactone. This is incorporated into an emulsion with mineral oil or vegetable oil, and is administered intramuscularly or subcutaneously. Individual birds thus receive a standard dose. There is no subsequent spread of virus or adverse respiratory reactions. Both virulent and avirulent strains are used as seed virus although, from the aspect of safety control, the use of the latter appears more suitable. As no virus multiplication takes place after administration, a much larger amount of antigen is required for immunisation than for live virus vaccination.

The duration of immunity depends on the vaccination programme chosen. One of the most important considerations affecting vaccination programmes is the level of maternal immunity in young chickens, which may vary considerably from farm to farm, batch to batch, and among individual chickens. For this reason, one of several strategies is employed. Either the birds are not vaccinated until 2–4 weeks of age when most of them will be susceptible, or 1-day-old birds are vaccinated by conjunctival instillation or by the application of a coarse spray. This will establish active infection in some birds that will persist until maternal immunity has waned. Revaccination is then carried out 2–4 weeks later. Vaccination of fully susceptible 1-day-old birds, even with live vaccines of the lowest virulence, may result in respiratory disease, especially if common pathogenic bacteria are present in significant numbers.

Re-vaccination of layers should be done at sufficiently frequent intervals to maintain an adequate immunity. Vaccination programmes often employ slightly more pathogenic live virus vaccines to boost immunity than those used initially. These more pathogenic live vaccines may also be used following initial vaccination with oil emulsion inactivated vaccines. Layers that have high serological titres for NDV are protected against drop in egg production and poor egg quality (shell-less, soft shelled eggs, off-coloured eggs) (Allan et al., 1978; Stone et al., 1975). The level of homology between the vaccine strain and the field virus can influence the degree of protection against reduced egg production (Cho et al., 2008).

When devising a vaccination programme, consideration should be given to the type of vaccine used, the immune and disease status of the birds to be vaccinated, and the level of protection required in relation to any possibility of infection with field virus under local conditions (Allan et al., 1978). Two examples of vaccination programmes that may be used in different disease circumstances are listed here. For the first
example, when the disease is mild and sporadic, it is suggested that the following order of vaccination be adopted: live Hitchner-B1 by conjunctival or spray administration at 1 day of age; live Hitchner-B1 or LaSota at 18–21 days of age in the drinking water; live LaSota in the drinking water at 10 weeks of age, and an inactivated oil emulsion vaccine at point of lay. For the second example, when the disease is severe and more widespread, the same protocol as above is adopted up to 21 days of age, and this is followed by revaccination at 35–42 days of age with live LaSota in the drinking water or as an aerosol; this revaccination is repeated at 10 weeks of age with an inactivated vaccine (or a mesogenic live vaccine) and again repeated at point of lay (Allan et al., 1978). The first protocol is generally applicable to countries were virulent NDV in not endemic and is intended to minimise productivity losses by using a milder vaccine during the initial vaccination. Considering possible constraints of ND vaccination, particularly applying to live vaccines, proper immunisation should be validated by serological testing of vaccinated flocks. Regardless of which test system would be applied, i.e. ELISA or HI, humoral immune response should be demonstrated at the flock level.

When HI is used to evaluate the immune response after vaccination, it should be taken into account that HI titres are greatly influenced by the quality of vaccine, the route and method of administration, environmental and individual factors, but also depend on the species (e.g. generally the HI response of some species, such as turkey and pigeon, is lower than that of chicken). It is also recommended to inactivate nonspecific haemagglutinating agents often present in the serum of some species such as game birds (pheasant, partridge, etc.), quails, ostriches and guinea fowl, by heat treatment in a water bath at 56°C for 30 minutes.

Single vaccinations with live lentogenic virus may produce a response in susceptible birds of about 4–6 log₂, but HI titres as high as 11 log₂ or more may be obtained following a vaccination programme involving oil-emulsion vaccines. The actual titres obtained and their relationship to the type of protection and duration of immunity for a given flock and programme are difficult to predict. Variation in HI titres may occur for nonspecific factors, for instance due to the antigenic correlations, infection with other AMPVs (e.g. APMV-3) may result in significant increased titres to NDV. The HI titre is also influenced by the characteristics of antigen used. For instance, the use of the homologous La Sota antigen in the HI assay after vaccination with this virus resulted in significantly higher titres than when heterologous Ulster virus was used (Maas et al., 1998). Furthermore, reference antigens produced with historic strains may reduce the sensitivity of HI assay when used for the detection of antibodies against ND viruses currently circulating. For this reason it is important to investigate the antigenic relationships between the antigen used in the laboratory and current circulating viruses, and between vaccine strains and reference HA antigens, to avoid misjudgements in estimating serum antibody titres.

2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed

i) Biological characteristics

The first principle to consider when selecting a strain for a live NDV vaccine is whether it is to be used as a primary or a secondary vaccine, the main consideration being its pathogenicity. The methods of application and frequency of use are valid considerations. In general, the more immunogenic live vaccines are more virulent, and are therefore more likely to cause adverse side effects. For example, vaccination with the LaSota strain will cause considerably greater problems in young susceptible birds than the Hitchner-B1 strain, the Ulster based vaccines, or specific LaSota clones, although in general the regular LaSota vaccine induces a stronger immune response. There is detectable variation in the antigenicity of different circulating strains, which may indicate a need to tailor vaccines more carefully to relate antigenically to any prevalent field virus (Miller et al., 2007).

Live vaccines using either of two avirulent Australian NDV strains selected for their heat stability, V4 or I-2, have been used with animal feed acting as carriers to combat the specific problems associated with village chicken rearing in developing countries with variable success. The intention is that this vaccine could be coated on food easily fed to roaming chickens while being slightly more resistant to inactivated by high ambient temperatures. Recently, vaccines with both viral strains have been formulated that produce sufficient HI antibody titres (Olabode et al., 2010) and in some instances prevent mortality after virulent challenge (Wambura, 2011).

The most important consideration in selecting a seed for the preparation of inactivated vaccine is the amount of antigen produced when grown in embryonated eggs; it is rarely cost-effective to concentrate virus. Both virulent and lentogenic strains have been used as inactivated vaccines, but the former offer an unnecessary risk because the manipulation of large quantities of virulent virus is involved, as well as the dangers of inadequate inactivation and possible subsequent contamination. Some lentogenic strains grow to very high titres in eggs.
ii) **Quality criteria (sterility, purity, freedom from extraneous agents)**

The master seed should be checked after preparation for sterility, safety, potency and extraneous agents. The master seed should be free of bacterial (including Salmonella), fungal, and mycoplasma contamination, and should be free of extraneous viruses. In addition to laboratory tests for the detection of avian lymphoid leukosis, cytopathic and hemadsorbing agents, chicken anaemia virus and reticuloendotheliosis virus, the master seed used in live vaccines should be evaluated for pathogens by inoculation into embryonating chicken eggs as well as by inoculation into healthy chickens that have not been vaccinated against ND.

b) **Method of manufacture**

i) **Procedure**

The vaccine production facility should operate under the appropriate bio-security procedures and practices. If ND, as defined in Section B.1.f of this chapter, is used for vaccine production or for vaccine–challenge studies, that part of the facility where this work is done should meet the requirements for Containment Group 4 pathogens as outlined in chapter 1.1.3 of this *Terrestrial Manual*.

A master seed is established, and from this a working seed. If the strain has been cloned through a limiting dilution or plaque selection, the establishment of a master culture may only involve producing a large volume of infective allantoic fluid (minimum 100 ml), which can be stored as lyophilised aliquots (0.5 ml). Seed viruses of unknown pedigree should be passed through SPF eggs and cloned before producing the master seed. Some passage through SPF chickens may also be desirable (Allan *et al.*, 1978).

For vaccine production, a working seed, from which batches of vaccine are produced, is first established by expansion of an aliquot of master seed to a sufficient volume to allow vaccine production for 12–18 months. It is best to store the working seed in liquid form at −60°C or lower as lyophilised virus does not always multiply to high titre on subsequent first passage (Allan *et al.*, 1978).

Most ND vaccines are produced in embryonating fowl eggs, and live virus vaccines should be produced in SPF eggs. The method of production is large-scale aseptic propagation of the virus with all procedures performed under sterile conditions. It is usual to dilute the working seed in sterile PBS, pH 7.2, so that roughly 10^3–10^6 EID₅₀/0.1–0.2 ml is inoculated into the allantoic cavity of 9- or 10-day-old embryonating SPF fowl eggs. These are then incubated at 37°C. Eggs containing embryos that die within 24 hours should be discarded. The incubation time will depend on the virus strain being used and will be predetermined to ensure maximum yield with the minimum number of embryo deaths.

The infected eggs should be chilled at 4°C before being harvested. The tops of the eggs are removed and the allantoic fluids aspirated after depression of the embryo. The inclusion of any yolk material and albumin should be avoided. All fluids should be stored immediately at 4°C and tested for bacterial contamination before large pools are made for lyophilisation or inactivation. Live vaccines are usually lyophilised. The methodology depends on the machinery used and the expertise of the manufacturers, but this is a very important step as inadequate lyophilisation results in both loss of titre and a reduced shelf life.

In the manufacture of inactivated vaccines, the harvested allantoic fluid is treated with either formaldehyde (a typical final concentration is 1/1000) or beta-propiolactone (a typical final concentration is 1/2000–1/4000). The time required must be sufficient to ensure freedom from live virus. Most inactivated vaccines are not concentrated; the inactivated allantoic fluid is usually emulsified with mineral or vegetable oil. The exact formulations are generally commercial secrets.

Generally, oil-based inactivated vaccines are prepared as primary emulsions of water-in-oil. The oil phase usually consists of nine volumes of highly refined mineral oil, such as Marcel 52, Drakeol 6VR or BayolF, plus one volume of emulsifying agent, such as Arlacel A, Montanide 80 or Montanide 888. The aqueous phase is the inactivated virus to which a non-ionic emulsifier such as Tween 80 has been added. The oil phase to aqueous phase ratio is usually 1:1 to 1:4. Manufacturers strive to reach a balance between adjuvant effect, viscosity and stability. If the viscosity is too high viscosity and the vaccine is difficult to inject; too low viscosity and the vaccine is unstable.

ii) **Requirements for substrates and media**

Most live virus vaccines are grown in the allantoic cavity of embryonated fowl eggs but some, notably some mesogenic strains, have been adapted to a variety of tissue culture systems. In the USA, both live and killed ND vaccines are prepared in SPF eggs.

iii) **In-process controls**

For those produced in eggs, the most important process control is testing for bacterial and fungal contamination. This is necessary because of the occasional occurrence of putrefying eggs, which may
Chapter 2.3.14. – Newcastle disease

remain undetected at the time of harvest. In the USA, passage is not required unless the results are inconclusive.

iv) Final product batch tests

Sterility/purity
Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7. In the USA, several purity tests are conducted on each serial of a live vaccine. Most of these may be omitted for killed products if the inactivating agent renders the test results meaningless.

Safety
Some countries also require back passage studies for live NDV vaccine to ensure that the pathogenicity is not increased by cycling through birds (Code of Federal Regulations [CFR], 2009).

Batch potency
Each batch of live vaccine virus should be tested for viability and potency. For inactivated vaccines, the efficacy of the process of inactivation should be tested in embryonated eggs, taking 25 aliquots (0.2 ml) from each batch and passing each three times through SPF embryos (Allan et al., 1978).

Most countries have published specifications for the control of production and testing of NDV vaccines, which include the definition of the obligatory tests on vaccines during and after manufacture. In Europe, the European Pharmacopoeia states that it is not necessary to repeat the potency test on each batch if it has been shown that a representative batch of the final product from the master seed has passed the potency test.

In the USA, each serial batch of inactivated ND vaccine is tested for potency by vaccination-challenge (CFR, 2009), as described in C.4.c above. At least ten vaccinates and ten control birds, 2–6 weeks of age, must be used. At least 90% of the control birds must show typical signs of Newcastle disease or die, and at least 90% of the vaccinates must remain normal during the 14 day post-challenge period. In the USA, each serial batch and each subserial of live ND vaccine must have a virus titer that is at least \(10^{0.7} \text{EID}_{50}\) greater than the titer of the virus used in the immunogenicity study described above (CFR, 2009). The minimum titer shall not be less than \(10^{5.5} \text{EID}_{50}\).

The infectivity of live virus vaccines is tested by titrating the virus in embryonated fowl eggs to calculate the EID

\(50\). This involves making tenfold dilutions of virus; 0.1 ml of each dilution is inoculated into five 9 to 10-day-old embryonated fowl eggs. After 5–7 days of incubation at 37°C, the eggs are chilled and tested for the presence of haemagglutinin activity, which is an indication of the presence of live virus. The EID

\(50\) end-point is calculated using a standard formula such as Spearman–Kärber or Reed Muench (Thayer & Beard, 2008).

c) Requirements for authorisation

i) Safety requirements

Target and non-target animal safety
Live NDV vaccines may represent a hazard to humans. ND viruses, both virulent and of low virulence for chickens have been reported to have infected humans, usually causing acute conjunctivitis following direct introduction to the eye. Infections are usually transient and the cornea is not involved.

Mineral oil emulsion vaccines represent a serious hazard to the vaccinator. Accidental injection of humans should be treated promptly by washing of the site with removal of the material, including incision of tissues, as for a ‘grease-gun’ injury.

Reversion-to-virulence for attenuated/live vaccines

The 9CFR 113.329.768 states that in the USA the use of chickens for the testing of NDV vaccines involves the inoculation of twenty-five SPF birds, five days of age or younger. Ten doses of live vaccine are administered supraconjunctivally to each bird and the birds are then observed for 21 days. No chicken should show serious clinical signs and none should die from causes attributable to the vaccine. An alternative is to use the prechallenge part of the potency test, described below, as a safety test and if unfavourable reactions that are attributable to the product occur, the test is declared inconclusive and the safety test is repeated. If not repeated satisfactorily, the batch is declared unsatisfactory (CFR, 2009). In the USA the safety test is done with a single dose, administered to chickens 2–6 weeks old (CFR, 2009); the prechallenge part of the potency test can serve as the safety test.

In view of the finding that virulent NDV can emerge by mutation from virus of low virulence (Gould et al., 2001), the introduction of wholly new strains of ND in live vaccines should be considered carefully and the vaccines subjected to evaluation before use. Recombinant strains that are used in live vaccines in the USA are subject to additional safety requirements. The genetic stability of the virus
should be demonstrated at the highest passage level to be used in production. The phenotypic effect of any genetic modification(s) should be thoroughly assessed to ensure that the genetic modifications have not resulted in any unexpected effects in vivo. Studies should be performed in chickens to evaluate possible alterations in tissue tropism, as well as to evaluate whether the vaccine virus is shed. Recombinant strains that are shed into the environment must be evaluated for safety in non-target avian species as well as in mammalian species, and the ability to persist in the environment under field conditions should be addressed.

**Environmental consideration**

None.

ii) **Efficacy requirements**

**For animal production**

Various methods for the testing of NDV vaccines for potency have been proposed. The importance of using a suitable challenge strain for assessment has been stressed (Allan et al., 1978). Challenge strains used in Europe and the USA are Herts 33 or GB Texas, respectively. For live vaccines, the method recommended involves the vaccination of 10 or more SPF or other fully susceptible birds, some countries specify 20 birds, at the minimum recommended age by the suggested route using the minimum recommended dose. After 14–28 days, each vaccinated bird and ten control birds are challenged intramuscularly with at least 10⁴ EID (50% egg infectious dose) or 10⁵ LD₅₀ (50% lethal dose) of ND challenge virus. Challenged birds are observed for 14 days; at least 90% of the control birds must develop clinical signs and die within 6 days of Newcastle disease. If at least 90–95% of the vaccinates do not remain free of clinical signs, the master seed is unsatisfactory.

For inactivated vaccines, in Europe 21–28-day-old SPF or susceptible chickens are used. Three groups of 20 birds are injected intramuscularly with volumes of vaccine equivalent to 1/25, 1/50 and 1/100 of a dose. A group of ten chickens is kept as controls. All the birds are challenged by intramuscular injection of 10⁶ LD₅₀ of ND challenge virus, 17–21 days later. Chickens are observed for 21 days. The PD₅₀ (50% protective dose) is calculated by standard statistical methods. The test is only valid if challenged control birds all die within 6 days. The vaccine complies with the test if the PD₅₀ is not less than 50 per dose and if the lower confidence limit is not less than 35 PD₅₀ per dose. Some control authorities accept a test at 1/50 only, for animal welfare reasons. It is not necessary to repeat the potency test on each batch if it has been shown that a representative batch of the final product from the master seed has passed the test.

The recommended efficacy test for inactivated vaccines in the USA is a vaccination–challenge study (CFR, 2009). At least ten SPF chickens, 2–6 weeks old, are vaccinated with the minimum recommended dose. The 9CFR 113.205.727 states that after 14 days post-vaccination, the vaccinates and at least ten unvaccinated controls are challenged with the GB Texas strain of Newcastle disease virus and the vaccinates are observed for 14 days. At least 90% of the control birds must develop clinical signs of Newcastle disease during the observation period. If at least 90% of the vaccinates do not remain free of clinical signs, the master seed is unsatisfactory.

**For control and eradication**

The level of immunity reached with any single dose or regimen of ND vaccination will vary enormously with both vaccine and host species. The level of immunity required in a given host (i.e. to protect against death, disease, meat or egg production losses) is extremely complex and difficult to evaluate. Generally some assessment of the longevity of serum antibodies should be made and vaccine regimens adopted to maintain these above an acceptable level (Allan et al., 1978). Most commercial vaccines have been designed to control clinical signs however they do not prevent viral replication and are not suitable for eradication.

Transmission of the ND virus in an area might be interrupted only if a very high percentage of the resident susceptible population (> 85%) is sufficiently immunised showing an Ab titre ≥ 1:8 (Boven et al., 2008)

iii) **Stability**

When stored under the recommended conditions the final vaccine product should maintain its potency for at least the designated shelf life of the product. Accelerated stability tests such as reduction of infectivity following incubation at 37°C for 7 days (Lensing, 1974) may be used as a guide to the storage capabilities of a batch of live vaccine. Oil emulsion vaccines should also be subjected to accelerated ageing by storing at 37°C, for a minimum of 1 month, without separation of the aqueous and oil phases. The USA requires real-time stability to be demonstrated on at least three sequential serials of NDV vaccine (CFR, 2009). Each serial should be evaluated at multiple intervals until the expiration date has been reached in order to develop a degradation profile for the product.
Live virus vaccines must be used immediately after reconstitution. Inactivated vaccines must not be frozen. In most countries, preservatives must not be included in the freeze-dried live product, but antimicrobial preservatives may be incorporated in the diluent used to reconstitute the vaccine. An alternative used in the USA is to allow the use of certain preservatives, but they must be indicated on the labelling.

3. Vaccines based on biotechnology

a) Vaccines available and their advantages

The advent of recombinant DNA technology has resulted in the development of novel NDV vaccines. One class consists of vector vaccines, which consist of a suitable carrier virus that expresses one or more immunogenic NDV proteins (usually F and/or HN), thereby inducing an immune response against both NDV and the vector virus itself. Examples of such vector vaccines are recombinants based on Vaccinia virus (Meulemans, 1988), Fowlpox virus (Boursnell et al., 1990; Karaca et al., 1998; Olabode et al., 2010), Pigeonpox virus (Letellier et al., 1991), Herpesvirus of turkeys (Heckert et al., 1996; Morgan et al., 1992; Reddy et al., 1996), Marek’s disease virus (Sakaguchi et al., 1998) and avian adeno-associated virus (Perozo et al., 2008).

Other approaches include the development of subunit vaccines based on the large scale expression of NDV proteins (usually F and/or HN) using baculovirus vectors (Fukanoki et al., 2001; Lee et al., 2008; Mori et al., 1994; Nagy et al., 1991) or plants (Berinstein et al., 2005; Yang et al., 2007) and the use of DNA vaccines, i.e. plasmid DNA encoding relevant immunogenic NDV proteins (Loke et al., 2005; Rajawat et al., 2008).

The establishment of a reverse genetics system for NDV (Peeters et al., 1999; Romer-Oberdorfer et al., 1999) has made it possible to genetically modify the NDV genome and to develop NDV strains with new properties. These include the implementation of serological differentiation (DIVA vaccines (Mebatsion et al., 2002; Peeters et al., 2001) and the incorporation and expression of foreign genes, thereby making NDV itself a vaccine vector for application in poultry (Nakaya et al., 2007; Schroer et al., 2009; Steel et al., 2008) and other species, including primates (Dinapoli et al., 2007).

The desired profile for NDV vaccines include: 1) prevention of transmission; 2) differentiation of infected from vaccinated animals (DIVA); 3) induction of protection with a single dose; 4) maternal antibody override; 5) mass vaccination; 6) cross-protection against variant strains, 7) increased safety and minimal side effects. Some of the above mentioned recombinant vaccines reach or surpass the efficiency of conventional vaccines in terms of antibody induction or protection against a virulent challenge strain, and thus they show great promise for future use. Moreover, they offer a number of advantages compared to conventional NDV live vaccines, such as i) improved safety for vaccinated birds due to the absence of residual virulence, ii) implementation of the DIVA principle, and iii) closer immunogenic match with outbreak strains.

Only few of the above mentioned biotechnological vaccines have been licensed in certain countries for application in poultry (VectorVax FP-N, Trovac-NDV, Innovax-ND). A problem for some of the vaccines mentioned here may be that existing immunity against the vector might interfere with generic application of such vaccines in the field. As most vector vaccines are based on viruses that are themselves potential avian pathogens, it is difficult to guarantee complete safety under field circumstances. In addition, the fact that most of these vaccines are genetically modified organisms (GMO) means that they have to go through a rigorous and tedious testing and registration process. Furthermore, the production of biotechnological vaccines is likely more expensive than that of classical NDV vaccines. As currently used classical vaccines are cheap and adequate, at least for the protection of poultry against clinical signs and death, a real incentive for veterinary pharmaceutical companies to develop new vaccines is lacking. It is likely that poultry farmers would be willing to pay a higher price for a vaccine only if it offers significant advantages over conventional vaccines. It is unlikely that this situation will soon change unless national or international authorities modify the requirements for ND vaccines such as a minimum requirement for the reduction of shedding of challenge virus or the implementation of the DIVA principle.

Recurrent outbreaks of ND in the face of vaccination has raised the question whether currently used ND vaccines are still adequate, not only for the protection against clinical disease, but also for the inhibition of virus transmission (Kapczynski & King, 2005). Indeed, it has been shown that the extent of homology between vaccine and challenge strain is important in reducing the shedding of virulent virus (Hu et al., 2009; Miller et al., 2007). Exchanging the F and HN genes of a vaccine strain with the corresponding genes of an outbreak strain resulted in a vaccine that was much better able to reduce virus shedding of the outbreak strain than the unmodified vaccine. These results argue for an adaptation of classical vaccine strains to improve the antigenic match between the vaccine and currently circulating virulent NDV strains.
b) Special requirements for biotechnological vaccines

Once registered and licensed, biotechnological vaccines have to fulfill the same or similar requirements as classical vaccines as detailed above (Section C: requirements for vaccines and diagnostic biologicals).

REFERENCES


Chapter 2.3.14. – Newcastle disease


OIE Terrestrial Manual 2012 571
Chapter 2.3.14. — Newcastle disease


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**NB:** There are OIE Reference Laboratories for Newcastle disease (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Newcastle disease.
CHAPTER 2.3.15.
TURKEY RHINOTRACHEITIS
(avian metapneumovirus infections)

SUMMARY

Avian metapneumovirus (aMPV) causes an acute highly contagious upper respiratory tract infection primarily of turkeys and chickens. The disease produced by aMPV was originally referred to as avian pneumovirus infection and avian rhinotracheitis; it also has been referred to as turkey rhinotracheitis in turkeys and swollen head syndrome (SHS) in chickens. The aMPV is a single-stranded non-segmented negative-sense RNA virus belonging to the family Paramyxoviridae, genus Metapneumovirus. The disease can cause significant economic losses in turkey and chicken flocks, particularly when exacerbated by secondary pathogens. The only avian species known to support the replication of aMPVs, other than turkeys and chickens, are pheasants, Muscovy ducks, Peking ducks and guinea fowl. The disease has global distribution in poultry-producing regions with only Oceania and Canada, reported to be free of aMPV infection. Four antigenically distinct subtypes, A, B, C and D, of aMPV have been identified by neutralisation with monoclonal antibodies, possible limited cross reactivity in enzyme-linked immunosorbent assay (ELISA), and sequence analysis of the attachment glycoprotein, G.

In susceptible turkeys, infection of the respiratory tract can occur at any age although young poults appear to be more severely affected. The severity of disease and mortality rate are largely influenced by secondary bacterial infection, but other husbandry factors can also exacerbate the severity of disease. Variability in the severity of clinical signs and morbidity can also be apparent between birds in a flock and between flocks. Infection with aMPV can result in serious egg production problems in turkey and duck breeding flocks. Other clinical signs include sneezing, depression, snicking, tracheal rales, gasping, nasal and ocular discharge, swollen infraorbital sinuses and conjunctivitis. The onset of clinical signs and spread of infection through a flock can be rapid occurring as quickly as 2–4 hours. In chickens, aMPV infections are less well understood and are typically associated with upper respiratory tract signs with decreased egg production in breeders and layers. Disease may also be characterised by the development of swellings of the periorbital and infraorbital sinuses and face, known as SHS. Cerebral disorientation, torticollis, and opisthotonus may frequently follow. In broilers, aMPV is not considered as either a sole or a primary pathogen, but instead is involved with other agents in SHS or other multi-factorial respiratory disease complexes.

Identification of the agent: Virus isolation in cell cultures, embryonating chicken eggs, and tracheal organ cultures, as well as molecular methods for identification of the nucleic acid, have all been used successfully to detect aMPV. The degree of success depends on the strain of virus, type and timeliness of sample collection, as well as storage and handling of specimens. Electron microscopy, virus neutralisation and molecular techniques are widely used to identify the virus. Virus detection and identification can be difficult unless samples are taken early in the course of the disease. Both attenuated and virulent strains of aMPV replicate to the highest titre in the upper respiratory tract tissues of young turkeys; the virus can only be isolated for approximately 10 days.

Monoclonal antibodies to the spike glycoprotein, G, have been used in virus neutralisation tests to differentiate subtypes A and B, while neutralisation tests using polyclonal antiserum have shown that subtypes A and B belong to a single serotype. Subtype C is neutralised poorly by subtype A or B monospecific antiserum, and not by monoclonal antibodies that differentiate subtype A and B. These data suggest that subtype C represents a second serotype of aMPV. Monospecific antiserum and monoclonal antibodies can be used for agent identification by virus neutralisation.
Infection with aMPV can occur from a very young age in turkeys and is characterised by snicking, rales, sneezing, nasal discharge, foaming conjunctivitis, swelling of the infraorbital sinuses and submandibular oedema (Pringle, 1998). Secondary agents that have been shown to exacerbate and prolong clinical disease are secondary bacterial infection occurs, airsacculitis, pericarditis, pneumonia, and perihepatitis may prolong the disease and there may be an increase in morbidity and mortality (Cook et al., 1998). Secondary adventitious agents can dramatically exacerbate the clinical signs. In an uncomplicated infection, recovery is rapid and the birds appear normal in approximately 14 days. When husbandry is poor or infection is weak when compared with the response in turkeys.

Serological tests: Due to difficulties in isolating and identifying aMPV, confirmation of infection is often achieved by serological methods, particularly in unvaccinated flocks. The most commonly employed method is the ELISA. Other methods that have been used are virus neutralisation (VN), immunofluorescence and immunodiffusion tests. The VN test can be performed in primary tracheal organ cultures, chicken embryo fibroblast (CEF) and chicken embryo liver (CEL); several cell lines such as Vero, MA104 or QT35 have also been used successfully. However, the VN and ELISA show similar sensitivity and the ELISA is the most commonly used assay. Numerous commercial ELISA kits, as well as in-house assays, have been developed. Differences in sensitivity and specificity between tests have been reported to occur between commercial kits. A homologous strain of aMPV should be used as antigen because of variations in antigenicity. In many countries where the disease is endemic, vaccination is also practised, complicating interpretation of the results. Ideally, serum samples from birds in the acute phase of disease and also from convalescent birds should be obtained for testing. In chickens the serological response to aMPV infection is weak when compared with the response in turkeys.

Requirements for vaccines and diagnostic biologicals: Two types of vaccine are commercially available for the control of TRT: live attenuated vaccines, and inactivated oil-emulsion adjuvanted vaccines.

A. INTRODUCTION

Avian metapneumovirus (aMPV), previously referred to as avian pneumovirus (APV) and avian rhinotracheitis (ART) virus, causes an acute, highly contagious upper respiratory tract infection of turkeys and chickens. In turkeys, the virus causes a disease known as turkey rhinotracheitis (TRT). The aetiological agent is an enveloped virus with an unsegmented single-stranded negative-sense RNA virus of approximately 14 kilo bases contained in a nucleocapsid with a helical symmetry (Gough, 2003). The virus exhibits some characteristics of a pneumovirus, but differs from mammalian pneumoviruses at the molecular level and has recently been classified as the type strain of a new genus, Metapneumovirus, in the family Paramyxoviridae (Padersen et al., 2000). Metapneumoviruses have been detected in humans and are associated with respiratory tract infection in children (Naylor & Jones, 1993; Toquin et al., 2003; Van Den Hoogen et al., 2001). Avian metapneumovirus has no non-structural NS1 and NS2 proteins and the gene order (3′-NS1-NS2-N-P-M-F-M2-L-5′) is different from that of mammalian pneumoviruses (3′-NS1-NS2-N-P-M-F-M2-L-5′) (Tanaka et al., 1995). The avian metapneumovirus has been classified into four subtypes: A, B, C and D based on nucleotide sequence analysis. Using monoclonal antibodies limited cross reactivity between subtypes has been observed in enzyme-linked immunosorbent assay (ELISA) and neutralisation test (Collins et al., 1993; Cook et al., 1993a). Other subtypes may exist, but have not yet been detected and identified.

Infection with aMPV can occur from a very young age in turkeys and is characterised by snicking, rales, sneezing, nasal discharge, foaming conjunctivitis, swelling of the infraorbital sinuses and submandibular oedema (Pringle, 1998). Secondary adventitious agents can dramatically exacerbate the clinical signs. In an uncomplicated infection, recovery is rapid and the birds appear normal in approximately 14 days. When husbandry is poor or secondary bacterial infection occurs, airsacculitis, pericarditis, pneumonia, and perihepatitis may prolong the disease and there may be an increase in morbidity and mortality (Cook et al., 1991; Mekkes & De Wit, 1999). Secondary agents that have been shown to exacerbate and prolong clinical disease are Bordetella avium,
**Chapter 2.3.15. — Turkey rhinotracheitis (avian metapneumovirus infections)**

**Pasteurella-like organisms, Mycoplasma gallisepticum, Chlamydophila and Ornithobacterium rhinotracheale** (Alkhalaf et al., 2002; Cook et al., 1991; Jirjis et al., 2004; Senne et al., 1997; Van Loock et al., 2006). Morbidity can be as high as 100%, with mortality ranging from 0.5% in adult turkeys to 80% in young poults (Buys et al., 1989; Gough, 2003; Van De Zande et al., 1999). Clinical signs of infection in chickens are less characteristic than those in turkeys. Severe respiratory distress may occur in broiler chickens particularly when exacerbated by secondary pathogens such as infectious bronchitis, mycoplasmas, and *Escherichia coli* (O’Brien, 1985; Pattison et al., 1989). Unlike subtype A and B, the United States of America (USA) strain – Colorado, or subtype C – has not been shown to naturally induce disease in chickens, although experimentally infected chickens were shown to be susceptible to a subtype C turkey isolate of aMPV (Shin et al., 2000). Different strains of aMPV have been shown to have a specific tropism for chickens or turkeys (Cook et al., 1993b). Other species of birds have been reported to have been infected with aMPV, however clinical signs have rarely been reported (Gough et al., 1988). Viruses characterised as subtype C aMPV and shown to have 75–83% nucleotide identity to the US Colorado subtype C aMPV have been associated with respiratory signs and decreased egg production in ducks in France (Toquin et al., 1999; 2006). Retrospective molecular analysis of viruses isolated in the 1980s from turkeys in France indicates the presence of a fourth subtype of aMPV designated subtype D (Bàyôn-Auboyer et al., 2000). The results of experimental studies suggest that direct contact is necessary for bird-to-bird spread of the virus (Alkhalaf et al., 2002; Cook et al., 1991). In commercial conditions aerogenous infection following airborne transmission is also likely as the disease is restricted to the respiratory tract. Following experimental infection of 2-week-old turkeys with aMPV alone, the virus was detected in the respiratory tract for only a few days (Bàyôn-Auboyer et al., 1999). However, in birds inoculated with aMPV and *B. avium*, virus was detected for up to 7 days post-inoculation (dpi) (Collins & Gough, 1988; Cook et al., 1993b; Naylor & Jones, 1993). There is no evidence that aMPV can result in a latent infection and no carrier state is known to exist. Although neonatal turkeys are occasionally infected (Shin et al., 2002a), there have been no reports of vertical transmission of aMPV.

In growing turkeys, virus replication is limited to the upper respiratory tract with a short viraemia. Replication of both attenuated and virulent strains of aMPV persists for approximately 10 dpi (Cook et al., 1991; Van De Zande et al., 1999). Limited replication occurs in the trachea, and lung, but virus has not been shown to replicate in other tissues following natural infection (Cook, 2000a; 2000b). Sequential histopathological and immunocytochemical studies have shown viral replication in the turbinates causing a serious rhinitis with increased glandular activity, epithelial exfoliation, focal loss of cilia, hyperaemia and mild mononuclear infiltration in the submucosa and eosinophilic intracytoplasmic inclusions in the ciliated cells of the turbinates at 2 dpi. A catarrhal rhinitis with mucopurulent exudate, damage to the epithelial layer and a copious mononuclear inflammatory infiltration in the submucosa was seen 3–4 dpi. Transient lesions were seen in the trachea, with little or no lesions present in the conjunctiva and other tissues (Giraud et al., 1988; Majo et al., 1995). Respiratory infection is less severe in laying turkeys; however, there may be a drop in egg production of up to 70% (Stuart, 1989) and the quality of eggs during the recovery period, up to 3 weeks, may be poor. In experimentally infected laying turkeys, viral replication has been demonstrated in both the respiratory and genital tracts up to 9 dpi (Jones et al., 1988).

In chickens, there is strong evidence to suggest aMPV is one of the aetiological agents of swollen head syndrome (SHS). The syndrome is characterised by respiratory disease, apathy, swelling of infrabiborial sinuses and unilateral or bilateral periorbital and facial swelling, extending over the head. These signs are frequently followed by cerebral disorientation, torticollis and opisthotonos. Although mortality does not usually exceed 1–2%, morbidity may reach 10%, and egg production is frequently affected (Gough et al., 1994; Morley & Thomson, 1984; O’Brien, 1985; Pattison et al., 1989; Picault et al., 1987; Tanaka et al., 1995).

Serological evidence suggests aMPV is widespread throughout the world and of considerable economic importance, particularly in turkeys. Oceania and Canada are the only regions that have not reported aMPV (Cook, 2000a; 2000b; Gough, 2003). There is serological and molecular evidence that aMPV occurs in a variety of other avian species, including pheasants, guinea fowl, ostriches, passerines and various waterfowl (Bennett et al., 2004; Gough, 2003; Lee et al., 2007; Shin et al., 2002b), but there is no evidence of disease.

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

To maximise the chances of successfully isolating the virus, a multiple approach to diagnosis is recommended. This is particularly relevant when dealing with different subtypes or genotypes that may require varied *in-vitro* virus isolation methods. This was illustrated in the USA with the failure of the first attempts to isolate subtype C aMPV. The USA subtype C has not been associated with ciliostasis, in tracheal organ cultures (Cook et al., 1999; Senne et al., 1997), and the agent was only cultured following multiple embryo and cell culture passages. This was in contrast to the experience in Europe and elsewhere in which tracheal organ cultures and/or Vero cells were shown to be the most reliable method for the primary isolation of subtypes A, B, C and D of aMPV (Cook & Cavanagh, 2002; Giraud et al., 1988; Toquin et al., 1999; 2000).
• **Collection and selection of diagnostic specimens**

It is very important to take samples for attempted virus isolation in the early stages of infection as the virus may be present only in the sinuses and turbinates for a short period. Ideally, the upper respiratory tract of live birds in the acute phase of the disease should be sampled using sterile swabs (Gough, 2003; Stuart, 1989). The most successful samples have been nasal exudates, choanal clef swabs and scrapings of sinus and turbinate tissue. The virus has also been isolated from trachea and lungs, and occasionally viscera of affected turkey pouls (Buys et al., 1989; Cook et al., 1999). Isolation of virus is rarely successful from birds showing severe chronic signs as the extreme clinical signs are usually due to secondary adventitious agents. This certainly applies to SHS of chickens in which the characteristic signs appear to be due to secondary Escherichia coli infection. Furthermore, for reasons that are unclear, virus isolation from chickens may be more difficult than from turkeys.

It is essential that samples should be sent immediately on ice to the diagnostic laboratory. When delays of more than 3 days are expected, the samples should be frozen prior to dispatch. Swabs for attempted virus isolation should be sent on ice fully immersed in viral transport medium, but those for polymerase chain reaction (PCR) analysis can be sent dry, but on ice or frozen.

For virus isolation, a 20% (v/v) suspension of the nasal exudate or homogenised tissue is made in phosphate-buffered saline (PBS) or brain–heart infusion (BHI) broth containing antibiotics, at pH 7.0–7.4. This is then clarified by centrifugation at 1000 g for 10 minutes and the supernatant is passed through a 450 nm membrane filter.

• **Culture and Identification of avian metapneumovirus (aMPV)**

The best method for primary virus isolation from infected birds is in tracheal organ cultures or embryonating turkey or chicken eggs with subsequent cultivation in cell cultures (Buys et al., 1989; Cook et al., 1999); serial passage on Vero cells has also been found to be a sensitive method for the isolation of aMPV (Giraud et al., 1988). The original isolation of aMPV in South Africa in the late 1970s and the more recent Colorado aMPV were carried out in embryonating eggs, however subtype A and B aMPV isolations have routinely been made in tracheal organ cultures (Cook & Cavanagh, 2002). Subtype C aMPV, and perhaps other non-identified APV, do not cause ciliostasis in organ cultures; for this reason: embryonating chicken eggs and subsequent passage on to cell culture are the preferred method for virus isolation (Gough, 2003; Senne et al., 1997). All four aMPV subgroups can be isolated using Vero cells (Toquin et al., 1999; 2000).

Tracheal organ cultures are prepared from turkey embryos or very young turkeys obtained from flocks free of specific antibodies to aMPV. Tracheas from chicken embryo or 1- to 2-day-old chicks may also be used. Transverse sections of trachea are rinsed in PBS (pH 7.2), placed one section per tube in Eagles medium with antibiotics, and held at 37°C. After incubation, the media is removed from the cultures and 0.1 ml of bacteria-free inoculum is added. After incubation for 1 hour at 37°C, growth medium is added and the cultures are incubated at 37°C on a roller apparatus, rotating at 30 revolutions per hour. Cultures are examined daily after agitation on a laboratory mixer to remove debris from the lumen. Ciliostasis may occur within 7 days of inoculation on primary passage, but usually is produced rapidly and consistently only after several blind passages (Gough, 2003).

Six-to-8-day-old embryonating chicken or turkey eggs from flocks known to be free of aMPV antibodies are inoculated by the yolk-sac route with 0.2–0.3 ml of bacteria-free material from infected birds and incubated at 37°C. If there is no evidence of infection (embryo stunting or mortality) after the first passage, yolk sac material should be processed for a second blind embryo passage. Within 7–10 days, there is usually evidence of stunting of the embryos with few deaths. Serial passage is often required before the agent causes consistent embryo mortality. Isolation in embryonating eggs is a slow, expensive, labour intensive process and requires multiple subsequent cell culture passages for identification (Gough, 2003).

Various cell cultures have been used for the primary isolation of aMPV, including chicken embryo cells, Vero cells and more recently the QT-35 cells, with varying degrees of success. Primary isolation of the USA subtype C has been made after multiple (5–6 serial passages) in Vero cell cultures (Bennett et al., 2004). However, once the virus has been adapted to growth in embryonating eggs or tracheal organ cultures, in which it grows only to low titres, the virus will readily replicate to moderate titres following multiple passages in a variety of primary chicken or turkey embryo cells, Vero cells, and QT-35 cells (Buys et al., 1989; Cook, 2000a; 2000b; Goyzm et al., 2000). The primary isolation of all four subgroups of aMPV has proven successful following serial passage on Vero cells (Toquin et al., 2000). The virus produces a characteristic cytopathic effect (CPE) with syncytial formation within 7 days. Identification of virus-infected cell cultures can be by immunofluorescence staining of infected cells or molecular methods.

Paramyxovirus-like morphology of the virus can be observed by negative-contrast electron microscopy. Pleomorphic fringed particles, roughly spherical and 80–200 nm in diameter are commonly seen. Occasionally much larger filamentous forms are present, which may be up to 1000 nm in length. The surface
projections are 13–14 nm in length and the helical nucleocapsid that can sometimes be seen emerging from disrupted particles, is 14 nm in diameter with an estimated pitch of 7 nm per turn (Collins & Gough, 1988; Giraud et al., 1988).

- **Molecular Identification**

Reverse-transcription PCR (RT-PCR) is a significantly more sensitive and rapid method for the detection of aMPV than standard virus isolation methods because of the fastidious nature of aMPV (Cook & Cavanagh, 2002; Gough, 2003). RT-PCR procedures targeted to the F, M, N and G genes are used for the detection of aMPV; however, because of molecular heterogeneity between aMPV strains, most RT-PCR procedures are subtype specific or do not detect all subtypes (Båyon-Auboyer et al., 1999; Cook & Cavanagh, 2002; Padersen et al., 2000; 2001). Subtype specific assays are successfully used for the detection and diagnosis of endemic strains (Båyon-Auboyer et al., 2000; Cook & Cavanagh, 2002; Mase et al., 2003; Naylor et al., 1997; Padersen et al., 2001). However, limitations of subtype-specific assays need to be recognised when conducting diagnostic testing for respiratory disease. Primers directed to conserved regions of the N gene have been shown to have broader specificity, detecting representative isolates from A, B, C, and D subtypes (Båyon-Auboyer et al., 1999). RT-PCR assays directed to the G gene have also been successfully used for genotype or subtype identification (Båyon-Auboyer et al., 2000; Juhasz & Easton, 1994; Lwamba et al., 2005; Mase et al., 1996). A variety of RT-PCR techniques have been developed and evaluated and these have been extensively reviewed elsewhere (Cook & Cavanagh, 2002; Njenga et al., 2003).

Nasal exudates, choanal cleft swabs, and turbinate specimens collected 2–7 days post-exposure are the preferred specimen (Cook et al., 1993b; Gough, 2003; Padersen et al., 2001; Stuart, 1989). It is imperative to collect specimens when clinical signs are first exhibited as recent studies have shown that the maximum amount of virus is present in the trachea and nasal turbinates at 3 days post-inoculation and viral RNA persists for 9 days in the trachea and up to 14 days in the nasal turbinates (Velayudhan et al., 2005). It has been shown that aMPV can be detected from specimens collected 7–10 days post-exposure, however the viral concentration is considerably less thus reducing success of detection (Alkhalaf et al., 2002; Padersen et al., 2001). Swabs from a single flock can be pooled in groups of not more than five to allow the processing of samples from a larger number of birds and therefore increasing the potential recovery rate.

Template RNA for RT-PCR can be extracted from homogenised tissue, dry swabs or wet swab pools with silica column or magnetic bead commercial RNA extraction reagents according to the manufacturer’s protocol. Tracheal swab supernatant and sinus fluid (140 µl/600 µl lysis buffer) specimens can also be processed with the RNeasy® (Qiagen, Valencia, CA) procedure.

**Table 1.** Example of primers that can be used for the detection of the N gene of subgroups A, B, C and D of aMPV (Båyon-Auboyer et al., 1999; Toquin et al., 1999)

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer ID</th>
<th>Sequence 5’–3’</th>
<th>Position</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>N Gene</td>
<td>Nc</td>
<td>5’–TTC-TTT-GAA-TTG-TTT-GAG-A–3’</td>
<td>632–653</td>
<td>RT primer</td>
</tr>
<tr>
<td></td>
<td>Nx</td>
<td>5’–CAT-GGC-CCA-ACA-TTA-TGT-T–3’</td>
<td>830–812</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>Nd</td>
<td>5’–AGC-AGG-ATG-GAG-AGC-CTC-TTT-G–3’</td>
<td>716–737</td>
<td>115</td>
</tr>
</tbody>
</table>

i) Synthesis of the cDNA can be carried out in 20 µl volume with the Nc RT primer (or any convenient primer, such as an oligoD or the reverse primer of the primer pair that will be used in the PCR) and SuperScript II® Rnase H-RT (Invitrogen, Carlsbad, CA) enzyme. Heat 1 µl RT primer (2 pmol), 1 µl dNTP mix (10 mM each), with extracted RNA and sterile distilled water (QS to 20 µl) to 65°C for 5 minutes.

ii) Chill quickly and pulse centrifuge.

iii) Add 4 µl 5× First-Strand buffer, 2 µl 0.1 M DTT, and 1 µl RNaseOUT® (Invitrogen, Carlsbad, CA).

iv) Heat contents to 42°C for 2 minutes and add 1 µl (200 units) of SuperScript II®, mix gently.

v) RT is conducted at 42°C for 50 minutes followed by 70°C for 15 minutes for inactivation of RT enzyme.

vi) PCR amplification can be conducted with Amplitaq Gold® polymerase (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions. Amplification conditions are as follows: 94°C for 15 minutes and 30 cycles of 94°C for 20 seconds, 51.0°C for 45 seconds (for the Nd/Nx primer pair, if another pair is used, the annealing temperature should be adapted), 72°C for 45 seconds with a final extension of 72°C for 10 minutes.
Several RT-PCR assays directed to the F, G and M genes have been successfully used for subtype identification and detection or diagnosis of endemic aMPV (Goyzm et al., 2000; Jirjis et al., 2004; Majo et al., 1995). Nucleotide sequence and phylogenetic analysis of the G gene has been used to genotype subtype A, B, C and D aMPV and is the recommended procedure for subtype identification of an unidentified virus. Recommended RT-PCR procedures for sequence analysis of the G gene have been described (Bayon-Auboyer et al., 2000; Juhasz & Easton, 1994; Lwamba et al., 2005; Toquin et al., 2006). Real-time RT-PCR has been demonstrated recently to allow the specific detection, identification and quantification of aMPV subgroups A, B, C and D (Guionie et al., 2007.)

Procedures for the identification of subtype A and B RNA in diagnostic specimens have also been described (Naylor et al., 1997), as have procedures for the detection of subtype A and C viruses (Padersen et al., 2001). Isolation of aMPV from chickens is difficult and has succeeded only in a limited number of cases; for this reason, molecular tests have been used primarily for the detection of aMPV in chickens (Cook & Cavanagh, 2002; Mase et al., 1996). It is important to remember that RT-PCR detects viral RNA, not live virus, so the significance of a positive PCR result in terms of detecting an active infection has to be established.

2. Serological tests

Serology is the most common method of diagnosis of aMPV infections, particularly in unvaccinated flocks, because of difficulties in isolating and identifying aMPV. The most commonly employed method is the ELISA; however, virus neutralisation, microimmunofluorescence and immunodiffusion tests have been used. A number of commercial and in-house ELISA kits are available that are suitable for testing both turkey and chicken serum; however, differences in sensitivity and specificity between commercial kits have been reported (Eterradossi et al., 1995; McFarlane-Toms & Jackson, 1998; Meekes & De Wit, 1999). Competitive or blocking ELISA kits incorporating an aMPV-specific monoclonal antibody have been developed. These kits claim to have a broad spectrum of sensitivity and specificity for all subtypes of aMPV and can be used for testing sera from a variety of avian species. In-house ELISA antigens, as described below, have been prepared in a variety of substrates including various cell cultures and tracheal organ cultures (Chiang et al., 2000; Cook & Cavanagh, 2002). Generally, aMPV antibodies are less well detected when a heterologous strain of aMPV is used as antigen, even though the strains appear closely related by virus neutralisation test (Cook & Cavanagh, 2002; Mase et al., 1996). The situation is further complicated by discrepancies in the ability of different ELISAs to detect vaccinal antibody when different aMPV strains are used as coating antigens (Eterradossi et al., 1995). In-house assays using a homologous antigen have been used extensively for the surveillance of endemic aMPV strains. Ideally, both acute and convalescent serum samples should be obtained for testing. In chickens, the serological response to aMPV infection is weak when compared to the response in turkeys (Cook et al., 1991).

- **Enzyme-linked immunosorbent assay**
  
  The following protocol (Chiang et al., 2000), or alternative methods with well documented results (Giraud et al., 1987; Grant et al., 1987), may be used.

  Virus is propagated in chicken embryo fibroblast (CEF) or Vero cell cultures until 70–100% of the monolayer is simultaneously infected (3–4 days). The cell culture fluid is decanted and the monolayer washed with PBS (pH 7.2). The monolayer is lysed with 0.5 ml (per 75 cm² flask) of a 0.5% non-ionic detergent solution (IGEPAL CA-630 or Nonidet P-40) on a rocking platform for 1 hour at 4°C. Following physical disruption of lysed cells, the whole virus antigen lysate is clarified at 3000 g for 15 minutes. Uninfected cell cultures are treated in the same manner for a negative control antigen. Serial dilutions of antigen are tested against serial dilutions of anti-species IgG horseradish peroxidise conjugate in a checker-board fashion to determine the optimal antigen/conjugate dilution. A working dilution of the aMPV antigen and normal antigen (100 µl) are coated onto flat-bottom microtitre plates with a carbonate/bicarbonate coating buffer (Chiang et al., 2000). Each serum is tested against aMPV and normal antigen for determination of the S/P ratio. Coated plates are incubated at 4°C overnight and washed a total of five times with a Tween 20 wash solution (Chiang et al., 2000) prior to use or three times prior to long-term storage at −70°C. Residual wash solution remains on the plate when the plates are frozen. Following storage and equilibration to room temperature, the plates are washed twice and blotted dry prior to use.

  i) Dilute test sera 1/40 in dilution/blocking buffer (Chiang et al., 2000).
  
  ii) Apply 50 µl test sera and working dilutions of positive and negative sera to aMPV antigen and normal antigen-coated wells.
  
  iii) Incubate at room temperature for 1 hour.
  
  iv) Wash plates five times with Tween 20 wash solution.
  
  v) Apply 50 µl of the working dilution of anti-species IgG horseradish peroxidase conjugate to each well and incubated for 1 hour at room temperature.
C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Two types of vaccine are commercially available for the control of TRT: live attenuated vaccines, or inactivated oil-emulsion adjuvanted vaccines (Cook, 2000a). The vaccines are intended for use only in turkeys. The possibility exists of developing live recombinant vaccines based on a fowlpox vector expressing the F protein of aMPV (Stuart, 1989), DNA vaccines encoding various aMPV proteins (Tanaka et al., 1995) and, more recently, genetically attenuated aMPV produced by reverse genetics (Toquin et al., 1999).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

- Live vaccines: methods of use

Live TRT vaccines are produced from virus strains that have been attenuated by serial passage in embryonating eggs, tracheal organ cultures or cell culture (various cell lines or chicken embryo fibroblasts), or by alternate passages using a combination of these methods. Commercially available live attenuated TRT vaccines have been derived from subgroup A or subgroup B aMPV isolates in Europe, and from a subgroup C aMPV isolate in the USA. The aMPV subgroup to which the vaccine belongs should be mentioned in the vaccine label, as this information is relevant to the development of efficient post-vaccination serological monitoring. Live TRT vaccines are intended for use in young birds to induce an active immune response that will help to prevent the respiratory disease caused by aMPV. Additionally, live TRT vaccines are also used in parent turkeys to produce a primary response prior to vaccination near to point-of-lay using inactivated vaccine (see below).

Live TRT vaccines are usually applied several times by coarse spray, in the drinking water, or by oculonasal administration. There is a published report on the use of a single in-ovo injection (Shin et al., 2002b), but, more often, the first TRT live vaccination is administered to turkeys at day-old or up to 7 days of age. The second TRT live vaccine is either applied around 6 weeks of age (when only two vaccinations are performed), or around 3 weeks of age (when there is a third application) or after 6 weeks of age. The rationale for these repeated vaccinations is linked first to the difficulties of inducing a prolonged antibody response lasting for the whole life of the meat turkeys, and second to the need to avoid TRT vaccination in young turkeys when they have recently been vaccinated against haemorrhagic enteritis (vaccines against haemorrhagic enteritis virus [HEV], are usually administered at around 28 days of age to avoid interference with maternally derived antibodies [MDA] to HEV). Although it has been published that MDA to TRTV do not prevent infection of day-old turkeys by virulent aMPV strains (Toquin et al., 2003), it has been observed that some interference between MDA and some live TRT vaccines may occur and result in lower vaccine take in young turkeys with higher MDA levels. Clinical cross-protection between live vaccine and challenge virus belonging to subgroups A or B (and vice versa) has been reported (Cook et al., 1993b; Velayudhan et al., 2005; Worthington et al., 2003). Protective immunity was also observed when birds immunised against aMPV subgroups A or B were subsequently challenged with a subgroup C virulent virus, but not in the converse experiment (Worthington et al., 2003).

Avian metapneumoviruses are very easily neutralised in the environment by physical and chemical agents and ensuring good live vaccination against these viruses may be demanding. If the vaccine is given in the drinking water, clean water with a neutral pH must be used and it must be free from smell or taste of chlorine or metals. Skimmed milk powder may be added at a rate of 2 g per litre. Care must be taken to ensure that all birds receive...
their dose of vaccine. To this end, all water should be removed (cut off) for 2–3 hours before the medicated water is made available and care must be taken that no residual water remains in the water adduction pipes or in the drinkers. If the vaccine is given by spray, high quality water with a neutral pH and with no disinfectant residues should be used. A specific nebuliser should be used that will be used for no other purpose but vaccination. This apparatus should ideally allow for constant pressure throughout the vaccination process (and thus for a constant size of the vaccine droplets). The turkeys to be vaccinated should be grouped together prior to vaccination and several passes with the nebuliser should be performed to ensure that all birds are indeed exposed to the spray. The ventilation and heating of the poultry house should be turned as low as practical, so that the nebulised vaccine is neither eliminated by ventilation, nor inactivated by overheating (heating moreover favours evaporation, which decreases the size of the nebulised vaccine droplets and cause an increase proportion of the vaccine to reach the lower respiratory tract, a phenomenon that has been suspected to contribute to adverse reactions to live vaccination). It is important that the birds are allowed to calm down immediately after spraying as a non-negligible amount of the vaccine may be absorbed when the birds preen their feathers after being exposed to the vaccine spray.

AMPV vaccines have been reported not to interfere with Newcastle disease vaccines in chickens (Van De Zande et al., 1999; Yu et al., 1992); however the compatibility of TRT vaccines is not documented in turkeys. As with other vaccines, only healthy birds should be vaccinated. Vials of lyophilised vaccine should be kept at temperatures between 2°C and 8°C up to the time of use.

**Inactivated vaccines: method of use**

Inactivated aMPV vaccines are mostly used to produce high, long-lasting and uniform levels of antibodies in breeder turkeys that have previously been primed by live vaccine or by natural exposure to field virus during rearing. As the rationale to use inactivated vaccines in breeders is to improve their protection not only against the respiratory signs of TRT, but also against the reproductive signs (egg-drops) associated with aMPV infection, it is not uncommon that the inactivated aMPV vaccines also associate this virus with several other viruses also involved in respiratory and/or reproductive disorders. The usual programme is to administer the inactivated vaccine at least 4–6 weeks after the last live vaccination, up to 28 weeks of age in turkeys, avoiding the 4 last weeks before lay. The inactivated vaccine is manufactured as a water-in-oil emulsion, and has to be injected into each bird. The preferred routes are intramuscular in the leg muscle, avoiding proximity to joints, tendons or major blood vessels or the subcutaneous route. A multidose syringe may be used, subject to the apparatus being in full working order and in accordance with manufacturers’ instructions and recommended hygiene practices. All equipment should be cleaned and sterilised between flocks, and vaccination teams should exercise strict hygiene when going from one flock to another. The vaccine should not be frozen; it should be stored at between 4°C and 8°C instead (but should be allowed to reach room temperature before injection). Inactivated vaccines should not be exposed to bright light or high temperatures.

Only healthy birds, known to be sensitised by previous exposure to aMPV, should be vaccinated. Used in this way the inactivated vaccine should produce a good antibody response that will protect the breeders against respiratory and reproductive signs during the period of lay (Van De Zande et al., 2000). The precise level and duration of immunity conferred by inactivated vaccines will depend mainly on the concentration of antigen present per dose. The manufacturing objective should be to obtain a high antigen concentration and hence a highly potent vaccine.

**1. Seed management**

**a) Characteristics of the seed**

- **Live vaccine**

  The seed virus must be shown to be free from extraneous viruses, bacteria, mycoplasma and fungi, particularly from avian pathogens.

  The seed virus must be shown to be stable, with no tendency to revert to virulence. This can be confirmed by carrying out at least five consecutive turkey-to-turkey passages at 2–6-day intervals. Use turkeys not older than 3 weeks and free of MDAs against aMPV. Passage may be achieved by natural spreading or by inoculating a suspension prepared from the mucosa of the turbinates and upper trachea of the previously inoculated birds, or from tracheal swabs. Care must be taken to avoid contamination by viruses from previous passages. It must be shown that the virus was transmitted. The stability should be evaluated by demonstrating that there is no indication of an increased severity in the clinical signs when comparing the maximally passaged virus with the unpassaged vaccine. A scoring system may be used to quantify the severity of the signs.

- **Killed vaccine**

  For killed vaccines, the most important characteristics are high yield and good antigenicity. The seed virus must be shown to be free from extraneous viruses, bacteria, mycoplasma and fungi, particularly from avian pathogens.
Chapter 2.3.15. – Turkey rhinotracheitis (avian metapneumovirus infections)

b) Method of culture

Avian metapneumovirus seed virus may be propagated in various cell culture systems. The bulk is distributed in aliquots and freeze-dried in sealed containers.

c) Validation as a vaccine

Data on efficacy should be obtained before bulk manufacture of vaccine begins. The vaccine should be administered to birds in the way in which it will be used in the field. Live vaccine can be given to young birds and the response measured serologically and by resistance to experimental challenge. In the case of killed vaccines, a test must be carried out in older birds that go on to lay, using the recommended vaccination schedule, so that the prolonged seroconversion can be demonstrated. A scoring system may be used to quantify the severity of the signs.

- **Live vaccine**

  *Efficacy test:* efficacy should be tested for each of the recommended routes of vaccination. Use turkeys that are not older than the minimum age recommended for vaccination and are free of antibodies against aMPV. Administer one field dose of vaccine of the minimum recommended titre by one of the recommended routes to each of 20 turkeys, keeping 10 turkeys as non-vaccinated controls. After 21 days, challenge all turkeys by ocularonasal administration of a suitable dose of a virulent strain of aMPV (suitable challenge viruses can be provided by the OIE Reference Laboratory for TRT; see Table given in Part 4 of this *Terrestrial Manual*). Observe the turkeys daily for 10 days and register their clinical signs individually. The vaccine fails the test unless at least 90% of the vaccinated turkeys survive without showing either clinical signs or lesions evocative of aMPV infection. A scoring system may be used to quantify the severity of the signs. If less than 80% of the non-vaccinated turkeys exhibit clinical signs following challenge, or more than 10% of the control or inoculated birds die from causes not attributable to the test, the test is invalid. Providing results are satisfactory, this test need be carried out on only one batch of all those batches prepared from the same seed lot.

- **Killed vaccine**

  *Efficacy test:* as drops in egg production are not easily reproduced experimentally, vaccine-induced protection against egg drop following virulent aMPV challenge may be difficult to document and thus protocols aimed at demonstrating the reduction in excretion levels are also acceptable. Alternatively, the induction of a long-lasting immune response following injection of the inactivated vaccine may also be used. For the latter experiment, at least 20 unprimed turkeys are given one dose of vaccine at the recommended age (near to point-of-lay) by one of the recommended routes, and the antibody response is measured between 4 and 6 weeks after vaccination by ELISA or serum neutralisation. If a primary vaccination with a live vaccine is recommended, an additional group of turkeys is given only the primary vaccination so that the actual effect of the inactivated vaccine can indeed be assessed individually.

2. Method of manufacture

The vaccine must be manufactured in suitable clean and secure accommodation, well separated from diagnostic facilities or commercial poultry.

Production of the vaccine should be on a seed-lot system using a suitable strain of virus of known origin and passage history. Specific pathogen free eggs must be used for all materials employed in propagation and testing of the vaccine. Live vaccines are made by growth in eggs or cell cultures. Inactivated vaccines may be made using virulent virus grown in cell culture or embryonating eggs. A high virus concentration is required. These vaccines are made as water-in-oil emulsions. A typical formulation is to use 80% mineral oil to 20% virus suspension, with suitable emulsifying and preservative agents.

3. In-process control

*Antigen content:* having grown the virus to a high concentration, its titre should be assayed by use of tracheal organ culture or cell cultures, as appropriate, to the strain of virus being used. The antigen content required to produce satisfactory batches of vaccine should be based on determinations made on test vaccine that has been shown to be effective in laboratory and field trials.

*Inactivation of killed vaccines:* this is often done with either β-propiolactone or formalin. The inactivating agent and the inactivation procedure must be chosen under the conditions of vaccine manufacture to inactivate the vaccine virus and any potential contaminants, e.g. bacteria that may arise from the starting materials.

Prior to inactivation, care should be taken to ensure a homogeneous suspension free from particles that may not be penetrated by the inactivating agent. A test for inactivation of the vaccine should be carried out on each batch.
of both the bulk harvest after inactivation and the final product. The test selected should be appropriate to the vaccine virus being used and should consist of at least two passages in susceptible cell cultures, embryos or turkeys, with ten replicates per passage. No evidence of the presence of any live virus or microorganism should be observed.

Sterility of killed vaccines: oil used in the vaccine must be sterilised by heating at 160°C for 1 hour, or by filtration, and the procedure must be shown to be effective. Tests appropriate to oil-emulsion vaccines are carried out on each batch of final vaccine as described, for example, in the European Pharmacopoeia.

4. Batch control
   a) Sterility
      Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.
   b) Safety
      • Live vaccine safety test
         Ten field doses of vaccine are administered by the oculonasal route to each of 10 turkeys of the minimum age recommended for vaccination and free from antibodies to aMPV. Observe the turkeys at least daily for 21 days. The vaccine fails the test if any turkey dies or shows signs of disease attributable to the vaccine. If more than two turkeys show abnormal clinical signs or die due to causes not related to the vaccine, the test must be repeated. This test is performed on each batch of final vaccine.
      • Killed vaccine safety test
         Ten turkeys, free of maternal antibodies to aMPV and 14–28 days of age, are inoculated by the recommended routes with twice the field dose. The birds are observed for 3 weeks. No abnormal local or systemic reaction should develop. The test is performed on each batch of final vaccine.
   c) Potency
      • Live vaccine potency test
         A potency test (virus titration) in embryonating eggs, tracheal organ cultures or suitable cell cultures, as appropriate to the vaccine virus, must be carried out on each serial (batch) of vaccine produced. The vaccine titre at the time of issue must be high enough to guarantee that the minimum virus titre per dose will be maintained at least until the expiry date. In addition, the method described in Section C.1.c Live vaccine (efficacy test) must be used on one batch representative of all the batches prepared from the same seed lot.
      • Killed vaccine potency test
         The potency test for inactivated vaccines is developed from the results of the efficacy test on a representative batch of vaccine the master seed virus, by measuring antibody production.
   d) Stability
      Evidence should be provided on at least one representative batch of vaccine to show that the vaccine passes the batch potency test at 3 months beyond the requested shelf life.
   e) Preservatives
      A preservative is normally required for vaccine in multidose containers. The concentration of the preservative in the final vaccine and its persistence throughout shelf life should be checked. A suitable preservative already established for such purposes should be used. There are maximum level requirements for antibiotics, preservatives and residual inactivating agents.
   f) Precautions (hazards)
      Avian metapneumovirus is not recognised as a zoonotic agent, however precautions should be implemented in the manufacturing steps or during vaccination to minimise the exposure of staff to vaccine aerosols.

Oil-emulsion vaccines cause serious injury to the vaccinator if accidentally injected into the hand or other tissues. In the event of such an accident the person should go at once to a hospital, taking the vaccine package and manufacturer’s datasheet with them. Each vaccine bottle and package should be clearly marked with a warning of the serious consequences of accidental self-injury.
5. Tests on the final product

a) Safety
   See Section C.4.b.

b) Potency
   See Section C.4.c.

REFERENCES


Chapter 2.3.15. — Turkey rhinotracheitis (avian metapneumovirus infections)


Chapter 2.3.15. – Turkey rhinotracheitis (avian metapneumovirus infections)


* * *

NB: There is an OIE Reference Laboratory for Turkey rhinotracheitis (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Turkey rhinotracheitis.
SECTION 2.4.

BOVINAE

CHAPTER 2.4.1.

BOVINE ANAPLASMOSIS

SUMMARY

Definition of the disease: Bovine anaplasmosis results from infection with Anaplasma marginale. A second species, A. centrale, has long been recognised. Anaplasma marginale is responsible for almost all outbreaks of clinical disease. A third species, A. phagocytophilum, has been reported rarely to infect cattle, and does not cause clinical disease. The organism is classified in the genus Anaplasma belonging to the family Anaplasmataceae of the order Rickettsiales.

Description of the disease: Anaemia and jaundice are characteristic signs of anaplasmosis, but the clinical disease can only be confirmed by identifying the organism. Once infected, cattle may remain carriers for life, and identification of these animals depends on the detection of specific antibodies using serological tests, or of rickettsial DNA using amplification techniques.

Identification of the agent: Microscopic examination of blood or organ smears stained with Giemsa stain is the most common method of identifying Anaplasma in clinically affected animals. In these smears, A. marginale appear as dense, rounded, intraerythrocytic bodies approximately 0.3–1.0 μm in diameter with most situated on or near the margin of the erythrocyte. Anaplasma centrale is similar in appearance, but most of the organisms are situated away from the margin of the erythrocyte. It can be difficult to differentiate A. marginale from A. centrale in a stained smear, particularly with low levels of rickettsaemia. Commercial stains that give very rapid staining of Anaplasma are available in some countries.

It is important that smears be well prepared and free from foreign matter. Smears from live cattle should preferably be prepared from blood drawn from the jugular vein or another large vessel. For post-mortem diagnosis, smears should be prepared from internal organs (including liver, kidney, heart and lungs) and from blood retained in peripheral vessels. The latter are particularly desirable if post-mortem decomposition is advanced.

Serological tests: A competitive enzyme-linked immunosorbent assay (C-ELISA) has been demonstrated to have good sensitivity in detecting carrier animals. Card agglutination is the next most frequently used assay. The complement fixation (CF) test is no longer considered a reliable test for disease certification of individual animals due to variable sensitivity. Cross reactivity between Anaplasma spp. can complicate interpretation of serological tests. In general, the C-ELISA has the best specificity, with cross-reactivity described between A. marginale, A. centrale, A. phagocytophilum and Ehrlichia spp.

Nucleic-acid-based tests have been used experimentally, and are capable of detecting the presence of low-level infection in carrier cattle and tick vectors. A nested reaction is necessary to identify low-level carriers using conventional polymerase chain reaction (PCR) and nonspecific amplification can occur. Recently, real-time PCR assays with analytical sensitivity equivalent to nested conventional PCR have been described.

Requirements for vaccines and diagnostic biologicals: Live vaccines are used in several countries to protect cattle against A. marginale infection. A vaccine consisting of live A. centrale is most widely used and gives partial protection against challenge with virulent A. marginale.
Anaplasma centrale vaccine is provided in chilled or frozen forms. Quality control is very important as other blood-borne agents that may be present in donor cattle can contaminate vaccines and be disseminated broadly. For this reason, frozen vaccine is recommended as it allows thorough post-production quality control, which limits the risk of contamination with other pathogens.

Anaplasma centrale vaccine is not entirely safe. A practical recommendation is to restrict its use, as far as possible, to calves, as nonspecific immunity will minimise the risk of some vaccine reactions that may require treatment with tetracycline or imidocarb. Partial immunity develops in 6–8 weeks and lasts for several years after a single vaccination.

A. INTRODUCTION

Outbreaks of bovine anaplasmosis are due to infection with Anaplasma marginale. Anaplasma centrale is capable of producing a moderate degree of anaemia, but clinical outbreaks in the field are extremely rare. A third species, A. phagocytophilum, with a primary reservoir in rodents, has been reported rarely to infect cattle, and does not cause clinical disease (Dreher et al., 2005; Hofmann-Lehmann et al., 2004).

The most marked clinical signs of anaplasmosis are anaemia and jaundice, the latter occurring late in the disease. Haemoglobinaemia and haemoglobinuria are not present, and this may assist in the differential diagnosis of anaplasmosis from babesiosis, which is often endemic in the same regions. The disease can only be confirmed, however, by identification of the organism.

Anaplasma marginale occurs in most tropical and subtropical countries, and in some more temperate regions. Anaplasma centrale was first described from South Africa. The organism has since been imported by other countries – including Australia and some countries in South America, South-East Asia and the Middle East – for use as a vaccine against A. marginale.

Anaplasma species were originally regarded as protozoan parasites, but later research showed they had no significant attributes to justify this description. Since the last major accepted revision of the taxonomy in 2001 (Dumler et al., 2001), the Family Anaplasmataceae (Order Rickettsiales) is now composed of four genera, Anaplasma, Ehrlichia, Neorickettsia, and Wolbachia. The genus Aegyptianella is retained within the Family Anaplasmataceae as genus incertae sedis. The revised genus Anaplasma now contains Anaplasma marginale as the type species, A. phagocytophilum (formerly Ehrlichia phagocytophila, E. equi and the unclassified agent of human granulocytic ehrlichiosis), A. platys, and A. bovis. Haemobartonella and Eperythrozoon are now considered most closely related to the mycoplasmas.

Anaplasma species are transmitted either mechanically or biologically by arthropod vectors. Reviews based on careful study of reported transmission experiments list up to 19 different ticks as capable of transmitting A. marginale experimentally (Kocan et al., 2004). These are: Argas persicus, Ornithodoros lahorensis, Boophilus annulatus, B. calcaratus, B. decoloratus, B. microplus, Dermacentor albipictus, D. andersoni, D. hunteri, D. occidentalis, D. variabilis, Hyalomma excavatum, H. rufipes, Hodes ricinus, I. scapularis, Rhipicephalus bursa, R. evertsi, R. sanguineus and R. simus. However, the classification of several ticks in these reports has been questioned. Intrastadial or transstadial transmission is the usual mode, even in the one-host Boophilus species. Male ticks may be particularly important as vectors; they can become persistently infected and serve as a reservoir for infection. Experimental demonstration of vector competence does not necessarily imply a role in transmission in the field. However, Boophilus species are clearly important vectors of anaplasmosis in countries such as Australia and countries in Africa, and some species of Dermacentor are efficient vectors in the United States of America (USA).

Various other biting arthropods have been implicated as mechanical vectors, particularly in the USA. Experimental transmission has been demonstrated with a number of species of Tabanus (horseflies), and with mosquitoes of the genus Psorophora (Kocan et al., 2004). The importance of biting insects in the natural transmission of anaplasmosis appears to vary greatly from region to region. Anaplasma marginale also can be readily transmitted during vaccination against other diseases unless a fresh or sterilised needle is used for injecting each animal. Similar transmission by means of unsterilised surgical instruments has been described.

The main biological vectors of A. centrale appear to be multistock ticks peculiar to Africa, including R. simus. The common cattle tick (B. microplus) has not been shown to be a vector. This is of relevance where A. centrale is used as a vaccine in B. microplus-infested regions.

Anaplasma marginale infection has not been reported in humans. Thus, there is no risk of laboratory transmission to workers, and laboratories working with A. marginale may operate at the lowest biosafety level, equivalent to BSL1.
B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Samples from live cattle should include thin blood smears and blood collected into an anticoagulant. Air-dried thin blood smears will keep satisfactorily at room temperature for at least 1 week. The blood sample in anticoagulant should be held and transferred at 4°C, unless it can reach the laboratory within a few hours. This sample is useful for preparing fresh smears if those submitted are not satisfactory. In addition, a low packed cell volume and/or erythrocyte count can help to substantiate the involvement of *A. marginale* when only small numbers of the parasites are detected in smears, such as may occur in the recovery stage of the disease.

In contrast to *Babesia bovis*, *A. marginale* does not accumulate in capillaries, so blood drawn from the jugular or other large vessel is satisfactory. Because of the rather indistinctive morphology of *Anaplasma*, it is essential that smears be well prepared and free from foreign matter, as specks of debris can confuse diagnosis. Thick blood films as used for the diagnosis of babesiosis are not appropriate for the diagnosis of anaplasmosis, as *Anaplasma* are difficult to identify once they become dissociated from erythrocytes.

Samples from dead animals should include air-dried thin smears from the liver, kidney, heart and lungs and from a peripheral blood vessel. The latter is particularly recommended should there be a significant delay before post-mortem examination because, under these circumstances, bacterial contamination of organ smears often makes identification of *Anaplasma* equivocal. Brain smears, which are useful for the diagnosis of some forms of babesiosis, are of no direct value for diagnosing anaplasmosis, but should be included for differential diagnosis where appropriate.

Blood from organs, rather than organ tissues per se, is required for smear preparation, as the aim is to be able to examine microscopically intact erythrocytes for the presence of *Anaplasma*. Organ-derived blood smears will store satisfactorily at room temperature for several days.

Both blood and organ smears can be stained in 10% Giemsa stain for approximately 30 minutes after fixation in absolute methanol for 1 minute. After staining, the smears are rinsed three or four times with tap water to remove adhering stain, and are then air-dried. Conditions for Giemsa staining vary from laboratory to laboratory. Commercial stains that give very rapid staining of *Anaplasma* are available in some countries. Smears are examined under oil immersion at a magnification of ×700–1000.

*Anaplasma marginale* appear as dense, rounded and deeply stained intraerythrocytic bodies, approximately 0.3–1.0 µm in diameter. Most of these bodies are located on or near the margin of the erythrocyte. This feature distinguishes *A. marginale* from *A. centrale*, as in the latter most of the organisms have a more central location in the erythrocyte. However, particularly at low levels of rickettsaemia, differentiation of these two species in smears can be difficult. Appendages associated with the *Anaplasma* body have been described in some isolates of *A. marginale* (Kreier & Ristic, 1963).

The percentage of infected erythrocytes varies with the stage and severity of the disease. Maximum rickettsaemias in excess of 50% may occur with *A. marginale*. Multiple infections of individual erythrocytes are common during periods of high rickettsaemias.

The infection becomes visible microscopically 2–6 weeks following transmission. During the course of clinical disease, the rickettsaemia approximately doubles each day for up to about 10 days, and then decreases at a similar rate. Quite severe anaemia may persist for some weeks after the parasites have become virtually undetectable in blood smears. Following recovery from initial infection, most cattle remain latently infected for life.

An expensive procedure, but one that may occasionally be justified to confirm infection, particularly in persistently infected carrier cattle, is the inoculation of blood from the suspect animal into a splenectomised calf. A quantity (up to 500 ml) of the donor’s blood in anticoagulant is inoculated intravenously into the splenectomised calf, which is then tested by blood smear examination at least every 2–3 days. If the donor is infected, *Anaplasma* will be observed in smears from the splenectomised calf generally within 4 weeks, but this period may extend up to 8 weeks.

Nucleic-acid-based tests to detect *A. marginale* infection in carrier cattle have been developed although not yet fully validated. The analytical sensitivity of polymerase chain reaction (PCR)-based methods has been estimated at 0.0001% infected erythrocytes, but at this level only a proportion of carrier cattle would be detected. A nested PCR has been used to identify *A. marginale* carrier cattle with a capability of identifying as few as 30 infected

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1 Commercial stains include Camco-Quik and Diff-Quik, Baxter Scientific Products, McGaw Park, Illinois, USA, and Hema 3 and Hema-Quik, Curtin-Matheson, Houston, Texas, USA.
erythrocytes per ml of blood, well below the lowest levels in carriers. However, nested PCR poses significant quality control and specificity problems for routine use (Torioni De Echaide et al., 1998). Real-time PCR has also been described for identification of A. marginale (Carelli et al., 2007; Decaro et al., 2008; Reinbold et al., 2010), and should be considered in place of the nested PCR. Two advantages of this technique, which uses a single closed tube for amplification and analysis, are reduced opportunity for amplicon contamination and a semi-quantitative assay result. Equipment needed for real-time PCR is expensive, requires preventive maintenance, and may be beyond the capabilities of some laboratories. Real-time PCR assays may target one of several genes (Carelli et al., 2007; Decaro et al., 2008), or 16S rRNA (Reinbold et al., 2010), and are reported to achieve a level of analytical sensitivity equivalent to nested conventional PCR (Carelli et al., 2007; Decaro et al., 2008; Reinbold et al., 2010).

In general, unless animals have been treated or are at a very early stage of infection (<14 days), serology using the competitive enzyme-linked immunosorbent assay (C-ELISA) or card agglutination test (CAT) (see below) may be the preferred method of identifying infected animals in most laboratories.

2. Serological tests

Anaplasma infections usually persist for the life of the animal. However, except for occasional small recrudescences, Anaplasma cannot readily be detected in blood smears after acute rickettsaemia. Thus, a number of serological tests have been developed with the aim of detecting persistently infected animals.

A feature of the serological diagnosis of anaplasmosis is the highly variable results with regard to both sensitivity and specificity reported for many of the tests from different laboratories. This is due at least in part to inadequate evaluation of the tests using significant numbers of known positive and negative animals. Importantly, the capacity of several assays to detect known infections of long-standing duration has been inadequately addressed. An exception is C-ELISA (see below), which has been validated using true positive and negative animals defined by nested PCR (Torioni De Echaide et al., 1998), and the card agglutination assay, for which relative sensitivity and specificity in comparison with the C-ELISA has been evaluated (Molloy et al., 1999). Therefore, while most of the tests described in this section are useful for obtaining broad-based epidemiological data, caution is advised on their use for disease certification. Both the C-ELISA and card agglutination test are described in detail below.

It should be noted that there is a high degree of cross-reactivity between A. marginale and A. centrale, as well as cross-reactivity with both A. phagocytophilum and Ehrlichia spp. in serological tests (Al-Adhami et al., 2011; Dreher et al., 2005). While the infecting species can sometimes be identified using antigens from homologous and heterologous species, equivocal results are obtained on many occasions.

a) Competitive enzyme-linked immunosorbent assay

A C-ELISA using a recombinant antigen termed rMSP5 and MSP5-specific monoclonal antibody (MAb) has proven very sensitive and specific for detection of Anaplasma-infected animals (Hofmann-Lehmann et al., 2004; Reinbold et al., 2010; Strik et al., 2007). All A. marginale strains tested, along with A. ovis and A. centrale, express the MSP5 antigen and induce antibodies against the immunodominant epitope recognised by the MSP5-specific MAb. A recent report suggests that antibodies from cattle experimentally infected with A. phagocytophilum will test positive in the C-ELISA (Dreher et al., 2005). However, in another study no cross-reactivity could be demonstrated, and the MAb used in the assay did not react with A. phagocytophilum MSP5 in direct binding assays (Strik et al., 2007). Recently, cross reactivity has been demonstrated between A. marginale and Ehrlichia spp, in naturally and experimentally infected cattle (Al-Adhami et al, 2011). Earlier studies had shown that the C-ELISA was 100% specific using 261 known negative sera from a non-endemic region, detecting acutely infected cattle as early as 16 days after experimental tick or blood inoculation, and was demonstrated to detect cattle that have been experimentally infected as long as 6 years previously (Knowles et al., 1996). In detecting persistently infected cattle from an anaplasmosis-endemic region that were defined as true positive or negative using a nested PCR procedure, the rMSP5 C-ELISA had a sensitivity of 96% and a specificity of 95% (Torioni De Echaide et al., 1998).

Test results using the rMSP5 C-ELISA are available in less than 2.5 hours. A test kit available commercially contains specific instructions. In general, however, it is conducted as follows.

- **Kit reagents**
  - A 96-well microtitre plate coated with rMSP5 antigen,
  - A 96-well coated adsorption/transfer plate for serum adsorption to reduce background binding,
  - 100 x MAB/peroxidase conjugate,
  - 10 x wash solution and ready-to-use conjugate-diluting buffer,
  - Ready-to-use substrate and stop solutions,
  - Positive and negative controls
• Test procedure
  i) Add 70 µl of undiluted serum sample to the coated adsorption/transfer plate and incubate at room temperature for 30 minutes.
  ii) Transfer 50 µl per well of the adsorbed serum to the rMSP5-coated plate and incubate at room temperature for 60 minutes.
  iii) Discard the serum and wash the plate twice using diluted wash solution.
  iv) Add 50 µl per well of the 1 × diluted MAb/peroxidase conjugate to the rMSP5-coated plate, and incubate at room temperature for 20 minutes.
  v) Discard the 1 × diluted MAb/peroxidase conjugate and wash the plate four times using diluted wash solution.
  vi) Add 50 µl per well of the substrate solution, cover the plate with foil, and incubate for 20 minutes at room temperature.
  vii) Add 50 µl per well of stop solution to the substrate solution already in the wells and gently tap the sides of the plate to mix the wells.
  viii) Immediately read the plate in the plate reader at 620 nm.
• Test validation
  The mean optical density (OD) of the negative control must range from 0.40 to 2.10. The per cent inhibition of the positive control must be ≥30%.
• Interpretation of the results
  The % inhibition is calculated as follows:

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\text{Per cent inhibition} = \left(1 - \frac{\text{Sample OD}}{\text{Mean negative control OD}}\right) \times 100
\]

Samples with <30% inhibition are negative. Samples with ≥30% inhibition are positive.

Specificity of the MSP5 C-ELISA may be increased by using a higher percentage inhibition cut-off value (Bradway et al., 2001); however the effect of this change on sensitivity has not been thoroughly evaluated.

b) Card agglutination test

The advantages of the CAT are that it is sensitive, may be undertaken either in the laboratory or in the field, and gives a result within a few minutes. Nonspecific reactions may be a problem, and subjectivity in interpreting assay reactions can result in variability in test interpretation. In addition, the CAT antigen, which is a suspension of A. marginale particles, can be difficult to prepare and can vary from batch to batch and laboratory to laboratory. Splenectomised calves are infected by intravenous inoculation with blood containing Anaplasma-infected erythrocytes. When the rickettsaemia exceeds 50%, the animal is exsanguinated, the infected erythrocytes are washed, lysed, and the erythrocyte ghosts and Anaplasma particles are pelleted. The pellets are sonicated, washed, and then resuspended in a stain solution to produce the antigen suspension.

A test procedure that has been slightly modified from that originally described (Amerault & Roby, 1968; Amerault et al., 1972) is as follows:

i) Ensure all test components are at a temperature of 25–26°C before use (this constant temperature is critical for the test).

ii) On each circle of the test card (a clear perspex/plastic or glass plate marked with circles that are 18 mm in diameter), place next to each other, but not touching, 10 µl of bovine serum factor (BSF), 10 µl of test serum, and 5 µl of CAT antigen. Negative and low positive control sera must be tested on each card.

BSF is serum from a selected animal with high known conglutinin level. If the conglutinin level is unknown, fresh serum from a healthy animal known to be free from Anaplasma can be used. The Jersey breed is often suitable. The BSF must be stored at –70°C in small aliquots, a fresh aliquot being used each time the tests are performed. The inclusion of BSF improves the sensitivity of the test.

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2 The test as conducted in the USA and Mexico uses larger volumes of reagents: antigen (15 µl), serum (30 µl), and bovine serum factor (30 µl), and a 4-minute reaction time (see step iv).
iii) Mix well with a glass stirrer. After mixing each test, wipe the stirrer with clean tissue to prevent cross-contamination.

iv) Place the test card in a humid chamber and rock at 100–110 rpm for 7 minutes.

v) Read immediately against a backlight. Characteristic clumping of the antigen (graded from +1 to +3) is considered to be a positive result. The test is considered to give a negative result when there is no characteristic clumping.

c) Complement fixation test

The complement fixation (CF) test has been used extensively for many years; however, it shows variable sensitivity (ranging from 20 to 60%), possibly reflecting differences in techniques for antigen production, and poor reproducibility. In addition, it has been demonstrated that the CF assay fails to detect a significant proportion of carrier cattle (Bradway et al., 2001). It is also uncertain as to whether or not the CF test can identify antibodies in acutely infected animals prior to other assays (Coetzee et al., 2007; Molloy et al., 1999). Therefore, the CF test is no longer recommended as a reliable assay for detecting infected animals.

d) Indirect fluorescent antibody test

Because of the limitations on the number of indirect fluorescent antibody (IFA) tests that can be performed daily by one operator, other serological tests are generally preferred to the IFA test. The IFA test is performed as described for bovine babesiosis in chapter 2.4.2, except that A.-marginae-infected blood is used for the preparation of antigen smears. A serious problem encountered with the test is nonspecific fluorescence. Antigen made from blood collected as soon as adequate rickettsaemia (5–10%) occurs is most likely to be suitable. Nonspecific fluorescence due to antibodies adhering to infected erythrocytes can be reduced by washing the erythrocytes in an acidic glycine buffer before antigen smears are prepared. Infected erythrocytes are washed twice in 0.1 M glycine buffer (pH 3.0, centrifuged at 1000 g for 15 minutes at 4°C) and then once in PBS, pH 7.4. Recently published data show that the IFA, like the C-ELISA, can cross react with other members of the Anaplasmataceae family (Al-Adhami et al., 2011).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Background

Several immunisation methods have been used to protect cattle against anaplasmosis in countries where the disease is endemic, but none is ideal (McHardy, 1984). A review of A. marginale vaccines and antigens has been published (Kocan et al., 2003) Use of the less pathogenic A. centrale, which gives partial cross-protection against A. marginale, is the most widely accepted method, although not used in North America.

In this section, the production of live A. centrale vaccine is described. It involves infection of a susceptible, splenectomised calf and the use of its blood as a vaccine. Detailed accounts of the production procedure are available and reference should be made to these publications for details of the procedures outlined here (Bock et al., 2004; de Vos & Jorgensen, 1992; Pipano, 1995).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

Anaplasma centrale vaccine can be provided in either frozen or chilled form depending on demand, transport networks, and the availability of liquid nitrogen or dry ice supplies. Frozen vaccine is recommended in most instances, as it allows for thorough post-production quality control of each batch. It is, however, more costly to produce and more difficult to transport than chilled vaccine. The risk of contamination makes post-production control essential, but may be prohibitively expensive.

2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed

i) Biological characteristics

Anaplasma centrale was isolated in 1911 in South Africa, and has been used as a vaccine in South America, Australia, Africa, the Middle East, and South-East Asia. It affords only partial, but adequate, protection in regions where the challenging strains are of moderate virulence (e.g. Australia) (Bock & de Vos, 2001). In the humid tropics where A. marginale appears to be a very virulent rickettsia, the protection afforded by A. centrale may be inadequate to prevent disease in some animals.
Anaplasma centrale usually causes benign infections, especially if used in calves under 9 months of age. Severe reactions following vaccination have been reported when adult cattle are inoculated. The suitability of an isolate of *A. centrale* as a vaccine can be determined by inoculating susceptible cattle, monitoring the subsequent reactions, and then challenging the animals and susceptible controls with a virulent local strain of *A. marginale*. Both safety and efficacy can be judged by monitoring rickettsaemias in stained blood films and the depression of packed cell volumes of inoculated cattle during the vaccination and challenge reaction periods.

Infected material for preparing the vaccine is readily stored as frozen stables of infected blood in liquid nitrogen or dry ice. Dimethyl sulphoxide (DMSO) and polyvinylpyrrolidone M.W. 40,000 (Bock *et al.*, 2004) are the recommended cryopreservatives, as they allow for intravenous administration after thawing of the stablate. A detailed account of the freezing technique using DMSO is reported elsewhere (Mellors *et al.*, 1982), but briefly involves the following: infected blood is collected, chilled to 4°C, and cold cryoprotectant (4 M DMSO in PBS) is added slowly with stirring to a final blood:protectant ratio of 1:1, to give a final concentration of 2 M DMSO. The entire dilution procedure is carried out in an ice bath and the diluted blood is dispensed into suitable containers (e.g. 5 ml cryovials), and frozen, as soon as possible, in the vapour phase of a liquid nitrogen container.

**ii) Quality criteria**

Evidence of purity of the *A. centrale* isolate can be determined by serological testing of paired sera from the cattle used in the safety test for possible contaminants that may be present (Bock *et al.*, 2004; Pipano, 1997). Donor calves used to expand the seed for vaccine production should be examined for all blood-borne infections prevalent in the vaccine-producing country, including Babesia, Anaplasma, Ehrlichia, Theileria and Trypanosoma. This can be done by routine examination of stained blood films after splenectomy, and preferably also by serology. Any calves showing evidence of natural infections of any of these agents should be rejected. The absence of other infective agents should also be confirmed. These may include the agents of enzootic bovine leukosis, mucosal disease, infectious bovine rhinotracheitis, ephemeral fever, Akabane disease, bluetongue, foot and mouth disease, and rinderpest. The testing procedures will depend on the diseases prevalent in the country and the availability of tests, but should involve serology of paired sera at the very least and, in some cases, virus isolation, antigen, or DNA/RNA detection (Bock *et al.*, 2004; Pipano, 1981; 1997).

**b) Method of manufacture**

**i) Procedure**

**Production of frozen vaccine**

Quantities of the frozen stablate (5–10 ml) are thawed by immersing the vials in water preheated to 40°C. The thawed material is kept on ice and used as soon as possible (within 30 minutes) to infect a susceptible, splenectomised calf by intravenous inoculation.

The rickettsaemia of the donor calf is monitored daily by examining stained films of jugular blood, and the blood is collected for vaccine production when suitable rickettsaemias are reached. A rickettsaemia of $1 \times 10^8$/ml (approximately 2% rickettsaemia in jugular blood) is the minimum required for production of vaccine. If a suitable rickettsaemia is not obtained, passage of the strain by subinoculation of 100–200 ml of blood to a second splenectomised calf may be necessary.

Blood from the donor is collected by aseptic jugular or carotid cannulation using heparin as an anticoagulant (5 International Units [IU] heparin/ml blood).

In the laboratory, the infective blood is mixed in equal volumes with 3 M glycerol in PBS supplemented with 5 mM glucose at 37°C (final concentration of glycerol 1.5 M). The mixture is then equilibrated at 37°C for 30 minutes and dispensed into suitable containers (e.g. 5 ml cryovials). The vials are cooled at approximately 10°C/minute in the vapour phase of liquid nitrogen and, when frozen, stored in the liquid phase (Bock *et al.*, 2004).

DMSO can be used as a cryoprotectant in the place of glycerol. This is done in the same way as outlined for the preparation of seed stablate (Mellors *et al.*, 1982; Pipano, 1981).

If glycerolised vaccine is to be diluted, the diluent should consist of PBS with 1.5 M glycerol and 5 mM glucose (Jorgensen *et al.*, 1989). Vaccine cryopreserved with DMSO should be diluted with diluent containing the same concentration of DMSO as in the original cryopreserved blood (Pipano *et al.*, 1986).

**Production of chilled vaccine**

Infected material for chilled vaccine is prepared in the same way as for frozen vaccine, but it must be issued and used as soon as possible after collection. The infective blood can be diluted to provide $1 \times 10^7$ parasites per dose of vaccine. A suitable diluent is 10% sterile bovine serum in a glucose/balanced...
Chapter 2.4.1. — Bovine anaplasmosis

Salt solution containing the following quantities per litre: NaCl (7.00 g), MgCl₂\(\cdot\)6H₂O (0.34 g), glucose (1.00 g), Na₂HPO₄ (2.52 g), KH₂PO₄ (0.90 g), and NaHCO₃ (0.52 g).

If diluent is not available, acid citrate dextrose (20% [v/v]) or citrate phosphate dextrose (20% [v/v]) should be used as anticoagulant to provide the glucose necessary for survival of the organisms.

Use of vaccine

In the case of frozen vaccine, vials should be thawed by immersion in water, preheated to 37°C to 40°C, and the contents mixed with suitable diluent to the required dilution. If glycerolised vaccine is prepared, it should be kept cool and used within 8 hours (Bock et al., 2004). If DMSO is used as a cryoprotectant, the prepared vaccine should be kept on ice and used within 15–30 minutes (Pipano, 1981). The vaccine is most commonly administered subcutaneously.

Chilled vaccine should be kept refrigerated and used within 4–7 days of preparation.

The strain of A. centrale used in vaccine is of reduced virulence, but is not entirely safe. A practical recommendation is, therefore, to limit the use of vaccine to calves, where nonspecific immunity will minimise the risk of vaccine reactions. When older animals have to be vaccinated, there is a risk of severe reactions. These reactions occur infrequently, but valuable breeding stock or pregnant animals obviously warrant close attention, and should be observed daily for 3 weeks post-vaccination. Clinically sick animals should be treated with oxytetracycline or imidocarb at dosages recommended by the manufacturers. Protective immunity develops in 6–8 weeks and usually lasts for several years.

Anaplasmosis and babesiosis vaccines are often used concurrently, but it is not advisable to use any other vaccines at the same time (Bock et al., 2004).

ii) Requirements for substrates and media

Anaplasma centrale cannot be cultured in vitro. No substrates or media other than buffers and diluents are used in vaccine production. DMSO or glycerol should be purchased from reputable companies.

iii) In-process controls

Source and maintenance of vaccine donors

A source of calves free from natural infections of Anaplasma and other tick-borne diseases should be identified. If a suitable source is not available, it may be necessary to breed the calves under tick-free conditions specifically for the purpose of vaccine production.

The calves should be maintained under conditions that will prevent exposure to infectious diseases and to ticks and biting insects. In the absence of suitable facilities, the risk of contamination with the agents of infectious diseases present in the country involved should be estimated, and the benefits of local production of vaccine weighed against the possible adverse consequences of spreading disease (Bock et al., 2004).

Surgery

Donor calves should be splenectomised to allow maximum yield of organisms for production of vaccine. This is best carried out in young calves and under general anesthesia.

Screening of vaccine donors before inoculation

As for preparation of seed stabilate, donor calves for vaccine production should be examined for all blood-borne infections prevalent in the vaccine-producing country, including Babesia, Anaplasma, Cowdria, Theileria and Trypanosoma. This can be done by routine examination of stained blood films after splenectomy, and preferably also by serology. Any calves showing evidence of natural infections of any of these agents should be rejected. The absence of other infective agents should also be confirmed. These may include the agents of enzootic bovine leukosis, mucosal disease, infectious bovine rhinotracheitis, ephemeral fever, Akabane disease, bluetongue, foot and mouth disease, and rinderpest. The testing procedures will depend on the diseases prevalent in the country and the availability of tests, but should involve serology of paired sera at the very least and, in some cases, virus isolation, antigen, or DNA/RNA detection (Bock et al., 2004; Pipano, 1981; 1997).

Monitoring of rickettsaemias following inoculation

It is necessary to determine the concentration of rickettsia in blood being collected for vaccine. The rickettsial concentration can be estimated from the erythrocyte count and the rickettsaemia (percentage of infected erythrocytes).
Collection of blood for vaccine

All equipment should be sterilised before use (e.g. by autoclaving). Once the required rickettsaemia is reached, the blood is collected in heparin using strict aseptic techniques. This is best done if the calf is sedated and with the use of a closed-circuit collection system.

Up to 3 litres of heavily infected blood can be collected from a 6-month-old calf. If the calf is to live, the transfusion of a similar amount of blood from a suitable donor is indicated. Alternatively, the calf should be killed immediately after collection of the blood.

Dispensing of vaccine

All procedures are performed in a suitable environment, such as a laminar flow cabinet, using standard sterile techniques. Use of a mechanical or magnetic stirrer will ensure thorough mixing of blood and diluent throughout the dispensing process. Penicillin (500,000 IU/litre) and streptomycin (370,000 µg/litre) are added to the vaccine at the time of dispensing.

iv) Final product batch tests

The potency, safety and sterility of vaccine batches cannot be determined in the case of chilled vaccine, and specifications for frozen vaccine depend on the country involved. The following are the specifications for frozen vaccine produced in Australia.

Sterility and purity

Standard tests for sterility are employed for each batch of vaccine and diluent (see Chapter 1.1.7 Tests for sterility and freedom from contamination of biological materials).

The absence of contaminants is determined by doing appropriate serological testing of donor cattle, by inoculating donor lymphocytes into sheep and then monitoring them for evidence of viral infection, and by inoculating cattle and monitoring them serologically for infectious agents that could potentially contaminate the vaccine. Cattle inoculated during the test for potency (see Section C.4.c) are suitable for the purpose. Depending on the country of origin of the vaccine, these agents include the causative organisms of enzootic bovine leukosis, infectious bovine rhinotracheitis, mucosal disease, ephemeral fever, Akabane disease, Aino virus, bluetongue, parainfluenza, foot and mouth disease, lumpy skin disease, rabies, Rift Valley fever, rinderpest, contagious bovine pleuropneumonia, Jembrana disease, heartwater, pathogenic Theileria and Trypanosoma spp., Brucella abortus, Coxiella, and Leptospira (Bock et al., 2004; Pipano, 1981; 1997).

Safety

Vaccine reactions of the cattle inoculated in the test for potency (see Section C.4.c) are monitored by measuring rickettsaemia and depression of packed cell volume. Only batches with pathogenicity levels equal to or lower than a predetermined standard are released for use.

Potency

Vaccine is thawed and diluted 1/5 with a suitable diluent (Bock et al., 2004). The diluted vaccine is then incubated for 8 hours at 4°C, and five cattle are inoculated subcutaneously with 2 ml doses. The inoculated cattle are monitored for the presence of infections by examination of stained blood smears. All should become infected for a batch to be accepted. A batch proving to be infective is recommended for use at a dilution of 1/5 with isotonic diluent.

c) Requirements for authorisation

i) Safety

The strain of *A. centrale* used in vaccine is of reduced virulence, but is not entirely safe. A practical recommendation is, therefore, to limit the use of vaccine to calves, where nonspecific immunity will minimise the risk of vaccine reactions. When older animals have to be vaccinated, there is a risk of severe reactions. These reactions occur infrequently, but valuable breeding stock or pregnant animals obviously warrant close attention, and should be observed daily for 3 weeks post-vaccination. Clinically sick animals should be treated with oxytetracycline or imidocarb at dosages recommended by the manufacturers.

*Anaplasma centrale* is not infective to other species, and the vaccine is not considered to have other adverse environmental effects. The vaccine is not infective for humans. When the product is stored in liquid nitrogen, the usual precautions pertaining to the storage, transportation and handling of deep-frozen material applies.
ii) **Efficacy requirements**

Partial but long-lasting immunity results from one inoculation. There is no evidence that repeated vaccination will have a boosting effect. The vaccine is used for control of clinical anaplasmosis in endemic areas. It will not provide sterile immunity, and should not be used for eradication of *A. marginale*.

iii) **Stability**

The vaccine can be kept for 5 years when stored in liquid nitrogen. Once thawed, it rapidly loses its potency. Thawed vaccine cannot be refrozen.

### 3. Vaccines based on biotechnology

#### a) Vaccines available and their advantages

There are no vaccines based on biotechnology available for anaplasmosis.

#### b) Special requirements for biotechnological vaccines, if any

This section is not applicable to vaccines for anaplasmosis.

## REFERENCES


* * *

**NB:** There is an OIE Reference Laboratory for Anaplasmosis (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)). Please contact the OIE Reference Laboratory for any further information on diagnostic tests, reagents and vaccines for bovine anaplasmosis
SUMMARY

Babesiosis is a tick-borne disease of cattle caused by the protozoan parasites Babesia bovis, B. bigemina, B. divergens and others. Rhipicephalus (Boophilus) spp., the principal vectors of B. bovis and B. bigemina, are widespread in tropical and subtropical countries. The major vector of B. divergens is ixodes ricinus. Other important vectors include Haemaphysalis and other Rhipicephalus spp.

Identification of the agent: In the case of live animals, thick and thin films of capillary blood should be taken from, for example, the tip of the tail. Demonstration of parasites in dead animals is possible by microscopic examination of smears of peripheral blood, brain, kidney, heart muscle, spleen and liver, provided decomposition is not advanced. The smears are fixed with methanol, stained with 10% Giemsa for 15–30 minutes, and examined at ×800–1000 magnification under oil immersion. Sensitive polymerase chain reaction assays are available that can detect and differentiate Babesia species in cattle.

Serological tests: Enzyme-linked immunosorbent assays (ELISAs) have replaced the indirect fluorescent antibody (IFA) test as the most widely used test for the detection of antibodies to Babesia spp., because of processing efficiency and objectivity in interpretation of results. The IFA test has been used for detection of antibodies to B. bigemina, but serological cross-reactions make species diagnosis difficult. The complement fixation (CF) test has also been used to detect antibodies against B. bovis and B. bigemina.

Requirements for vaccines: Vaccines consisting of live, attenuated strains of B. bovis, B. bigemina or B. divergens are produced in several countries from the blood of infected donor animals or from in-vitro culture. The vaccines are provided in frozen or chilled forms. Frozen vaccine has the advantage of allowing thorough post-production control of each batch, but has a much reduced post-thaw shelf life compared with chilled vaccine. The risk of contamination of this blood-derived vaccine makes thorough quality control essential, but this may be prohibitively expensive.

Whilst in-vitro production methods offer obvious advantages in terms of animal welfare, vaccine can also be successfully produced using in-vivo production systems under strict animal welfare guidelines. With either in-vivo or in-vitro systems, strict adherence to production protocols is essential to ensure consistency of vaccine and to avoid potential changes in virulence, immunogenicity and consequent protectiveness associated with continued passage of Babesia spp. organisms in both culture and splenectomised calves.

Live Babesia vaccines are not entirely safe. A practical recommendation is to limit their use to calves, preferably less than 1 year old, when nonspecific immunity will minimise the risk of vaccine reactions. When older animals are to be vaccinated, the risk of reaction warrants close surveillance and treatment with a babesiacide if severe reactions do occur.

Protective immunity develops in 3–4 weeks. A single vaccination usually provides lifelong immunity.

A. INTRODUCTION

Bovine babesiosis is caused by protozoan parasites of the genus Babesia, order Piroplasmida, phylum Apicomplexa. Of the species affecting cattle, two – Babesia bovis and Babesia bigemina – are widely distributed
and of major importance in Africa, Asia, Australia, and Central and South America. Babesia divergens is economically important in some parts of Europe.

Tick species are the vectors of Babesia (Bock et al., 2008). Rhipicephalus (Boophilus) microplus is the principal vector of B. bigemina and B. bovis and is widespread in the tropics and subtropics. The vector of B. divergens is Ixodes ricinus. Other important vectors include Haemaphysalis, and other species of Rhipicephalus.

Babesia bigemina has the widest distribution but B. bovis is generally more pathogenic than B. bigemina or B. divergens. Babesia bovis infections are characterised by high fever, ataxia, anorexia, general circulatory shock, and sometimes also nervous signs as a result of sequestration of infected erythrocytes in cerebral capillaries. Anaemia and haemoglobinuria may appear later in the course of the disease. In acute cases, the maximum parasitaemia (percentage of infected erythrocytes) in circulating blood is less than 1%. This is in contrast to B. bigemina infections, where the parasitaemia often exceeds 10% and may be as high as 30%. In B. bigemina infections, the major signs include fever, haemoglobinuria and anaemia. Intravascular sequestration of infected erythrocytes does not occur with B. bigemina infections. The parasitaemia and clinical appearance of B. divergens infections are somewhat similar to B. bigemina infections (Zintl et al., 2003).

Infected animals develop a life-long immunity against reinfection with the same species. There is also evidence of a degree of cross-protection in B. bigemina-immune animals against subsequent B. bovis infections. Calves rarely show clinical signs of disease after infection regardless of the Babesia spp. involved or the immune status of the dams (Bock et al., 2008; Zintl et al., 2003).

**B. DIAGNOSTIC TECHNIQUES**

1. Identification of the agent

a) Direct microscopic examination

The traditional method of identifying the agent in infected animals is by microscopic examination of thick and thin blood films stained with Giemsa, a Romanowsky type stain (10% Giemsa in phosphate buffered saline (PBS) or Sorenson’s buffer at pH 7.4). The sensitivity of thick films is such that it can detect parasitaemias as low as 1 parasite in 10<sup>5</sup> red blood cells (RBCs) (Bose et al., 1995). Species differentiation is good in thin films but poor in the more sensitive thick films. This technique is usually adequate for detection of acute infections, but not for detection of carriers where the parasitaemias are mostly very low. Parasite identification and differentiation can be improved by using a fluorescent dye, such as acridine orange, instead of Giemsa (Bose et al., 1995).

Samples from live animals should preferably be films made from fresh blood taken from capillaries, such as those in the tip of the ear or tip of the tail, as B. bovis is more common in capillary blood. Babesia bigemina and B. divergens parasites are uniformly distributed through the vasculature. If it is not possible to make fresh films from capillary blood, sterile jugular blood should be collected into an anticoagulant such as lithium heparin or ethylene diamine tetra-acetic acid (EDTA). The sample should be kept cool, preferably at 5°C, until delivery to the laboratory. Thin blood films are air-dried, fixed in absolute methanol for 10–60 seconds and then stained with 10% Giemsa for 15–30 minutes. It is preferable to stain blood films as soon as possible after preparation to ensure proper stain definition. Thick films are made by placing a small droplet of blood (approximately 50 µl) on to a clean glass slide and spreading this over a small area using a circular motion with the corner of another slide. This droplet is not fixed in methanol, but simply air-dried, heat-fixed at 80°C for 5 minutes, and stained in 10% Giemsa. This is a more sensitive technique for the detection of Babesia spp., as RBCs are lysed and parasites concentrated, but species differentiation is more difficult. Unstained blood films should not be stored with formalin solutions as formalin fumes affect staining quality. Moisture also affects staining quality.

Samples from dead animals should consist of thin blood films, as well as smears from cerebral cortex, kidney (freshly dead), spleen (when decomposition is evident), heart muscle, lung, and liver (Bock et al., 2006; de Vos et al., 2004). Organ smears are made by pressing a clean slide on to a freshly cut surface of the organ or by crushing a small sample of the tissue (particularly cerebral cortex) between two clean microscope slides drawn lengthwise to leave a film of tissue on each slide. The smear is then air-dried, fixed in absolute methanol for 10–60 seconds and then stained with 10% Giemsa for 15–30 minutes. It is preferable to stain blood films as soon as possible after preparation to ensure proper stain definition.

All stained films are examined under oil immersion using (as a minimum) a x8 eyepiece and a x60 objective lens. Babesia bovis is a small parasite, usually centrally located in the erythrocyte. It measures approximately 1–1.5 µm long and 0.5–1.0 µm wide, and is often found as pairs that are at an obtuse angle to each other. Babesia divergens is also a small parasite and is very similar morphologically to B. bovis. However, obtuse-angled pairs are...
are often located at the rim of the erythrocyte. *Babesia bigemina* is typically pear-shaped, but many diverse single forms are found. It is usually a much bigger parasite (3–3.5 µm long and 1–1.5 µm wide), and is often found as pairs at an acute angle to each other or almost parallel. In acute cases, the parasitaemia of *B. bovis* seldom reaches 1% (measured in general circulation, rather than capillary blood), but with *B. bigemina* and *B. divergens* much higher parasitaemias are usual.

b) Nucleic acid-based diagnostic assays

Nucleic acid-based diagnostic assays are very particular in detecting *B. bovis* and *B. bigemina* in carrier cattle (Buling et al., 2007; Costa-Junior et al., 2006; Criado-Fornelio, 2007). Polymerase chain reaction (PCR-based techniques) are reported to be as much as 1000 times more sensitive than microscopy for detection of *Babesia* spp., with detection at parasitaemia levels ranging from 0.001% to 0.0000001% (1 parasite in 10¹⁸ RBCs) (Criado-Fornelio, 2007). A number of PCR techniques have been described that can detect and differentiate species of *Babesia* in carrier infections (Buling et al., 2007; Criado-Fornelio, 2007). PCR assays to differentiate isolates of *B. bovis* have also been described. The application of the reverse line blot procedure, in which PCR products are hybridised to membrane-bound, species-specific oligonucleotide probes, to *Babesia* (Gubbels et al., 1999) and, more recently, two quantitative PCR methods (Criado-Fornelio et al., 2009) have enabled the simultaneous detection of multiple species, even in carrier state infections. However, current PCR assays generally do not lend themselves well to large-scale testing and at this time are unlikely to supplant serological tests as the method of choice for epidemiological studies. PCR assays are useful as confirmatory tests and in some cases for regulatory testing.

c) In-vitro culture

In-vitro culture methods have been used to demonstrate the presence of carrier infections of *Babesia* spp. (Holman et al., 1993), and *B. bovis* has also been cloned in culture. The minimum parasitaemia detectable by this method will depend, to a large extent, on the facilities available and the skills of the operator (Bose et al., 1995), but could be as low as 10⁻¹⁰ (Friedhoff & Bose, 1994), making it a very sensitive method, with 100% specificity, for the demonstration of infection. Details of the method are outlined in Section C.2.a (Preparation and storage of master seed).

d) Animal inoculation

Confirmation of infection in a suspected carrier animal can also be made by transfusing approximately 500 ml of jugular blood intravenously into a splenectomised calf known to be Babesia-free, and monitoring the calf for the presence of infection. This method is cumbersome and expensive, and obviously not suitable for routine diagnostic use. Mongolian gerbils (*Meriones unguiculatus*) have been used to demonstrate the presence of *B. divergens* (Zintl et al., 2003).

2. Serological tests

The indirect fluorescent antibody (IFA) test was widely used in the past to detect antibodies to *Babesia* spp., but the *B. bigemina* test has poor specificity. Cross-reactions with antibodies to *B. bovis* in the *B. bigemina* IFA test were a particular problem in areas where the two parasites coexist. The IFA test also has the disadvantages of low sample throughput and subjectivity (Anonymous, 1984). The complement fixation (CF) test has been described as a method to detect antibodies against *B. bovis* and *B. bigemina* (Anonymous, 2006). This test has been used to qualify animals for importation into some countries.

Enzyme-linked immunosorbent assays (ELISA) have largely replaced the IFA as the diagnostic test of choice for *Babesia* spp, because of the objectivity in interpretation of results and capacity to process high numbers of samples daily. An ELISA for the diagnosis of *B. bovis* infection that uses a whole merozoite antigen has undergone extensive evaluation (De Echaide et al., 1995, Molloy et al., 1998a). High sensitivity and specificity of this test was demonstrated in both Australia and Zimbabwe, although threshold values varied between laboratories (Molloy et al., 1998a). Indirect (Bono et al., 2008; Boonchit et al., 2006) and competitive ELISAs (Goff et al., 2003) using recombinant merozoite surface and rhoptry-associated antigens of *B. bovis* have recently been developed. The competitive ELISA has been more widely validated in different laboratories, with the antigen recognised by antibody from diverse regions around the world (Goff et al., 2006). Reduction in specificity of the indirect *B. bovis* ELISA using recombinant antigens has been noted in some situations (Bono et al., 2008).

There is still no well validated ELISA available for *B. bigemina* despite the efforts of several investigators in different laboratories. ELISAs for antibodies to *B. bigemina* crude antigen typically have poor specificity. Competitive ELISAs developed and validated in Australia (Molloy et al., 1998b) and USA (Goff et al., 2008) are apparently the only ELISAs in routine use. Unlike *B. bovis* where animals are thought to remain carriers for life after infection, *B. bigemina* may clear infection and antibody levels may decline below the negative threshold within months after infection (Goff et al., 2008). Inconclusive results may occur around the negative threshold values; and this phenomenon can provide a diagnostic challenge in animals where titres are declining if the animal clears infection.
ELISAs have also been developed for *B. divergens* using antigen derived from culture, *Meriones* or cattle, but there does not appear to be one that has been validated internationally (Zintl et al., 2003). An immunochromatographic test for simultaneous rapid serodiagnosis of bovine babesioses caused by *B. bovis* and *B. bigemina* was developed recently (Chul-Min et al., 2008).

a) **Babesia bovis** indirect enzyme-linked immunoabsorbent assay

Antigen preparation is based on a technique described by Waltisbuhl et al., 1987. Infected blood (usually 5–10% parasitaemia) is collected from a splenectomised calf into EDTA. The blood is first centrifuged and the plasma removed and stored for later use. The RBCs are then washed three times in five volumes of phosphate buffered saline (PBS), and infected cells are concentrated by differential lysis of uninfected cells in hypotonic saline solution. Infected cells are more resistant to lysis in hypotonic saline solutions than are uninfected cells.

To find the best concentration for particular infected blood, a series of hypotonic saline solutions are prepared, ranging from 0.35% to 0.50% NaCl in 0.025% increments. Five volumes of each saline solution is then added to one volume of packed RBCs, gently mixed and allowed to stand for 5 minutes. The mixtures are then centrifuged and the supernatants aspirated. An equal volume of plasma (retained from the original blood) is added to each tube containing packed RBCs, and the contents of the tubes are mixed. Thin blood films are prepared from each of these resuspended blood cell mixtures, fixed in methanol, and stained with Giemsa. These films are examined under a microscope to determine which saline solution lyses most uninfected RBCs but leaves infected RBCs intact. It should be possible to achieve >95% infection in the remaining intact RBCs.

The bulk of the packed RBCs are then differentially lysed with the optimal saline solution, centrifuged and the supernatant removed. The sediment (>95% infected RBCs) is lysed in distilled water at 4°C, and parasites are pellet at 12,000 g for 30 minutes. The pellet is washed at least three times in PBS by resuspension and centrifugation at 4°C until minimal haemoglobin is in the supernatant. It is then resuspended in one to two volumes of PBS at 4°C, and sonicated in appropriate volumes using medium power for 60–90 seconds. The sonicated material is ultra-centrifuged (105,000 g for 60 minutes at 4°C) and the supernatant containing the solubilised merozoite antigen is retained. The supernatant is mixed with an equal volume of glycerol and stored in 2–5 ml aliquots at −70°C. Short-term storage at −20°C is acceptable for the working aliquot.

- **Test procedure**

  The following test procedure is based on that described by Molloy et al. (1998a) with some modification.

  i) 100 µl of antigen solution (with the antigen typically diluted in the range from 1/400 to 1/1600 in 0.1 M carbonate buffer (pH 9.6) is added to each well of a polystyrene 96-well microtitre plate. The plate is covered and incubated overnight at 4°C.

  ii) The solution containing any unbound antigen is removed and the wells are then blocked for 1 hour at room temperature by the addition of 200 µl of a 2% solution of sodium caseinate in carbonate buffer (pH 9.6).

  iii) After blocking, the wells are rinsed three times with PBS containing 0.1% Tween 20 (PBST); then 100 µl of diluted test and control bovine serum (diluted 1/100 in PBST containing 2% skim milk powder) is added into each well, and the plates are incubated for 30 minutes at room temperature with shaking.

  iv) The washing step consists of five rinses with PBST. During the last rinse, the plate is shaken for 5 minutes.

  v) Next, 100 µl of peroxidase-labelled anti-bovine IgG diluted in PBST containing 2% skim milk powder is added and the plates are shaken for a further 30 minutes at room temperature. (NB: some batches of skim milk powder may contain immunoglobulins that can interfere with anti-bovine IgG conjugates and must be tested for suitability prior to use).

  vi) Wells are washed as described in step iv above, and 100 µl of peroxidase substrate (ABTS [2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)]) is added to each well (recommended working dilution contains 0.3 g/litre ABTS in a glycerine/citric acid buffer; with H2O2 concentration of 0.01%5). The substrate reaction is allowed to continue until the absorbance of a strong positive control serum included on each plate approaches 1.

  vii) At this point, the reaction is stopped using an equal volume (100 µl) of ABTS Peroxidase Stop Solution (working concentration of 1% sodium dodecyl sulphate2). Absorbance at 414 nm is read on a microtitre plate reader within 30 minutes. 3,3',5,5'-Tetramethyl benzidine (TMB) is also a suitable substrate, but is stopped with equal volumes of 1 M phosphoric acid (H3PO4) and is read at wavelength 450 nm.

  To control for inter-plate variation, known positive and negative sera are included in each plate. Test sera are then ranked relative to the positive control. ELISA absorbance results are expressed as a percentage of
this positive control (per cent positivity). Positive and negative threshold values should be determined in each laboratory by testing as many known positive and negative sera as possible.

Each batch of antigen and conjugate should be titrated using a checkerboard layout. The most suitable enzyme label for the conjugate is horseradish peroxidase. ABTS or TMB are suitable substrates. With this test, it is possible to detect antibodies at least 4 years after a single infection. There should be 95–100% positive reactions with B. bovis-immune animals, 1–2% false-positive reactions with negative sera, and <2% false-positive reactions with B. bigemina-immune animals.

b) Babesia bovis and Babesia bigemina competitive enzyme-linked immunosorbent assays

The format of these tests is based on that described by Goff et al. (2003). The tests are described together because of the similarity of the processes. They have also been developed with the intention of validation as international standard tests and include purified recombinant antigen dried onto microtitre wells for ease of standardisation, handling and distribution, and inter-laboratory use and comparison under varying conditions (Goff et al., 2006; 2008). The assays are based on a species-specific, broadly conserved, and tandemly repeated B-cell epitope within the C terminus of the rhoptry-associated protein 1 (RAP 1), expressed as a histidine-tagged thioredoxin fusion peptide. The expressed purified antigen is coated and then dried onto microtitre wells; optimal concentration of antigen and monoclonal antibody (MAb) is determined by block titration. In the case of B. bovis, positive sera inhibit the binding of the epitope-specific MAb BABB75A4; in the case of B. bigemina, positive sera inhibit the binding of MAb 64/04.10.3.

The specificity, sensitivity and predictive values of these competitive ELISAs have been calculated, and the test reliability compared across laboratories. For B bovis (Goff et al., 2006), based on random operator receiver (ROC) analysis, 21% inhibition was chosen as the threshold value to define positive and negative samples. Using this value, specificity was 100%, sensitivity 91.1%, and positive predictive value 100%; negative predictive value varied with prevalence, ranging from 99% at 10% prevalence to 55.6% at a prevalence of 90%. For B bigemina (Goff et al., 2008), using a hypothetical prevalence rate of 25% and threshold inhibition for a negative value at 16%, the assay had a specificity of 98.3% and sensitivity of 94.7%. When threshold inhibition was increased to 21%, specificity was 100% but sensitivity reduced to 87.2%; negative predictive value at 25% prevalence reduced from 98.2% to 95.9%; and positive predictive value increased to 100% from 94.9%. At 21% inhibition, negative predictive value varied from 97.0% at 10% prevalence to 48.2% with a prevalence of 95%; positive predictive values were 90.7% (10% prevalence), 95.7% (15% prevalence) and 100% at all higher prevalence rates. The attributes of both tests appear to meet standards required for international application.

It is expected that commercial kits based on this work will be available in about 2 years from the time of writing (after completion of the validation process and submission to the regulatory authorities). Detailed instructions will be available with those kits.

c) Indirect fluorescent antibody test

- Antigen preparation

Antigen slides are made from jugular blood, ideally when the parasitaemia is between 2% and 5%.

Blood is collected into a suitable anticoagulant (sodium citrate or EDTA), and is then washed at least three times in five to ten volumes of PBS to remove contaminating plasma proteins and, in particular, host immunoglobulins. After washing, the infected RBCs are resuspended in two volumes of PBS to which 1% bovine serum albumin (BSA) has been added. The BSA is used to enable RBCs to adhere to the glass slide. By preference, single-layered blood films are made by placing a drop of blood on to a clean glass slide, which is then spun in a cytocentrifuge. This produces very uniform smears. Alternatively, thin blood films may be made by the conventional manual technique (dragging with the end of another slide). The films are air-dried and fixed for 5 minutes in an oven at 80°C. Fixed blood films are then covered (e.g. with aluminium foil or brown paper sticking tape) so as to be airtight, and stored at −70°C until required (maximum 5 years).

- Test procedure

Test sera are diluted 1/30 in PBS. Sera may be used with or without heat inactivation at 56°C for 30 minutes. The slides are marked into 8–10 divisions with an oil pen to produce hydrophobic divisions. In each test square, 5–10 µl of each serum dilution is added to a filter paper disc using a fine pipette. The preparations are then incubated at 37°C for 30 minutes, in a humid chamber. For controls, negative and weak positive sera (at the same 1/30 dilution) are used on each test slide.

After incubation, the slides are rinsed with a gentle stream of PBS to remove the filter paper discs. The slides are soaked for 10 minutes in racks in PBS followed by 10 minutes in water. The PBS and water are circulated using a magnetic stirrer. Diluted anti-bovine IgG antibody labelled with fluorescein isothiocyanate
(FITC) is then added to each test square. The appropriate dilution is based on titration of every new batch of conjugate, with the working range usually being between 1/400 and 1/1200. Conjugated rabbit and chicken antibodies are usually more suitable for this purpose than goat antibodies. The slides with the conjugate are incubated again at room temperature for 30 minutes, and washed as above. The wet slides are mounted with cover-slips in a solution containing 1 part glycerol and 1 part PBS, and examined by standard fluorescence microscopy. A competent operator can examine approximately 150 samples per day.

d) Complement fixation test

The CF test is used by some countries for general diagnosis and to qualify cattle for importation. A brief description is provided here of antigen production and test protocols used by the United States Department of Agriculture (Anonymous, 2006). The test is based on the microtitration CF test procedure previously described and validated for the detection of antibody against Babesia caballi and Theileria equi (see Chapter 2.5.8 Equine piroplasmosis).

• Solutions

Alsever’s solution: prepare 1 litre of Alsever’s solution by dissolving 20.5 g glucose; 8.0 g sodium citrate; 4.2 g sodium chloride in sufficient distilled water. Adjust to pH 6.1 using citric acid, and make up the volume to 1 litre with distilled water. Sterilise by filtration.

Stock veronal buffer (5×): dissolve the following in 1 litre of distilled water: 85.0 g sodium chloride; 3.75 g sodium 5,5 diethyl barbituric acid; 1.68 g magnesium chloride (MgCl₂·6H₂O); 0.28 g calcium chloride. Dissolve 5.75 g of 5,5 diethyl barbituric acid in 0.5 litre hot (near boiling) distilled water. Cool this acid solution and add to the salt solution. Make up to 2 litres with distilled water and store at 4°C. To prepare a working dilution, add one part stock veronal buffer to four parts distilled water. The final pH should be from 7.4 to 7.6.

• Antigen production

Blood is obtained from cattle with a high parasitaemia (minimum 30% parasitised RBCs), and mixed with equal volumes of Alsever’s solution as an anticoagulant. The plasma/Alsever’s supernatant and buffy coat are removed when the RBCs have settled to the bottom of the flask. The RBCs are washed several times with cold veronal buffer and then disrupted. The antigen is recovered from the list by centrifugation at 30,900 g for 30 minutes.

The recovered antigen is washed several times in cold veronal buffer by centrifugation at 20,000 g for 15 minutes. Polyvinyl pyrrolidone (5% w/v) is added as a stabiliser and the preparation is mixed on a magnetic stirrer for 30 minutes, strained through two thicknesses of sterile gauze, dispensed into 2 ml volumes and freeze-dried. The antigen can then be stored at below –50°C for several years.

• Test procedure – Microtitration method

i) The specificity and potency of each batch of antigen should be checked against standard antisera of known specificity and potency. Optimal antigen dilutions are also determined in a preliminary checkerboard titration.

ii) Test sera are inactivated for 30 minutes at 58°C (±2°C) and tested in dilutions of 1/5 to 1/320. Veronal buffer is used for all dilutions.

iii) Complement is prepared and titrated and a spectrophotometer used to determine the 50% haemolytic dose (C’H50). Complement is used in the test at five times C’H50. The haemolytic system (sensitised RBCs) consists of equal parts of a 2% sheep RBC suspension and veronal buffer with optimally diluted haemolysin.

iv) The initial test process is made up using equal portions (0.025 ml) of antigen, complement (five times C’H50) and diluted serum. Incubation is performed for 1 hour at 37°C.

v) A double portion (0.05 ml) of the haemolytic system (sensitised sheep RBCs) is then added (bringing the total volume to 0.125 ml) and the plates are incubated for a further 45 minutes at 37°C with shaking after 20 minutes.

vi) The plates are centrifuged for 5 minutes at 300 g before being read over a mirror.

vii) The reaction in each well is recorded as follows: 100% lysis = 0 or negative, 75% lysis = 1+, 50% lysis = 2+, 25% lysis = 3+, 0% lysis = 4+. A 2+ reaction (50% lysis) or stronger at the 1/5 dilution is recorded as positive, with titre results reported as the reaction, if any, at the next dilution higher than the greatest serum dilution with a 4+ reaction (e.g. 1+ at 1/10, for a sample with a 4+ reaction at 1/5 and a 1+ reaction at 1/10). A full set of controls must be included in each test, including positive and negative
sera, as well as control antigen prepared from normal (uninfected) cattle RBCs. Anti-complementary samples may be examined by the IFA test or ELISA.

e) Other tests

Other serological tests have been described in recent years, and include a dot ELISA (Montenegro-James et al., 1992), a slide ELISA (Kung’u & Goodger, 1990), latex and card agglutination tests (Blidinno et al., 1991; Madruga et al., 1995) and an immunochromatographic test (Chul-Min et al., 2008). These tests show acceptable levels of sensitivity and specificity for B. bovis and, in the case of the dot ELISA, also for B. bigemina. However, none of these tests appears to have been adopted for routine diagnostic use in laboratories other than those in which the original development and validation took place. Adaptability of these tests to routine diagnostic laboratories is therefore unknown.

C. REQUIREMENTS FOR VACCINES

1. Background

Cattle develop a durable, long-lasting immunity after a single infection with B. bovis, B. divergens or B. bigemina. This feature has been exploited in some countries to immunise cattle against babesiosis (Bock et al., 2008; Mangold et al., 1996; Pipano, 1997). Most of these live vaccines contain specially selected strains of Babesia, mainly B. bovis and B. bigemina, and are produced in government-supported production facilities as a service to the livestock industries, in particular in Australia, Argentina, South Africa and Israel. Some other countries possess the ability to produce vaccine on a smaller scale. An experimental B. divergens vaccine prepared from the blood of infected Meriones has also been used successfully in Ireland (Zintl et al., 2003).

A killed B. divergens vaccine has also been prepared from the blood of infected calves (Zintl et al., 2003), but little information is available on the level and duration of the conferred immunity. Other experimental vaccines containing Babesia spp. antigens produced in vitro have also been developed (Montenegro-James et al., 1992; Schetters & Montenegro-James, 1995; Timms et al., 1983), but the level and duration of protection against heterologous challenge are unclear. Despite the characterisation of various parasite proteins and the B. bovis genome (Brayton et al., 2007) and considerable effort worldwide directed towards identification of candidate vaccine antigens, the prospects for recombinant vaccines against Babesia spp. remain challenging (Brown et al., 2006a: 2006b). To date no effective subunit vaccine is available commercially.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and those in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

This section will deal with the production of live babesiosis vaccines, mainly those against B. bovis and B. bigemina infections in cattle. Production involves infection of calves with selected strains, and use of the infected RBCs as vaccine (Bock et al., 2008); or in-vitro culture methods to produce parasites for vaccine (Jorgensen et al., 1992; Mangold et al., 1996). Calves used for infection with these strains or, in the case of in vitro methods, as a source of serum and RBCs for culture, must be free of infectious agents that can be transmitted by products derived from their blood. In the case of B. divergens, blood of infected gerbils (Meriones unguiculatus) can be used instead of bovine blood. Evidence that changes in immunogenicity occur with repeat passage in calves, and that possible antigenic drift occurs during long-term maintenance of B. bovis in culture, must be managed by limiting the number of repeat passages or subcultures made before returning to the vaccine working seed stablate. Whilst in-vitro production methods offer obvious advantages in terms of animal welfare, vaccine can also be successfully produced using in-vivo production systems under strict animal welfare guidelines. Close to 400,000 doses of vaccine per year have been successfully produced in Argentina under authority of SENASA (Servicio Nacional de Sanidad y Calidad Agroalimentaria, National Service for Agrifood Health and Quality) using in-vitro culture, and up to 850,000 doses per year have been successfully produced in Australia under authority of APVMA (Australian Pesticides and Veterinary Medicines Authority) using in-vivo techniques.

Babesia bovis and B. bigemina vaccines can be prepared in either frozen or chilled form depending on demand, transport networks and the availability of liquid nitrogen or dry ice supplies. Preparation of frozen vaccine (Bock et al., 2008; Mangold et al., 1996; Pipano, 1997) allows for thorough post-production testing of each batch. However, it has a much reduced shelf-life once thawed, is more costly to produce and more difficult to transport than chilled vaccine. The potential risk of contamination of this blood-derived vaccine makes pre- and post-production quality control essential, but may put production beyond the financial means of some countries in endemic regions.
2. Outline of vaccine production

a) Characteristics of the seed

- Internationally available strains
  
  Attenuated Australian strains of *B. bovis* and *B. bigemina* have been used effectively to immunise cattle in Africa, South America and South-East Asia (Bock et al., 2008). Tick-transmissible and non-transmissible strains are available. A strain of *B. divergens* with reduced virulence for *Meriones* has also been developed (Zintl et al., 2003).

- Isolation and purification of local strains
  
  Strains of *B. bovis*, *B. divergens* and *B. bigemina* that are free of contaminants, such as *Anaplasma*, *Eperythrozoon*, *Theileria*, *Trypanosoma* and various viral and bacterial agents are most readily isolated by feeding infected ticks on susceptible splenectomised cattle. The vectors and modes of transmission of the species differ, and these features can be used to separate the species (Friedhoff & Bose, 1994).

  *Babesia* spp. can also be isolated from infected cattle by subinoculation of blood into susceptible splenectomised calves. A major disadvantage of this method is the difficulty of separating the *Babesia* spp. from contaminants such as *Anaplasma* and *Eperythrozoon*. Isolation of *B. divergens* is a relatively simple process because of the susceptibility of *Meriones* (Jorgensen & Waldron, 1994) can be used to eliminate most contaminants, but not to separate *Babesia* spp. Selective chemotherapy (for example, 1% trypan blue to eliminate *B. bigemina*) can be used to obtain pure *B. bovis* from a mixed *Babesia* infection, while rapid passage in susceptible calves will allow isolation of *B. bigemina* (Anonymous, 1984).

- Attenuation of strains
  
  Various ways of attenuating *Babesia* spp. have been reported. The most reliable method of reducing the virulence of *B. bovis* involves rapid passage of the strain through susceptible splenectomised calves. Attenuation is not guaranteed, but usually follows after 8 to 20 calf passages (Bock et al., 2008). The virulence of *B. bigemina* decreases during prolonged residence of the parasite in latently infected animals. This feature has been used to obtain avirulent strains by infecting calves, splenectomising them 6–12 weeks after inoculation and then using the ensuing relapse parasites to repeat the procedure (Bock et al., 2008). Attenuation of *B. divergens for Meriones* followed long-term maintenance in vitro (Zintl et al., 2003).

Attenuation of *Babesia* spp. with irradiation has been attempted, but the results were variable. Similarly, maintenance in vitro in modified media has been used experimentally.

Avirulent strains should be stored as stabilate for safety testing and for future use as master seed in the production of vaccine.

- Preparation and storage of master seed
  
  Avirulent strains are readily stored as frozen infected blood in liquid nitrogen or dry ice. Dimethyl sulphoxide (DMSO) and polyvinylpyrrolidone (PVP) MW 40,000 (Bock et al., 2008) are the recommended cryopreservatives, as they allow for intravenous administration after thawing of the master seed. A detailed account of the freezing technique using DMSO is reported elsewhere (Mellors et al., 1982). Briefly, it involves the following:

  Infected blood is collected and chilled to 4°C. Cold cryoprotectant (4M DMSO in PBS) is then added, while stirring slowly, to a final blood:cryoprotectant ratio of 1:1 (final concentration of DMSO is 2 M). This dilution procedure is carried out in an ice bath, and the diluted blood is dispensed into suitable containers (e.g. 5 ml cryovials), and frozen, as soon as possible, in the vapour phase of a liquid nitrogen container. The vials are stored in the liquid phase in a designated tank to prevent loss of viability and contamination. Stored in this way, master seed lots of *Babesia* spp. have been known to remain viable for 20 years.

Unlike DMSO, it has not been found necessary to work with stabilates containing PVP in an ice-bath (Standfast & Jorgensen, 1997). Pre- and post-thaw storage at room temperate have not affected infectivity. PVP is a complex polymer that does not permeate intact cell membranes. It has low toxicity for vertebrates and parasites and stabilate-containing PVP is infective when inoculated intravenously. PVP with a molecular weight of 40,000 is made up to a 20% solution with PBS and autoclaved to sterilise. Blood from an infected calf is slowly mixed with an equal volume of the 20% PVP in PBS solution to produce a final concentration of 10% PVP. The mixture is then dispensed into 5 ml cryovials, frozen in the vapour phase of liquid nitrogen by cooling at a rate of about 10°C per minute for 15 minutes and then stored in liquid nitrogen (Standfast & Jorgensen, 1997).
In-vitro cultures are based on the microaerophilous stationary phase method (Levy & Ristic, 1980). Blood infected with avirulent strains of B. bovis or B. bigemina is harvested from splenectomised calves and washed with VYM phosphate-buffered saline solution (Vega et al., 1985) to remove the plasma and buffy coat. The VYM solution is composed of CaCl₂·2H₂O (16.0 mg), KCl (400.0 mg), KH₂PO₄ (1415.4 mg), MgSO₄·7H₂O (154.0 mg), Na₂HPO₄·7H₂O (1450 mg), NaCl (7077.0 mg) and dextrose (20.5 g) in 1 litre of double-distilled deionised water containing 0.25 mM adenine and 0.50 mM guanosine. Individual 5% suspensions of infected and uninfected RBCs are then prepared in basic culture medium consisting of commercial M199 medium and normal bovine serum (60/40). The basic medium is supplemented with 18 mM HEPES (4-[2-Hydroxyethyl] piperazine-1-ethanolsulfonic acid), 10 mM NaHCO₃, 100 µg/ml streptomycin sulphate and 100 U/ml penicillin G. The parasitised and normal RBC suspensions are mixed (1/1), dispensed into culture flasks and incubated under an atmosphere of 90% N₂, 5% O₂ and 5% CO₂ at 37°C. After 8 to 10 subcultures in different size culture flasks, the final complete cultures are spun at 1200 g for 10 minutes at 4°C and the supernatant removed. Packed parasitised RBCs are gently mixed with an equal volume (1/1) of 20% PVP in VYM solution, and dispensed in 2 ml cryovials. The parasitised RBCs are frozen in the vapour phase of liquid nitrogen by cooling at a rate of about 10°C per minute for 15 minutes and then stored in liquid nitrogen (Standfast & Jorgensen, 1997). Normal blood from donor cattle, which is used as a source of serum and uninfected RBCs for culture medium, is defibrinated with glass balls. The RBCs are washed and stored for up to 3 weeks in VYM solution at 4°C and normal serum is stored frozen at –20°C until use.

- Preparation and storage of working seed

Working seed is prepared in the same way as master seed (Section C.2.a) using master seed as starting material.

- Validation of safety and efficacy of working seed

The suitability of a working seed is determined by repeatability of infectivity in splenectomised calves, or in initiating cultures, and its safety and efficacy in non-splenectomised cattle. Repeatability is determined by inoculating several susceptible splenectomised calves and monitoring parasite progression by stained blood smears. The prepatent period and parasite progression should be relatively consistent between calves to allow inoculations to be scheduled with a degree of certainty.

In-vitro prepared working seed vials are thawed by immersing in water preheated to 40°C and directly dispensed in culture media. The in-vitro multiplication process starts with a 5% RBC suspension, which is progressively increased up to 10%. Working seed is considered acceptable when continuous cultures derived from it achieve 8–12% of RBCs parasitised by morphologically normal merozoites/trophozoites after the third subculture and growth in an atmosphere of 5% CO₂ in air at 37°C.

The safety and efficacy of the vaccine strain is determined by inoculating suitable numbers of susceptible adult cattle with vaccine prepared from RBCs from a calf that has been inoculated with the strain, or from an in-vitro culture process. Safety can be judged by monitoring body temperature, parasitaemia in stained blood films, and PCV depression following vaccination. Efficacy is judged by monitoring the same parameters following the inoculation of the vaccinated cattle with a heterologous strain. The purity of the working seed is tested by monitoring the cattle used in the safety test for evidence of possible contaminants or by thorough testing of the calf from which the stabilate was produced (see Section C.2.b.iii). Bovine donors of uninfected blood used for in-vitro cultures are maintained in isolated pens and their health status thoroughly monitored.

b) Method of manufacture

i) Production of frozen vaccine concentrate

First, 5–10 ml quantities of working seed are rapidly thawed by immersing the vials in water preheated to 37°C. The thawed material is used as soon as possible to infect a susceptible, splenectomised calf (free of potential vaccine contaminants) by intravenous inoculation. If DMSO is used as the cryopreservative, the thawed working seed must be kept on ice and inoculated within 30 minutes of thawing.

Blood suitable for vaccine is obtained by monitoring films of jugular blood and collecting the required volume of blood when a suitable parasitaemia is reached. A parasitaemia of 3.5 × 10⁹/ml for B. bovis in jugular blood, or 3 × 10⁷/ml for B. bigemina, is usually adequate for production of frozen vaccine. If a suitable B. bovis parasitaemia is not obtained, passage of the strain by subinoculation of 100–800 ml of blood into a second splenectomised calf may be necessary. Passage of B. bigemina through spleenectomised calves is not recommended because of the potential for the attenuated strain to increase in virulence.

Blood from the infected donor calf is collected by jugular cannulation using preservative-free heparin as anticoagulant (5 IU heparin/ml blood).
In the laboratory, the parasitised blood is held at room temperature and mixed in equal volumes with 3M glycerol in PBS supplemented with 5 mM glucose (final concentration of glycerol in blood mixture is 1.5 M) held at 37°C. The mixture is then equilibrated at 37°C for 30 minutes, and dispensed into suitable containers (e.g. 5 ml cryovials). The vials are cooled at approximately 10°C/minute in the vapour phase of liquid nitrogen and, when frozen, stored in the liquid phase (Bock et al., 2008).

DMSO can be used as cryoprotectant in the place of glycerol. This is carried out in the same way as outlined for the preparation of master seed (Pipano, 1997).

When glycerolised frozen vaccine is diluted for use as vaccine, the diluent should be iso-osmotic and consist of PBS containing 1.5 M glycerol and 5 mM glucose. Similarly, the diluent used in vaccine cryopreserved with DMSO should be iso-osmotic, and should contain the same concentration of DMSO in PBS as the concentration of DMSO in the vaccine concentrate.

Frozen vaccine containing both *B. bovis* and *B. bigemina* can be prepared by mixing equal volumes of blood containing each of the parasites obtained from different donors (Mangold et al., 1996). A trivalent vaccine containing RBCs infected with *B. bovis*, *B. bigemina* and *Anaplasma centrale* is also made in Australia. RBCs from three donors (one for each parasite) are concentrated by centrifugation and mixed with glycerol solution to produce the trivalent concentrate, which is thawed and mixed with a diluent before use (Bock et al., 2008).

The recommended dose of vaccine after reconstitution and dilution ranges from 1 to 2 ml depending on local practices and requirements, but aims to deliver a minimum infective dose of parasites, based on the parasitaemia prior to freezing.

ii) **Production of chilled vaccine**

Infective material used in the production of chilled vaccine is obtained in the same way as for frozen vaccine, but should be issued and used as soon as possible after collection. If it is necessary to obtain the maximum number of doses per calf, the infective material can be diluted to provide the required number of parasites per dose (usually from 2.5 to 10 × 10⁶). A suitable diluent is 10% sterile bovine serum in a balanced salt solution containing the following ingredients per litre: NaCl (7.00 g), MgCl₂.6H₂O (0.34 g), glucose (1.00 g), Na₂HPO₄ (2.52 g), KH₂PO₄ (0.90 g), and NaHCO₃ (0.52 g).

*In-vitro* multiplication is carried out in 225 cm² tissue culture flasks, where 115 ml of complete culture medium is dispensed to achieve a depth of 5.0–5.2 mm. Ninety ml of supernatant is removed daily by fresh medium and 50–75% of parasitised RBCs are replaced every 48 hours by uninfected RBCs (subculture). Parasitised RBCs containing *Babesia* spp are harvested when the parasites show typical morphology and achieve the maximum parasitaemia still inside the RBCs. Ninety per cent of the basic medium from each flask is removed without disturbing the settled RBCs. The *Babesia*-parasitised RBCs, still suspended in the remaining medium, are then mixed 1:1 with balanced salt solution, dispensed into one bottle and refrigerated to 5°C until use. The suspensions of each *Babesia* species, both with a high concentration of parasites, are finally diluted with the same balanced salt solution enriched with 10% bovine serum to achieve a concentration of 10⁷ *B. bovis* and 10⁶ *B. bigemina* parasitised RBCs per 2 ml dose.

Where anaplasmosis is of concern, *Anaplasma centrale* may also be incorporated into the vaccine to make a trivalent vaccine effective against *B. bovis*, *B. bigemina* and *Anaplasma marginale*.

iii) **In-process control**

- **Sources and maintenance of vaccine donors**

A source of donors free of natural infections with *Babesia*, other tick-borne diseases, and other infectious agents transmissible with blood, should be identified. If a suitable source is not available, it may be necessary to breed donor calves under tick-free conditions specifically for the purpose.

Donor calves should be maintained under conditions that will prevent exposure to infectious diseases and to ticks and biting insects. In the absence of suitable facilities, the risk of contamination with the agents of infectious diseases present in the country involved should be estimated, and the benefits of local production of vaccine (as opposed to importation of a suitable product) should be weighed against the possible adverse consequences of spreading disease (Bock et al., 2008).

- **Surgery**

Calves to be used as vaccine donors should be splenectomised to allow maximum yield of parasites for production of vaccine. This is easier in calves less than 3 months of age and is best performed under general anaesthesia.

- **Screening of vaccine donors before inoculation**

Donor calves should be examined for agents of all blood-borne infections prevalent in the country, including *Babesia*, *Anaplasma*, *Theileria*, *Eperythrozoon* and *Trypanosoma*. This can be done by routine examination of stained blood films after splenectomy, and preferably also by serological testing pre- and post-quarantine. Calves showing evidence of natural infections with any of these agents should be rejected or have infections chemically sterilised. The absence of other infective agents...
endemic in the country should also be confirmed; this may include the agents of enzootic bovine leucosis, bovine immunodeficiency virus, bovine syncytial virus, infectious bovine rhinotracheitis, Akabane disease, Aino virus, ephemeral fever, bluetongue, foot and mouth disease, bluetongue, *Brucella abortus*, *Leptospira* spp., heartwater, Jembrana disease, Rift Valley fever, rabies, lumpy skin disease, contagious bovine pleuropneumonia and rinderpest. The test procedures will depend on the diseases prevalent in the country and the availability of tests, but should involve serology of paired sera and, in some cases, virus isolation or antigen or DNA detection (Bock et al., 2008; Pipano, 1997).

- **Monitoring of parasitaemias following inoculation**
  It is necessary to determine the concentration of parasites in blood collected for vaccine or in RBCs harvested from culture. There are accurate techniques for determining the parasite count (Anonymous, 1984), but the parasite concentration can be adequately estimated from the RBC count and the parasitaemia (% infected RBCs).

- **Collection of blood for vaccine**
  All equipment should be sterilised before use (e.g. by autoclaving). The blood is collected in heparin using strict aseptic techniques when the required parasitaemia is reached. This is best done if the calf is sedated (for example, with xylazine) and with the use of a closed-circuit collection system. Up to 3 litres of heavily infected blood can be collected from a 6-month-old calf. If the calf is to live, the transfusion of a similar amount of blood from a suitable donor (or blood previously collected from the donor itself) is indicated. Alternatively, the calf should be killed immediately after collection of the blood. Using *in-vitro* culture, a 225 cm$^2$ flask can routinely provide 1800 doses. Starting from one 225 cm$^2$ flask containing 11 ml of 8–10% parasitised RBCs, it is possible to harvest about 45,000 doses after 6 days of continuous growth.

- **Dispensing of vaccine**
  All procedures are performed in a suitable environment, such as a laminar flow cabinet, using standard sterile techniques. Use of a mechanical or magnetic stirrer will ensure thorough mixing of infected RBCs and diluent throughout the dispensing process.

- **Batch control**
  The potency, safety and sterility of vaccine batches cannot be determined in the case of chilled vaccine, except by thorough testing of vaccine donors and adherence to the principles of a code of good manufacturing practice. Specifications of frozen vaccine depend on the code of practice of the country involved. The following are the specifications for frozen vaccine produced in Australia.

- **Sterility and freedom from contaminants**
  Standard tests for sterility are employed for each batch of vaccine and diluent. The absence of contaminants is determined by doing appropriate serological and molecular diagnostic testing of donor cattle for evidence of viral and bacterial infection. Potential contaminants include those agents listed in Section C.2.iii.

- **Safety**
  Vaccine reactions of the cattle inoculated in the test for potency may be monitored by measuring parasitaemia, temperature and depression of packed cell volume; or based on regular observation of the health and demeanour of vaccinated animals. Detailed monitoring is more usually associated with the development and testing of parasite strains as potential candidates for vaccine production. Only batches with pathogenicity levels equal to or lower than a predetermined standard are released for use. Vaccine is preferably used in calves less than 1 year of age. Non-target species are of no concern. Some attenuated vaccine strains of *B. bovis* are tick transmissible and evidence suggests that they may return to virulence after transmission by ticks. This is of little consequence in endemic situations.
  
No withholding periods for milk or meat are necessary following use of the vaccine.

- **Potency**
  Frozen, glycerolised vaccine concentrate is thawed and diluted 1/10 with isotonic diluent (Bock et al., 2008; Pipano, 1997). The prepared vaccine is then stored for 8 hours at 4°C, and 5 to 25 susceptible cattle (held in cattle tick-free areas) are each inoculated subcutaneously with a 2 ml dose of that vaccine batch. The inoculated cattle are then monitored for the presence of viable *Babesia* spp infections by examination of stained blood smears, by PCR techniques or by evidence of seroconversion. Only batches with acceptable infectivity are released for use at a working dilution of 1/10. Greater than 95% of vaccinated cattle would be expected to develop immunity to *Babesia* spp.
Chapter 2.4.2. – Bovine babesiosis

after a single inoculation with an adequate dose \((1 \times 10^7\) parasites\) in a chilled or frozen vaccine prepared, stored and transported according to appropriate protocols.

- **Duration of immunity**
  Long-lasting immunity usually results from one inoculation. Protective immunity develops in 3–4 weeks, and lasts at least 4 years in most cases (Bock & de Vos, 2001). Evidence of \(B.\) bovis vaccine failures have been reported and are related to the choice of vaccine strain, the presence of heterologous field strains and host factors (Bock et al., 2008). There is little evidence of time-related waning of immunity (Bock & de Vos, 2001).

- **Stability**
  When stored in liquid nitrogen, the frozen vaccine can be kept for 5 years. Sterile diluent can be kept for 2 years in a refrigerator. Thawed vaccine rapidly loses potency and cannot be refrozen.

- **Preservatives**
  Benzylpenicillin (500,000 IU/litre) and streptomycin (370,000 µg/litre) are added to the vaccine concentrate prior to dispensing into cryotubes. No preservative is used.

- **Use of vaccine**
  In the case of frozen vaccine, vials should be thawed by immersion in water preheated to 37°C. Glycerolised vaccine should be kept cool and used within 8 hours (Bock et al., 2008), while vaccine with DMSO as cryoprotectant should be kept on ice and used within 15–30 minutes of thawing (Pipano, 1997).
  Chilled vaccine should be kept refrigerated and used within 4–7 days of preparation, depending on the viability of the parasites and the recommendation of the vaccine production facility.
  The strains of \(B.\) bovis, \(B.\) divergens and \(B.\) bigemina used in the vaccine may be of reduced virulence, but may not be entirely safe. A practical recommendation is therefore to limit the use of vaccine to calves under the age of 1 year, when nonspecific immunity will minimise the risk of vaccine reactions. If older animals are to be vaccinated, there is a greater risk of vaccine reactions. These reactions occur infrequently, but valuable breeding stock or pregnant animals warrant due attention and should be observed daily for 3 weeks after vaccination. Ideally, rectal temperatures of vaccinated cattle should be taken and the animals should be treated if significant fever develops. Reactions to \(B.\) bigemina and \(B.\) divergens are usually seen by day 6–8 and those to \(B.\) bovis by day 14–18 (Bock et al., 2008).
  Babesiosis and anaplasmosis vaccines are often used concurrently, but it is preferable not to use any other vaccines at the same time (Bock et al., 2008).

- **Precautions**
  \(Babesia\) bovis and \(B.\) bigemina vaccines are not infective for humans. However, cases of \(B.\) divergens have been reported in splenectomised individuals. When the vaccine is stored in liquid nitrogen, the usual precautions pertaining to the storage, transportation and handling of liquid nitrogen and deep-frozen material applies.

  **c) Requirements for authorisation**
  Issues of safety, potency, stability of vaccine strains, non-target species and reversion to virulence are dealt with in preceding sections. The vaccine is only used to control babesiosis. Eradication of babesiosis is only undertaken through eradication of the tick vector and/or intensive chemotherapeutic regimes.

3. Vaccines based on biotechnology

No biotechnology-based vaccines are currently available.

**REFERENCES**


Chapter 2.4.2. — Bovine babesiosis


Chapter 2.4.2. — Bovine babesiosis


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NB: There are OIE Reference Laboratories for Bovine babesiosis (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for bovine babesiosis.
CHAPTER 2.4.3.

BOVINE BRUCELLOSIS

SUMMARY

Bovine brucellosis is usually caused by Brucella abortus, less frequently by B. melitensis, and occasionally by B. suis. Infection is widespread globally. Several countries in Northern and Central Europe, Canada, Japan, Australia and New Zealand are believed to be free from the agent.

Clinically, the disease is characterised by one or more of the following signs: abortion, retained placenta, orchitis, epididymitis and, rarely, arthritis, with excretion of the organisms in uterine discharges and in milk. Diagnosis depends on the isolation of Brucella from abortion material, udder secretions or from tissues removed at post-mortem. Presumptive diagnosis can be made by assessing specific cell-mediated or serological responses to Brucella antigens.

Brucella abortus, B. melitensis and B. suis are highly pathogenic for humans, and all infected tissues, cultures and potentially contaminated materials must be handled under appropriate containment conditions.

Identification of the agent: Presumptive evidence of Brucella is provided by the demonstration, by modified acid-fast staining of organisms, of Brucella morphology in abortion material or vaginal discharge, especially if supported by serological tests. The polymerase chain reaction methods provide additional means of detection. Whenever possible, Brucella spp. should be isolated using plain or selective media by culture from uterine discharges, aborted fetuses, udder secretions or selected tissues, such as lymph nodes and male and female reproductive organs. Species and biovars should be identified by phage lysis, and by cultural, biochemical and serological criteria. Polymerase chain reaction (PCR) can provide both a complementary and biotyping method based on specific genomic sequences.

Serological and allergic skin tests: The buffered Brucella antigen tests, i.e. Rose Bengal test and buffered plate agglutination test, the complement fixation test, the enzyme-linked immunosorbent assay (ELISA) or the fluorescence polarisation assay, are suitable tests for screening herds and individual animals. However, no single serological test is appropriate in each and all epidemiological situations. Therefore, the reactivity of samples that are positive in screening tests should be assessed using an established confirmatory and/or complementary strategy. The indirect ELISA or milk ring test performed on bulk milk samples are effective for screening and monitoring dairy cattle for brucellosis, but the milk ring test is less reliable in large herds. Another immunological test is the brucellin skin test, which can be used as a screening or as a confirmatory herd test when positive serological reactors occur in the absence of obvious risk factors in unvaccinated herds.

Requirements for vaccines and diagnostic biologicals: Brucella abortus strain 19 remains the reference vaccine to which any other vaccines are compared. It should be prepared from US-derived seed cultures with adequate residual virulence and immunogenicity to protect mice against challenge with a virulent strain of B. abortus. Moreover each batch must conform to minimum standards for viability, smoothness, and designated CFU (colony-forming units) per dose. Brucella abortus strain RB51 vaccine was produced from a laboratory-derived rough mutant of smooth B. abortus strain 2308. It has become the official vaccine for prevention of brucellosis in cattle in some countries. Brucellin preparations for the intradermal test must be free of smooth lipopolysaccharide and must not produce nonspecific inflammatory reactions or interfere with serological tests. Diagnostic antigens must be prepared from smooth strains of B. abortus, strain 1119-3 or strain 99 and comply with minimum standards for purity, sensitivity and specificity.
A. INTRODUCTION

Brucellosis in cattle is usually caused by biovars of *Brucella abortus*. In some countries, particularly in southern Europe and western Asia, where cattle are kept in close association with sheep or goats, infection can also be caused by *B. melitensis* (Jimenez et al., 1991; Verger, 1985). Occasionally, *B. suis* may cause a chronic infection in the mammary gland of cattle, but it has not been reported to cause abortion or spread to other animals (Ewalt et al., 1997). The disease is usually asymptomatic in nonpregnant females. Following infection with *B. abortus* or *B. melitensis*, pregnant adult females develop a placentaitis usually resulting in abortion between the fifth and ninth month of pregnancy. Even in the absence of abortion, profuse excretion of the organism occurs in the placenta, fetal fluids and vaginal discharges. The mammary gland and associated lymph nodes may also be infected, and organisms may be excreted in the milk. Subsequent pregnancies are usually carried to term, but uterine and mammary infection recurs, with reduced numbers of organisms in cyctic products and milk. In acute infections, the organism is present in most major body lymph nodes. Adult male cattle may develop orchitis and brucellosis may be a cause of infertility in both sexes. Hygromas, usually involving leg joints, are a common manifestation of brucellosis in some tropical countries and may be the only obvious indicator of infection; the hygroma fluid is often infected with *Brucella*.

Brucellosis has been reported in the one-humped camel (*Camelus dromedarius*) and in the two-humped camel (*C. bactrianus*), and in the South American cameldids, llama (*Lama glama*), alpaca (*L. pacos*), guanaco (*L. guinicoe*), and vicuna (*Vicugne vicugne*) related to contact with large and small ruminants infected with *B. abortus* or *B. melitensis*. In addition, brucellosis has been observed in the domestic buffalo (*Bubalus bubalus*), American and European bison (*Bison bison, Bison bonasus*), yak (*Bos grunniens*), elk/wapiti (*Cervus elaphus*) and also occurs in the African buffalo (*Syncerus caffer*) and various African antelope species. The clinical manifestations of brucellosis in these animals are similar to those in cattle.

The World Health Organization (WHO) laboratory biosafety manual classifies *Brucella* in Risk group III. Brucellosis is readily transmissible to humans, causing acute febrile illness – undulant fever – which may progress to a more chronic form and can also produce serious complications affecting the musculo–skeletal, cardiovascular, and central nervous systems. Precautions should be taken to prevent human infection. Infection is often due to occupational exposure and is essentially acquired by the oral, respiratory, or conjunctival routes, but ingestion of dairy products constitutes the main risk to the general public where the disease is endemic. There is an occupational risk to veterinarians and farmers who handle infected animals and aborted fetuses or placentas. Brucellosis is one of the most easily acquired laboratory infections, and strict safety precautions should be observed when handling cultures and heavily infected samples, such as products of abortion. Specific recommendations have been made for the biosafety precautions to be observed with *Brucella*-infected materials (for further details see Alton et al., 1988; Joint FAQ/WHO Expert Committee on Brucellosis, 1986; WHO, 2004 and Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities). Laboratory manipulation of live cultures or contaminated material from infected animals is hazardous and must be done under containment level 3 or higher, as outlined in chapter 1.1.3, to minimise occupational exposure. Where large-scale culture of *Brucella* is carried out (e.g. for antigen or vaccine production) then biosafety level 3 is essential.

Genetic and immunological evidence indicates that all members of the *Brucella* genus are closely related. Nevertheless, based on relevant differences in host preference and epidemiology displayed by the major variants, as well as molecular evidence of genomic variation, the International Committee on Systematics of Prokaryotes, Subcommittee on the Taxonomy of *Brucella* took a clear position in 2005 on a return to pre-1986 *Brucella* taxonomic opinion; the consequences of this statement imply the re-approval of the six *Brucella* nomenspecies with recognised biovars. The classical names related to the six *Brucella* nomenspecies are validly published in the Approved Lists of Bacterial Names, 1980, and the designated type strains are attached to these validly published names: *Brucella abortus*, *B. melitensis*, *B. suis*, *B. neotomae*, *B. ovis* and *B. canis* (http://www.the-icsp.org/subcoms/Brucella.htm). The first three of these are subdivided into biovars based on cultural and serological properties (see Tables 1 and 2). Strains of *Brucella* have been isolated in the last decade from marine mammals that cannot be ascribed to any of the above-recognised species. Investigations are continuing to establish their correct position in the taxonomy of that genus and it is proposed that they could be classified into two new species, *B. ceti* and *B. pinnipedialis* (Foster et al., 2007). A new strain, named *Brucella microti*, was recently isolated from the common vole (*Microtus arvalis*) in Central Europe (Scholz et al., 2008a; 2008b). Finally, *Brucella* shows close genetic relatedness to some plant pathogens and symbionts of the genera *Agrobacterium* and *Rhizobium*, as well as, animal pathogens (*Bartonella*) and opportunistic or soil bacteria (*Ochrobactrum*).
Table 1. Differential characteristics of species of the genus Brucella

<table>
<thead>
<tr>
<th>Species</th>
<th>Colony morphology</th>
<th>Serum requirement</th>
<th>RTD&lt;sup&gt;c&lt;/sup&gt;</th>
<th>10&lt;sup&gt;4&lt;/sup&gt;RTD</th>
<th>RTD</th>
<th>RTD</th>
<th>RTD</th>
<th>Oxidase</th>
<th>Urease activity</th>
<th>Preferred host</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tb</td>
<td>Wb</td>
<td>Iz&lt;sub&gt;1&lt;/sub&gt;</td>
<td>R/C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. abortus</em></td>
<td>S</td>
<td>–&lt;sup&gt;d&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+&lt;sup&gt;e&lt;/sup&gt;</td>
<td>+&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Cattle and other Bovidae</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>Biovar 1: swine</td>
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<td></td>
<td></td>
<td></td>
<td>Biovar 2: swine, hare</td>
<td></td>
</tr>
<tr>
<td><em>B. suis</em></td>
<td>S</td>
<td>–</td>
<td>–&lt;sup&gt;k&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+&lt;sup&gt;h&lt;/sup&gt;</td>
<td></td>
<td>+</td>
<td>Biovar 3: swine</td>
<td></td>
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<td>Biovar 4: reindeer</td>
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<td></td>
<td>Biovar 5: wild rodents</td>
<td></td>
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<tr>
<td><em>B. melitensis</em></td>
<td>S</td>
<td>–</td>
<td>–&lt;sup&gt;i&lt;/sup&gt;</td>
<td>+&lt;sup&gt;g&lt;/sup&gt;</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+&lt;sup&gt;j&lt;/sup&gt;</td>
<td>Sheep and goats</td>
<td></td>
</tr>
<tr>
<td><em>B. neotomae</em></td>
<td>S</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Desert wood rat&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
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</tr>
<tr>
<td><em>B. ovis</em></td>
<td>R</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>Rams</td>
<td></td>
</tr>
<tr>
<td><em>B. canis</em></td>
<td>R</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Dogs</td>
<td></td>
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<tr>
<td><em>B. ceti</em></td>
<td>S</td>
<td>+&lt;sup&gt;m&lt;/sup&gt;</td>
<td>+&lt;sup&gt;n&lt;/sup&gt;</td>
<td>+&lt;sup&gt;o&lt;/sup&gt;</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Cetaceans</td>
<td></td>
</tr>
<tr>
<td><em>B. pinnipedialis</em></td>
<td>S</td>
<td>+&lt;sup&gt;m&lt;/sup&gt;</td>
<td>+&lt;sup&gt;n&lt;/sup&gt;</td>
<td>+&lt;sup&gt;o&lt;/sup&gt;</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Pinnipeds</td>
<td></td>
</tr>
<tr>
<td><em>B. microti</em></td>
<td>S</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>Common vole</td>
<td></td>
</tr>
</tbody>
</table>


- a Phages: Tbilisi (Tb), Weybridge (Wb), Izatagar1 (Iz<sub>1</sub>) and R/C
- b Normally occurring phase: S: smooth, R: rough
- c RTD: routine test dilution
- d *B. abortus* biovar 2 generally requires serum for growth on primary isolation
- e Some African isolates of *B. abortus* biovar 3 are negative
- f Intermediate rate, except strain 544 and some field strains that are negative
- g Some isolates of *B. suis* biovar 2 are not or partially lysed by phage Wb or Iz<sub>1</sub>
- h Rapid rate
- i Some isolates are lysed by phage Wb
- j Slow rate, except some strains that are rapid
- k Minute plaques
- l *Neotoma lepida*
- m Some isolates are lysed by Tb
- n Most isolates are lysed by Wb
- o Most isolates are lysed by Iz
**Table 2. Differential characteristics of the biovars of Brucella species**

<table>
<thead>
<tr>
<th>Species</th>
<th>Biovar</th>
<th>CO₂ requirement</th>
<th>H₂S production</th>
<th>Growth on dyes&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Agglutination with monospecific sera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Thionin</td>
<td>Basic fuchsin</td>
</tr>
<tr>
<td>B. melitensis</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>3</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. abortus</td>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. suis</td>
<td>4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>–</td>
<td>+&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–</td>
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<tr>
<td></td>
<td>5</td>
<td>–</td>
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<td>+</td>
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<td>9</td>
<td>+ or –</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. neotomae</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–&lt;sup&gt;f&lt;/sup&gt;</td>
<td>–</td>
</tr>
<tr>
<td>B. ovis</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+&lt;sup&gt;e&lt;/sup&gt;</td>
<td>–</td>
</tr>
<tr>
<td>B. canis</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+&lt;sup&gt;e&lt;/sup&gt;</td>
<td>–</td>
</tr>
<tr>
<td>B. ceti</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. pinnipedialis</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>B. microti</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>


- **a** Dye concentration in serum dextrose medium: 20 µg/ml
- **b** Usually positive on primary isolation
- **c** Some basic fuchsin-sensitive strains have been isolated
- **d** Some basic fuchsin-resistant strains have been isolated
- **e** Negative for most strains
- **f** Growth at a concentration of 10 µg/ml thionin
B. DIAGNOSTIC TECHNIQUES

All abortions in cattle in late gestation, starting from the fifth month, should be treated as suspected brucellosis and should be investigated. The clinical picture is not pathognomonic, although the herd history may be helpful. Unequivocal diagnosis of *Brucella* infections can be made only by the isolation and identification of *Brucella*, but in situations where bacteriological examination is not practicable, diagnosis must be based on serological methods. There is no single test by which a bacterium can be identified as *Brucella*. A combination of growth characteristics, serological, bacteriological and/or molecular methods is usually needed.


a) **Staining methods**

*Brucella* are coccobacilli or short rods measuring from 0.6 to 1.5 µm long and from 0.5 to 0.7 µm wide. They are usually arranged singly, and less frequently in pairs or small groups. The morphology of *Brucella* is fairly constant, except in old cultures where pleomorphic forms may be evident. *Brucella* are nonmotile. They do not form spores, and flagella, pili, or true capsules are not produced. *Brucella* are Gram negative and usually do not show bipolar staining. They are not truly acid-fast, but are resistant to decolorisation by weak acids and thus stain red by the Stamp's modification of the Ziehl–Neelsen's method. This is the usual procedure for the examination of smears of organs or biological fluids that have been previously fixed with heat or ethanol, and by this method, *Brucella* organisms stain red against a blue background. A fluorochrome or peroxidase-labelled antibody conjugate based technique could also be used (Roop *et al.*, 1987). The presence of intracellular, weakly acid-fast organisms of *Brucella* morphology or immuno-specifically stained organisms is presumptive evidence of brucellosis. However, these methods have a low sensitivity in milk and dairy products where *Brucella* are often present in small numbers, and interpretation is frequently impeded by the presence of fat globules. Care must be taken as well in the interpretation of positive results in the Stamps's method because other organisms that cause abortions, e.g. *Chlamydothila abortus* (formerly *Chlamydia psittaci*) or *Coxiella burnetii*, are difficult to differentiate from *Brucella* organisms. The results, whether positive or negative, should be confirmed by culture.

DNA probes or polymerase chain reaction (PCR) methods can be used also to demonstrate the agent in various biological samples (Bricker, 2002).

b) **Culture**

i) **Basal media**

Direct isolation and culture of *Brucella* are usually performed on solid media. This is generally the most satisfactory method as it enables the developing colonies to be isolated and recognised clearly. Such media also limit the establishment of non-smooth mutants and excessive development of contaminants. However, the use of liquid media may be recommended for voluminous samples or for enrichment purpose. A wide range of commercial dehydrated basal media is available, e.g. *Brucella* medium base, tryptose (or trypticase)–soy agar (TSA). The addition of 2–5% bovine or equine serum is necessary for the growth of strains such as *B. abortus* biovar 2, and many laboratories systematically add serum to basal media, such as blood agar base (Oxoid) or Columbia agar (BioMérieux), with excellent results. Other satisfactory media, such as serum–dextrose agar (SDA) or glycerol dextrose agar, can be used (Alton *et al.*, 1986). SDA is usually preferred for observation of colonial morphology. A nonselective, biphasic medium, known as Castañeda’s medium, is recommended for the isolation of *Brucella* from blood and other body fluids or milk, where enrichment culture is usually advised. Castañeda’s medium is used because brucellae tend to dissociate in broth medium, and this interferes with biotyping by conventional bacteriological techniques.

ii) **Selective media**

All the basal media mentioned above can be used for the preparation of selective media. Appropriate antibiotics are added to suppress the growth of organisms other than *Brucella*. The most widely used selective medium is the Farrell’s medium (Farrell, 1974), which is prepared by the addition of six antibiotics to a basal medium. The following quantities are added to 1 litre of agar: polymyxin B sulphate (5000 units = 5 mg); bacitracin (25,000 units = 25 mg); natamycin (50 mg); nalidixic acid (5 mg); nystatin (100,000 units); vancomycin (20 mg).

A freeze-dried antibiotic supplement is available commercially (Oxoid). However, nalidixic acid and bacitracin, at the concentration used in Farrell’s medium, have inhibitory effects on some *B. abortus* and *B. melitensis* strains (Maring *et al.*, 1996). Therefore the sensitivity of culture increases significantly by the simultaneous use of both Farrell’s and the modified Thayer–Martin medium. Briefly, the modified Thayer–Martin’s medium can be prepared with GC medium base (38 g/litre; Biolife Laboratories, Milan, Italy) supplemented with haemoglobin (10 g/litre; Difco) and colistin methanesulphonate (7.5 mg/litre).
vancomycin (3 mg/litre), nitrofurantoin (10 mg/litre), nystatin (100,000 International Units [IU]/litre = 17.7 mg) and amphotericin B (2.5 mg/litre) (all products from Sigma Chemical, St Louis, United States of America [USA]) (Marin et al., 1996). Contrary to several biovars of B. abortus, growth of B. melitensis is not dependent on an atmosphere of 5–10% CO₂ (Table 2).

As the number of Brucella organisms is likely to be lower in milk, colostrum and some tissue samples than in abortion material, enrichment is advisable. In the case of milk, results are also improved by centrifugation and culture from the cream and the pellet, but strict safety measures should be implemented in this case to avoid aerosols. Enrichment can be carried out in liquid medium consisting of serum–dextrose broth, tryptose broth (or trypticase)–soy broth (TSA) or Brucella broth supplemented with an antibiotic mixture of at least amphotericin B (1 µg/ml), and vancomycin (20 µg/ml) (all final concentrations). The enrichment medium should be incubated at 37°C in air supplemented with 5–10% (v/v) CO₂ for up to 6 weeks, with weekly subcultures on to solid selective medium. If preferred, a biphasic system of solid and liquid selective medium in the same bottle (Castañeda’s method) may be used to minimise subculture. A selective biphasic medium composed of the basal Castañeda’s medium with the addition of the following antibiotics to the liquid phase, is sometimes recommended for isolation of Brucella in milk (quantities are per litre of medium): polymyxin B (sulphate) (6000 units = 6 mg); bacitracin (25,000 units = 25 mg); natamycin (50 mg); nalidixic acid (5 mg); amphotericin B (1 mg); vancomycin (20 mg); D-cycloserine (100 mg).

All culture media should be subject to quality control and should support the growth of Brucella strains from small inocula or fastidious strains, such as B. abortus biovar 2.

On suitable solid media, Brucella colonies can be visible after a 2–3-day incubation period. After 4 days’ incubation, Brucella colonies are round, 1–2 mm in diameter, with smooth margins. They are translucent and a pale honey colour when plates are viewed in the daylight through a transparent medium. When viewed from above, colonies appear convex and pearly white. Later, colonies become larger and slightly darker.

Smooth (S) Brucella cultures have a tendency to undergo variation during growth, especially with subcultures, and to dissociate to rough (R) forms. Colonies are then much less transparent, have a more granular, dull surface, and range in colour from matt white to brown in reflected or transmitted light. Checking for dissociation is easily tested by crystal violet staining: rough colonies stain red/violet and smooth colonies do not uptake dye or stain pale yellow. If the colonies are smooth, they should be checked against antisera to smooth B. abortus, or preferably against anti-A and -M monospecific sera. In the case of non-smooth colonies, isolates should be checked with antisera to Brucella R antigen. Changes in the colonial morphology are generally associated with changes in virulence, serological properties and/or phage sensitivity. Typical colonial morphology and positive agglutination with a Brucella antiserum provide presumptive identification of the isolate as Brucella. Subsequent full identification is best performed by a reference laboratory.

### iii) Collection and culture of samples

For the diagnosis of animal brucellosis by cultural examination, the choice of samples usually depends on the clinical signs observed. The most valuable samples include aborted fetuses (stomach contents, spleen and lung), fetal membranes, vaginal secretions (swabs), milk, semen and arthritis or hygroma fluids. From animal carcasses, the preferred tissues for culture are those of the reticulo-endothelial system (i.e. head, mammary and genital lymph nodes and spleen), the late pregnant or early post-parturient uterus, and the udder. Growth normally appears after 3–4 days, but cultures should not be discarded as negative until 8–10 days have elapsed.

**Tissues:** Samples are removed aseptically with sterile instruments. The tissue samples are prepared by removal of extraneous material (e.g. fat), cut into small pieces, and macerated using a ‘Stomacher’ or tissue grinder with a small amount of sterile phosphate buffered saline (PBS), before being inoculated on to solid media.

**Vaginal discharge:** A vaginal swab taken after abortion or parturition is an excellent source for the recovery of Brucella and far less risky for the personnel than abortion material. The swab is then streaked on to solid media.

**Milk:** Samples of milk must be collected cleanly after washing and drying the whole udder and disinfecting the teats. It is essential that samples should contain milk from all quarters, and 10–20 ml of milk should be taken from each teat. The first streams are discarded and the sample is milked directly into a sterile vessel. Care must be taken to avoid contact between the milk and the milker’s hands. The milk is centrifuged in conditions that avoid the risk of aerosol contamination to personnel, and the cream and deposit are spread on solid selective medium, either separately or mixed. If brucelae are present in bulk milk samples, their numbers are usually low, and isolation from such samples is very unlikely.

**Dairy products:** Dairy products, such as cheeses, should be cultured on the media described above. As these materials are likely to contain small numbers of organisms, enrichment culture is advised. Samples need to be carefully homogenised before culture, after they have been ground in a tissue grinder or macerated and pounded in a ‘Stomacher’ or an electric blender with an appropriate volume.
of sterile PBS. Superficial strata (rind and underlying parts) and the core of the product should be cultured. As brucellae grow, survive or disappear quite rapidly, their distribution throughout the different parts of the product varies according to the local physico-chemical conditions linked to specific process technologies.

All samples should be cooled immediately after they are taken, and transported to the laboratory in the most rapid way. On arrival at the laboratory, milk and tissue samples should be frozen if they are not to be cultured immediately.

Use of laboratory animals should be avoided unless absolutely necessary, but may sometimes provide the only means of detecting the presence of Brucella, especially when samples have been shown to be heavily contaminated or likely to contain a low number of Brucella organisms. Animal inoculation may be either subcutaneously or through abraded skin in guinea-pigs or, preferably, intravenously or intraperitoneally in mice. This work must be carried out under appropriate biosafety conditions as outlined in chapter 1.1.3. The spleens of mice are cultured 7 days after inoculation and, for guinea-pigs, a serum sample is subjected to specific tests 3 and 6 weeks after inoculation, then the spleens are cultured.

c) Identification and typing

Any colonies of Brucella morphology should be checked using a Gram-stained (or a Stamp-stained) smear. As the serological properties, dyes and phage sensitivity are usually altered in the non-smooth phases, attention to the colonial morphology is essential in the typing tests described below. The recommended methods for observing colonial morphology are Henry's method by obliquely reflected light, the acriflavine test described by Braun & Bonestell, or White & Wilson's crystal violet method of staining colonies (Alton et al., 1988).

Identification of Brucella organisms can be carried out by a combination of the following tests: organism morphology after Gram or Stamp’s staining, colonial morphology, growth characteristics, urease, oxidase and catalase tests, and the slide agglutination test with an anti-Brucella polyclonal serum. Species and biovar identification requires elaborate tests (such as phage lysis and agglutination with anti-A, -M or -R monospecific sera), the performance of which is left to reference laboratories with expertise in these methods. The simultaneous use of several phages e.g. Tbilissi (Tb), Weybridge (Wb), Izatnagar (Iz) and R/C provides a phage-typing system that, in experienced hands, allows a practical identification of smooth and non-smooth phases of Brucella. However, several characteristics, for example added CO₂ requirement for growth, production of H₂S (detected by lead acetate papers), and growth in the presence of basic fuchsin and thionin at final concentrations of 20 µg/ml, are revealed by routine tests that can be performed in moderately equipped nonspecialised laboratories (see Tables 1 and 2).

When sending Brucella strains to a reference laboratory for typing, it is essential that smooth colonies be selected. Cultures should be lyophilised and sealed in ampoules packed in screw-capped canisters or subcultured on to appropriate nutrient agar slopes contained in screw-capped bottles. The strains could also be sent suspended in transport media (e.g. Amies), but this could provide an opportunity for the establishment of rough mutants.

i) Brucella organisms are among the most dangerous bacteria with which to work in terms of the risk of producing laboratory-acquired infections. For transporting Brucella cultures, the caps of the bottles or canisters should be screwed tightly down and sealed with PVC tapes. Bottles should be wrapped in absorbent paper or cotton wool, sealed in polyethylene bags and packed into a rigid container in accordance with the requirements of the International Air Transport Association (IATA) for shipping dangerous goods (IATA, 2006). These regulations are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens, and they must be followed. As Brucella cultures are infectious agents, they are designated UN2814 and a Declaration of Dangerous Goods must be completed. There are also restrictions on submitting samples from suspected cases of brucellosis and the IATA regulations should be reviewed before sending samples (IATA, 2006). Other international and national guidelines should also be followed (WHO, 2005).

ii) Before dispatching cultures or diagnostic samples for culture, the receiving laboratory should be contacted to determine if a special permit is needed and if the laboratory has the capability to do the testing requested. If samples are to be sent across national boundaries, an import licence will probably be needed and should be obtained before the samples are dispatched (chapter 1.1.1).

d) Nucleic acid recognition methods

The PCR, including the real-time format, provides an additional means of detection and identification of Brucella sp. (Bricker, 2002; Bricker et al., 2003; Bricker & Halling, 1994; 1995; Garcia-Yoldi et al., 2006; Hinić et al., 2008; Ocampo-Sosa et al., 2005). Despite the high degree of DNA homology within the genus Brucella, several molecular methods, including PCR, PCR restriction fragment length polymorphism (RFLP)
and Southern blot, have been developed that allow, to a certain extent, differentiation between Brucella species and some of their biovars (for a review see Bricker, 2002 and Moreno et al., 2002). Pulse-field gel electrophoresis has been developed that allows the differentiation of several Brucella species (Jensen et al., 1999; Michaux-Charachon et al., 1997). Brucella biotyping and distinguishing vaccine strains by PCR can be accomplished satisfactorily but there has been limited validation of the PCR for primary diagnosis.

The first species-specific multiplex PCR assay for the differentiation of Brucella was described by Bricker & Halling (1994). The assay, named AMOS-PCR, was based on the polymorphism arising from species-specific localisation of the insertion sequence IS711 in the Brucella chromosome, and comprised five oligonucleotide primers that can identify without differentiating B. abortus, biovars 1, 2 and 4 but could not identify B. abortus biovars 3, 5, 6 and 9. Modifications to the assay have been introduced over time to improve performance, and additional strain-specific primers were incorporated for identification of the B. abortus vaccine strains, and other biovars and species (Bricker et al., 2003; Bricker & Halling, 1995; Ewalt & Bricker, 2000; 2003; Ocampo-Sosa et al., 2005).

A new multiplex PCR assay (Bruce-ladder) has been proposed for rapid and simple one-step identification of Brucella (Garcia-Yoldi et al., 2006). The major advantage of this assay over previously described PCRs is that it can identify and differentiate in a single step most Brucella species as well as the vaccine strains B. abortus S19, B. abortus RB51 and B melitensis Rev.1. In contrast to other PCRs, Bruce-ladder is able to detect also DNA from B. neotomae, B pinnipedialis and B ceti. In addition, B abortus biovars 3, 5, 6, 7, 9, and B. suis biovars 2, 3, 4, 5 can be identified by this new multiplex PCR. The only minor inconvenience of the Bruce-ladder is that some B canis strains can be identified erroneously as B suis (MacMillan & Cockrem, 1985). Further, this assay cannot positively identify the new B. microti species.

- **Test procedure (Bruce-ladder multiplex PCR)**
  i)  **Brucella DNA preparation**
  Prepare bacteria from agar plates: with a sterile inoculating loop, transfer bacteria from one colony to 200 µl of saline. Extract the bacterial DNA by boiling for 10 minutes and, after centrifugation (12,000 g for 20 seconds), use 1.0 µl of the supernatant as a DNA template for PCR amplification (between 0.1 and 0.05 µg/µl of DNA, approximately).
  ii)  **Bruce-ladder PCR mix preparation (per one reaction, final volume of 25 µl)**
<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR buffer 10×</td>
<td>1×</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>dNTPs (2 mM)</td>
<td>400 µM each one</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>Mg²⁺ (50 mM)</td>
<td>3.0 mM</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>Bruce-ladder eight pair primer cocktail (12.5 µM)</td>
<td>6.25 pmol each one</td>
<td>7.6 µl</td>
</tr>
<tr>
<td>H₂O (PCR-grade)</td>
<td>–</td>
<td>7.1 µl</td>
</tr>
<tr>
<td>DNA polymerase*</td>
<td>1.5 U</td>
<td>0.3 µl</td>
</tr>
</tbody>
</table>

  *As this assay is a multiplex PCR with eight pairs of primers in the same tube reaction, best results are obtained when high quality DNA polymerase is used (for instance, Immolase DNA polymerase [Bioline], Titanium Taq DNA polymerase [Clontech], or PFU DNA polymerase [Biotools B&M Labs.]).

  **NOTE**: include always a negative control without DNA and a positive control with B. suis DNA

  Add 1.0 µl of template DNA
  iii)  **Amplification by PCR**
  Initial denaturation at 95°C for 7 minutes
  35 seconds of template denaturation at 95°C
  45 seconds of primer annealing at 64°C for a total of 25 cycles
  3 minutes of primer extension at 72°C
  Final extension at 72°C for 6 minutes
  iv)  **Detection of amplified product and interpretation of results**
  Analyse the PCR products (7 µl) by electrophoresis (120 V for 1 hour) in a 1.5% agarose gel in TBE buffer (89 mM Tris/Cl, 89 mM boric acid, 2.0 mM ethylene diamino tetra-acetic acid [EDTA], pH 8.0). Use 1 kb plus DNA ladder as a molecular size marker. Visualise bands with UV light after staining with ethidium bromide. For interpretation of the results see Garcia-Yoldi et al., 2006.
**Table 3. Oligonucleotides used in the Bruce-ladder multiplex PCR assay**

<table>
<thead>
<tr>
<th>Primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence (5’–3’)</th>
<th>Amplicon six (bp)</th>
<th>DNA targets</th>
<th>Source of genetic differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMEI0998f</td>
<td>ATC-CTA-TTG-CCC-CGA-TAA-GG</td>
<td>1682</td>
<td>Glycosyltransferase, gene wboA</td>
<td>IS711 insertion in BMEI0998 in <em>B. abortus</em> RB51, and deletion of 15,079 bp in BMEI0993-BMEI1012 in <em>B. ovis</em></td>
</tr>
<tr>
<td>BMEI0997r</td>
<td>GCT-TCG-CAT-TTT-CAC-TGT-AGC</td>
<td>450 (1320&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>Immunodominant antigen, gene bp26</td>
<td>IS711 insertion in BMEI0535-BMEI0536 in <em>Brucella</em> strains isolated from marine mammals</td>
</tr>
<tr>
<td>BMEI0535f</td>
<td>GCG-ATT-CAT-TCT-TGC-GTT-ATG-AA</td>
<td>1071</td>
<td>Outer membrane protein, gene omp31</td>
<td>deletion of 25,061 bp in BMEI0826-BMEI0850 in <em>B. abortus</em></td>
</tr>
<tr>
<td>BMEI0536r</td>
<td>CGC-AGG-CGA-AAA-CAG-CTA-TAA</td>
<td>794</td>
<td>Polysaccharide deacetylase</td>
<td>deletion of 976 bp in BMEI1435 in <em>B. canis</em></td>
</tr>
<tr>
<td>BMEI1436f</td>
<td>ACG-CAG-ACC-ACC-TTC-GGT-AT</td>
<td>587</td>
<td>Erythritol catabolism, gene eryC (D-erythrulose-1-phosphate dehydrogenase)</td>
<td>deletion of 702 bp in BMEI1437-BMEI1438 in <em>B. abortus</em> S19</td>
</tr>
<tr>
<td>BMEI1435r</td>
<td>TTT-ATC-CAT-CGC-CCT-GTC-AC</td>
<td>218</td>
<td>Ribosomal protein S12, gene rpsL</td>
<td>point mutation in BMEI0752 in <em>B. melitensis</em> Rev.1</td>
</tr>
<tr>
<td>BR0953f</td>
<td>GGA-ACA-CTA-CGC-CAC-CTT-GT</td>
<td>272</td>
<td>ABC transporter binding protein</td>
<td>deletion of 2653 bp in BR0951-BR0955 in <em>B. abortus</em></td>
</tr>
<tr>
<td>BR0953r</td>
<td>GAT-GGA-GCA-AAC-GCT-GAA-G</td>
<td>218</td>
<td>Ribosomal protein S12, gene rpsL</td>
<td>point mutation in BMEI0752 in <em>B. melitensis</em> Rev.1</td>
</tr>
<tr>
<td>BMEI0752f</td>
<td>CAG-GCA-AAC-CCT-CAG-AAG-C</td>
<td>152</td>
<td>Transcriptional regulator, CRP family</td>
<td>deletion of 2,203 bp in BMEI0985-BMEI0986 in <em>B. neotomae</em></td>
</tr>
<tr>
<td>BMEI0752r</td>
<td>GAT-GTG-GTA-ACG-ACG-ACC-AA</td>
<td>152</td>
<td>Transcriptional regulator, CRP family</td>
<td>deletion of 2,203 bp in BMEI0985-BMEI0986 in <em>B. neotomae</em></td>
</tr>
<tr>
<td>BMEI10987f</td>
<td>CGC-AGA-CAG-TGA-CCA-TCA-CA</td>
<td>152</td>
<td>Transcriptional regulator, CRP family</td>
<td>deletion of 2,203 bp in BMEI0985-BMEI0986 in <em>B. neotomae</em></td>
</tr>
<tr>
<td>BMEI10987r</td>
<td>GTA-TTC-AGC-CCC-CGT-TAC-CT</td>
<td>152</td>
<td>Transcriptional regulator, CRP family</td>
<td>deletion of 2,203 bp in BMEI0985-BMEI0986 in <em>B. neotomae</em></td>
</tr>
</tbody>
</table>

<sup>a</sup>Designations are based on the *B. melitensis* (BME) or *B. suis* (BR) genome sequences. f: forward; r: reverse.

<sup>b</sup>Due to a DNA insertion in the bp26 gene, the amplicon size in *Brucella* strains isolated from marine mammals is 1320 bp.

Other tests such as as *omp25*, 2a and 2b PCR/RFLP (Cloeckaert et al., 2001; 2002a) are available and may be used to identify *Brucella* species.

Alternative approaches allowing identification of all *Brucella* species based on single nucleotide polymorphism (SNP) discrimination by either primer extension or real-time PCR have recently been described (Gopaul et al., 2008; Scott et al., 2007). These tests are rapid, simple and unambiguous and, being based on a robust phylogenetic analysis, overcome some problems seen with Bruce-ladder, such as the misidentification of some *B. canis* isolates.

A number of other methods have recently been described that can add useful epidemiological information. These include a multilocus sequencing scheme (Whatmore et al., 2007) and several typing schemes based on the use of multiple locus variable number of tandem repeats analysis (MLVA) (Bricker et al., 2003;
Identification of vaccine strains

Identification of the vaccine strains *B. abortus* S19, *B. abortus* RB51 and *B. melitensis* strain Rev.1, depends on further tests.

*Brucella abortus* S19 has the normal properties of a biovar 1 strain of *B. abortus*, but does not require CO₂ for growth, does not grow in the presence of benzylpenicillin (3 µg/ml = 5 IU/ml), thionin blue (2 µg/ml), and i-erythritol (1 mg/ml) (all final concentrations), and presents a high l-glutamate use (Alton et al., 1988). In some cases strain 19 will grow in the presence of i-erythritol, but does not use it.

*Brucella melitensis* strain Rev.1 has the normal properties of a biovar 1 strain of *B. melitensis*, but develops smaller colonies on agar media, does not grow in the presence of basic fuchsin, thionin (20 µg/ml) or benzylpenicillin (3 µg/ml) (final concentrations), but does grow in the presence of streptomycin at 2.5 or 5 µg/ml (5 IU/ml) (Alton et al., 1988; Corbel et al., 1979; Corbel & Hendry, 1983; Diaz et al., 1979).

*Brucella abortus* strain RB51 is identified by the following characteristics: rough morphology and growth in the presence of rifampicin (250 µg per ml of media).

Vaccine strains S19, Rev.1 and RB51 may also be identified using specific PCRs (Bricker & Halling, 1995; Garcia-Yoldi et al., 2006; Sangari et al., 1994; Vemulapalli et al., 1999; Villarroel et al., 2000).

2. Serological tests

No single serological test is appropriate in all epidemiological situations; all have limitations especially when it comes to screening individual animals (Godfroid et al., 2002; Nielsen et al., 2006). Consideration should be given to all factors that impact on the relevance of the test method and test results to a specific diagnostic interpretation or application. In epidemiological units where vaccination with smooth *Brucella* is practised, false-positive reactions may be expected among the vaccinated animals because of antibodies cross-reacting with wild strain infection. For the purposes of this chapter, the serological methods described represent standardised and validated methods with suitable performance characteristics to be designated as either prescribed or alternative tests for international trade. This does not preclude the use of modified or similar test methods or the use of different biological reagents. However, the methods and reagents described in this chapter represent a standard of comparison with respect to expected diagnostic performance.

It should be stressed that the serum agglutination test (SAT) is generally regarded as being unsatisfactory for the purposes of international trade. The complement fixation test (CFT) is diagnostically more specific than the SAT, and also has a standardised system of unitage. The diagnostic performance characteristics of some enzyme-linked immunosorbent assays (ELISAs) and the fluorescence polarisation assay (FPA) are comparable with or better than that of the CFT, and as they are technically simpler to perform and more robust, their use may be preferred (Nielsen et al., 1995; Wright et al., 1997). The performances of several of these tests have been compared.

For the control of brucellosis at the national or local level, the buffered *Brucella* antigen tests (BBATs), i.e. the Rose Bengal test (RBT) and the buffered plate agglutination test (BPAT), as well as the ELISA and the FPA, are suitable screening tests. Positive reactions should be retested using a suitable confirmatory and/or complementary strategy.

In other species, for example, buffaloes (*Bubalus bubalus*), American and European bison (*Bison bison, Bison bonasus*), yak (*Bos grunniens*), elkwapiti (*Cervus elaphus*), and camels (*Camelus bactrianus* and *C. dromedarius*), and South American camelids, *Brucella* sp. infection follows a course similar to that in cattle. The same serological procedures may be used for these animals (Nicoletti, 1992), but each test should be validated in the animal species under study (Gall et al., 2000; 2001).

- **Reference sera**

  The OIE reference standards are those against which all other standards are compared and calibrated. These reference standards are all available to national reference laboratories and should be used to establish secondary or national standards against which working standards can be prepared and used in the diagnostic laboratory for daily routine use.
These sera have been developed and designated by the OIE as International Standard Sera\(^1\). The use of these promotes international harmonisation of diagnostic testing and antigen standardisation (Wright \textit{et al}., 1997):

- For RBT and CFT, the OIE International Standard Serum (OIEISS, previously the WHO Second International anti-\textit{Brucella abortus} Serum) is used. This serum is of bovine origin and contains 1000 IU and ICFTU (international complement fixation test units).
- In addition, three OIE ELISA Standard Sera are available for use. These are also of bovine origin and consist of a strong positive (OIEELISASPSS), a weak positive (OIEELISAWPSS) and a negative (OIEELISANS) standard. Conditions for standardising FPA with these Standards need to be reviewed.

\begin{itemize}
  \item \textit{Production of cells}
  
  \textit{Brucella abortus} strain 99 (Weybridge) (S99) (see footnote 1 for address) or \textit{B. abortus} strain 1119-3 (USDA) (S1119-3)\(^2\) should always be used for diagnostic antigen production. It should be emphasised that antigen made with one of these \textit{B. abortus} strains is also used to test for \textit{B. melitensis} or \textit{B. suis} infection. The strains must be completely smooth and should not autoagglutinate in saline and 0.1% (w/v) acriflavine. They must be pure cultures and conform to the characteristics of CO\textsubscript{2}-independent strains of \textit{B. abortus} biovar 1. The original seed cultures should be propagated to produce a seed lot that must conform to the properties of these strains, and should be preserved by lyophilisation or by freezing in liquid nitrogen.

  \textbf{For antigen production}, the seed culture is used to inoculate a number of potato-infusion agar slopes that are then incubated at 37°C for 48 hours. SDA and TSA, to which 5% equine or newborn calf serum and/or 0.1% yeast extract may be added, are satisfactory solid media provided a suitable seed is used as recommended above. The growth is checked for purity, resuspended in sterile PBS, pH 6.4, and used to seed layers of potato-infusion agar or glycerol–dextrose agar in Roux flasks. These are then incubated at 37°C for 72 hours with the inoculated surface facing down. Each flask is checked for purity by Gram staining samples of the growth, and the organisms are harvested by adding 50–60 ml of phenol saline (0.5% phenol in 0.85% sodium chloride solution) to each flask. The flasks are gently agitated, the suspension is decanted, and the organisms are killed by heating at 80°C for 90 minutes. Following a viability check, the antigen is stored at 4°C.

  Alternatively, the cells may be produced by batch or continuous culture in a fermenter (Hendry \textit{et al}., 1995), using a liquid medium containing (per litre of distilled water) D-glucose (30 g), a high-grade peptone (30 g), yeast extract (Difco) (10 g), sodium dihydrogen phosphate (9 g) and disodium hydrogen phosphate (3.3 g). The initial pH is 6.6, but this tends to rise to pH 7.2–7.4 during the growth cycle. Care should be taken to check batches of peptone and yeast extract for capacity to produce good growth without formation of abnormal or dissociated cells. Vigorous aeration and stirring is required during growth, and adjustment to pH 7.2–7.4 by the addition of sterile 0.1 M HCl may be necessary. The seed inoculum is prepared as described above. The culture is incubated at 37°C for 48 hours. Continuous culture runs can be operated for much longer periods, but more skill is required to maintain them. In-process checks should be made on the growth from either solid or liquid medium to ensure purity, an adequate viable count and freedom from dissociation to rough forms. Cells for use in the preparation of all antigens should be checked for purity and smoothness at the harvesting stage.

  The culture is harvested by centrifugation to deposit the organisms, which are resuspended in phenol saline. The organisms are killed by heating at 80°C for 90 minutes and are stored at 4°C. They form stable suspensions in physiological saline solutions and show no evidence of autoagglutination. A viability check must be performed on the suspensions and no growth must be evident after 10 days’ incubation at 37°C. The packed cell volume (PCV) of the killed suspensions can be determined by centrifuging 1 ml volumes in Wintrobe tubes at 3000 \textit{g} for 75 minutes.

  \begin{itemize}
    \item \textbf{a) Buffered \textit{Brucella} antigen tests (prescribed tests for international trade)}
      \begin{itemize}
        \item \textbf{Rose Bengal test}
          
          This test is a simple spot agglutination test using antigen stained with Rose Bengal and buffered to a low pH, usually 3.65 ± 0.05 (Morgan \textit{et al}., 1969).

        \begin{itemize}
          \item \textbf{Antigen production}
            
            Antigen for the RBT is prepared by depositing killed \textit{B. abortus} S99 or S1119-3 cells by centrifugation at 23,000 \textit{g} for 10 minutes at 4°C, and uniformly resuspending in sterile phenol saline (0.5%) at the rate of 1 g
        \end{itemize}
      \end{itemize}
  \end{itemize}
\end{itemize}

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1 Obtainable from the OIE Reference Laboratory for Brucellosis at Animal Health and Veterinary Laboratories Agency (AHVLA) Weybridge, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom.
2 Obtainable from the United States Department of Agriculture (USDA), National Veterinary Services Laboratories (NVSL), 1800 Dayton Road, Ames, Iowa 50010, United States of America.
to 22.5 ml. (Note: if sodium carboxymethyl cellulose is used as the sedimenting agent during preparation of the cell concentrate, insoluble residues must be removed by filtering the suspension through an AMF-CUNO Zeta-plus prefilter [Type CPR 01A] before staining.) To every 35 ml of this suspension, 1 ml of 1% (w/v) Rose Bengal (Cl No. 45440) in sterile distilled water is added, and the mixture is stirred for 2 hours at room temperature. The mixture is filtered through sterile cotton wool, and centrifuged at 10,000 g to deposit the stained cells, which are then uniformly resuspended at the rate of 1 g cells to 7 ml of diluent (21.1 g of sodium hydroxide dissolved in 353 ml of sterile phenol saline, followed by 95 ml of lactic acid, and adjusted to 1056 ml with sterile phenol saline). The colour of this suspension should be an intense pink and the supernatant of a centrifuged sample should be free of stain; the pH should be 3.65 ± 0.05. After filtration through cotton wool, the suspension is filtered twice through a Sartorius No. 13430 glass fibre prefilter, adjusted to a PCV of approximately 8%, pending final standardisation against serum calibrated against the OIEISS, and stored at 4°C in the dark. The antigen should be stored as recommended by the manufacturer but usually should not be frozen.

When used in the standard test procedure, the RBT antigen should give a clearly positive reaction with 1/45 dilution, but not 1/55 dilution, of the OIEISS diluted in 0.5% phenol saline or normal saline. It may also be advisable to compare the reactivity of new and previously standardised batches of antigen using a panel of defined sera.

- **Test procedure**
  
i) Bring the serum samples and antigen to room temperature (22 ± 4°C); only sufficient antigen for the day’s tests should be removed from the refrigerator.

  ii) Place 25–30 µl of each serum sample on a white tile, enamel or plastic plate, or in a WHO haemagglutination plate.

  iii) Shake the antigen bottle well, but gently, and place an equal volume of antigen near each serum spot.

  iv) Immediately after the last drop of antigen has been added to the plate, mix the serum and antigen thoroughly (using a clean glass or plastic rod for each test) to produce a circular or oval zone approximately 2 cm in diameter.

  v) The mixture is agitated gently for 4 minutes at ambient temperature on a rocker or three-directional agitator (if the reaction zone is oval or round, respectively).

  vi) Read for agglutination immediately after the 4-minute period is completed. Any visible reaction is considered to be positive. A control serum that gives a minimum positive reaction should be tested before each day’s tests are begun to verify the sensitivity of test conditions.

The RBT is very sensitive. However, like all other serological tests, it could sometimes give a positive result because of S19 vaccination or of false-positive serological reactions (FPSR). Therefore positive reactions should be investigated using suitable confirmatory and/or complementary strategies (including the performance of other tests and epidemiological investigation). False-negative reactions occur rarely, mostly due to prozoning and can sometimes be detected by diluting the serum sample or retesting after 4–6 weeks. Nevertheless RBT appears to be adequate as a screening test for detecting infected herds or to guarantee the absence of infection in brucellosis-free herds.

- **Buffered plate agglutination test**

  - **Antigen production**

    Antigen for the BPAT is prepared from *B. abortus* S1119-3 according to the procedure described by Angus & Barton (1984).

    Two staining solutions are required: brilliant green (2 g/100 ml) and crystal violet (1 g/100 ml) both certified stains dissolved in distilled water. Once prepared, the two solutions should be stored separately for a period of 24 hours, and then mixed together in equal volumes in a dark bottle and stored in a refrigerator for a period of not less than 6 months before use. The mixed stain may only be used between 6 and 12 months after initial preparation.

    Buffered diluent is prepared by slowly dissolving sodium hydroxide (150 g) in 3–4 litres of sterile phenol saline. Lactic acid (675 ml) is added to this solution, and the final volume is adjusted to 6 litres by adding sterile phenol saline. The pH of the solution should be between 3.63 and 3.67.

    *Brucella abortus* S1119-3 packed cells are diluted to a concentration of 250 g/litre in phenol saline; 6 ml of stain is added per litre of cell suspension, and the mixture is shaken thoroughly before being filtered through sterile absorbent cotton. The cells are centrifuged at 10,000 g at 4°C, and the packed cells are then resuspended at a concentration of 50 g/100 ml in buffered diluent (as described above). This mixture is shaken thoroughly for 2 hours, and is then further diluted by the addition of 300 ml of buffered diluent per 100 ml of suspended cells (i.e. final concentration of 50 g packed cells/400 ml buffered diluent). The mixture
is stirred at room temperature for 20–24 hours before the cell concentration is adjusted to 11% (w/v) in buffered diluent. This suspension is stirred overnight before testing. Pending final quality control tests, the antigen is stored at 4°C until required for use. The antigen has a shelf life of 1 year and should not be frozen.

The pH of the buffered plate antigen should be 3.70 ± 0.03 and the pH of a serum:antigen mixture at a ratio of 8:3 should be 4.02 ± 0.04. The 11% stained-cell suspension should appear blue-green. Each batch of buffered plate antigen should be checked by testing at least 10 weakly reactive sera and comparing the results with one or more previous batches of antigen. If possible, the antigen batches should be compared with the standard antigen prepared by the NVSL, USDA (see footnote 2 for address). There is, however, no international standardisation procedure established for use with the OIEISS.

- **Test procedure**
  i) Bring the serum samples and antigen to room temperature (22 ± 4°C); only sufficient antigen for the day’s tests should be removed from the refrigerator.
  ii) Shake the sample well. Place 80 µl of each serum sample on a glass plate marked in 4 × 4 cm squares
  iii) Shake the antigen bottle well, but gently, and place 30 µl of antigen near each serum spot.
  iv) Immediately after the last drop of antigen has been added to the plate, mix the serum and antigen thoroughly (using a clean glass or plastic rod for each test) to produce a circular zone approximately 3 cm in diameter.
  v) After the initial mixing, the plate should be rotated three times in a tilting motion to ensure even dispersion of the reagents, and then incubated for 4 minutes in a humid chamber at ambient temperature.
  vi) The plate should be removed and rotated as above, and then returned for a second 4-minute incubation.
  vii) Read for agglutination immediately after the 8-minute period is completed. Any visible reaction is considered to be positive. A control serum that gives a minimum positive reaction should be tested before each day’s tests are begun to verify the sensitivity of test conditions.

Like the RBT, the test is very sensitive, especially for detection of vaccine-induced antibody, and positive samples should be retested using a confirmatory and/or complementary test(s). False-negative reactions may occur, usually due to prozoning, which may be overcome by diluting the serum or retesting after a given time.

b) **Complement fixation test (a prescribed test for international trade)**

The CFT is widely used and accepted as a confirmatory test although it is complex to perform, requiring good laboratory facilities and adequately trained staff to accurately titrate and maintain the reagents. There are numerous variations of the CFT in use, but this test is most conveniently carried out in a microtitre format. Either warm or cold fixation may be used for the incubation of serum, antigen and complement: either 37°C for 30 minutes or 4°C for 14–18 hours. A number of factors affect the choice of the method: anti-complementary activity in serum samples of poor quality is more evident with cold fixation, while fixation at 37°C increases the frequency and intensity of prozones, and a number of dilutions must be tested for each sample.

Several methods have been proposed for the CFT using different concentrations of fresh or preserved sheep red blood cells (SRBCs) (a 2, 2.5% or 3% suspension is usually recommended) sensitised with an equal volume of rabbit anti-SRBC serum diluted to contain several times (usually from two to five times) the minimum concentration required to produce 100% lysis of SRBCs in the presence of a titrated solution of guinea-pig complement. The latter is independently titrated (in the presence or absence of antigen according to the method) to determine the amount of complement required to produce either 50% or 100% lysis of sensitised SRBCs in a unit volume of a standardised suspension; these are defined as the 50% or 100% haemolytic unit of complement/minimum haemolytic dose (C'H50 or MHD50 or C'H or MHD100), respectively. It is generally recommended to titrate the complement before each set of tests, a macromethod being preferred for an optimal determination of C'H50. Usually, 1.25–2 C'H100 or 5–6 C'H50 are used in the test.

Barbital (veronal) buffered saline is the standard diluent for the CFT. This is prepared from tablets available commercially; otherwise it may be prepared from a stock solution of sodium chloride (42.5 g), barbituric acid (2.875 g), sodium diethyl barbiturate (1.875 g), magnesium sulphate (1.018 g), and calcium chloride (0.147 g) in 1 litre of distilled water and diluted by the addition of four volumes of 0.04% gelatin solution before use.
Chapter 2.4.3. – Bovine brucellosis

- **Antigen production**

Numerous variations of the test exist but, whichever procedure is selected, the test must use an antigen that has been prepared from an approved smooth strain of *B. abortus*, such as S99 or S1119-3, and standardised against the OIEISS. Antigen for the CFT can be prepared by special procedures (Alton *et al*., 1988; Hendry *et al*., 1995) or a whole cell antigen can be used after diluting the stock suspension such that the PCV of the concentrated antigen suspension for CFT should be approximately 2% before standardisation against the OIEISS. The antigen should be standardised to give 50% fixation at a dilution of 1/200 of the OIEISS and must also show complete fixation at the lower serum dilutions, because too weak (or too strong) a concentration of antigen may not produce 100% fixation at the lower dilutions of serum. When two dilutions of antigen are suitable, the more concentrated antigen suspension must be chosen in order to avoid prozone occurrence.

The appearance of the antigen when diluted 1/10 must be that of a uniform, dense, white suspension with no visible aggregation or deposit after incubation at 37°C for 18 hours. It must not produce anti-complementary effects at the working strength for the test. The antigen is stored at 4°C and should not be frozen.

- **Test procedure (example)**

The undiluted test sera and appropriate working standards should be inactivated for 30 minutes in a water bath at 60°C ± 2°C. If previously diluted with an equal volume of veronal buffered saline these sera could be inactivated at 58°C ± 2°C for 50 minutes. Usually, only one serum dilution is tested routinely (generally 1/4 or 1/5 depending on the CF procedure chosen), but serial dilutions are recommended for trade purposes in order to detect prozone.

Using standard 96-well microtitre plates with round (U) bottoms, the technique is usually performed as follows:

i) Volumes of 25 µl of diluted inactivated test serum are placed in the well of the first, second and third rows. The first row is an anti-complementary control for each serum. Volumes of 25 µl of CFT buffer are added to the wells of the first row (anti-complementary controls) to compensate for lack of antigen. Volumes of 25 µl of CFT buffer are added to all other wells except those of the second row. Serial doubling dilutions are then made by transferring 25 µl volumes of serum from the third row onwards; 25 µl of the resulting mixture in the last row are discarded.

ii) Volumes of 25 µl of antigen, diluted to working strength, are added to each well except in the first row.

iii) Volumes of 25 µl of complement, diluted to the number of units required, are added to each well.

iv) Control wells containing diluent only, complement + diluent, antigen + complement + diluent, are set up to contain 75 µl total volume in each case. A control serum that gives a minimum positive reaction should be tested in each set of tests to verify the sensitivity of test conditions.

v) The plates are incubated at 37°C for 30 minutes or at 4°C overnight, and a volume (25 or 50 µl according to the technique) of sensitised SRBCs is added to each well. The plates are re-incubated at 37°C for 30 minutes.

vi) The results are read after the plates have been centrifuged at 1000 g for 10 minutes at 4°C or left to stand at 4°C for 2–3 hours to allow unlysed cells to settle. The degree of haemolysis is compared with standards corresponding to 0, 25, 50, 75 and 100% lysis. The absence of anti-complementary activity is checked for each serum in the first row.

vii) **Standardisation of results of the CFT:**

There is a unit system that is based on the OIEISS. This serum contains 1000 ICFTU (international complement fixation test units) per ml. If this serum is tested in a given method and gives a titre of, for example 200 (50% haemolysis), then the factor for an unknown serum tested by that method can be found from the formula: 1000 × 1/200 × titre of test serum = number of ICFTU of antibody in the test serum per ml. The OIEISS contains specific IgG; national standard sera should also depend on this isotype for their specific complement-fixing activity. Difficulties in standardisation arise because different techniques selectively favour CF by different immunoglobulin isotypes. It is recommended that any country using the CFT on a national scale should obtain agreement among the different laboratories performing the test to use the same method in order to obtain the same level of sensitivity. To facilitate comparison between countries, results should always be expressed in ICFTUs, calculated in relation to those obtained in a parallel titration with a standard serum, which in turn may be calibrated against the OIEISS.

vii) **Interpretation of the results:** Sera giving a titre equivalent to 20 ICFTU/ml or more are considered to be positive.
This procedure is an example, other volumes and quantities of reagents could be chosen provided that the test is standardised against the OIESS as described above and the results expressed in ICFTU/ml.

The CFT is usually very specific. However, like all other serological tests, it could sometimes give a positive result due to S19 vaccination or due to FPSR. Therefore positive reactions should be investigated using suitable confirmatory and/or complementary strategies. Females that have been vaccinated with \(B. \text{abortus}\) S19 between 3 and 6 months are usually considered to be positive if the sera give positive fixation at a titre of 30 or greater ICFTU/ml when the animals are tested at an age of 18 months or older.

c) Enzyme-linked immunosorbent assays (prescribed tests for international trade)

- **Indirect ELISA**

Numerous variations of the indirect ELISA (I-ELISA) have been described employing different antigen preparations, antiglobulin-enzyme conjugates, and substrate/chromogens. Several commercial I-ELISAs using whole cell, smooth lipopolysaccharide (sLPS) or the O-polysaccharide (OPS) as antigens that have been validated in extensive field trials are available and are in wide use. In the interests of international harmonisation, the three OIE ELISA Standard Sera should be used by national reference laboratories to check or calibrate the particular test method in question.

These assays should be calibrated such that the optical density (OD) of the strong positive OIE ELISA Standard Serum should represent a point on the linear portion of a typical dose–response curve just below the plateau. The weak positive OIE ELISA Standard Serum should consistently give a positive reaction that lies on the linear portion of the same dose–response curve just above the positive/negative threshold. The negative serum and the buffer control should give reactions that are always less than the positive/negative threshold (Wright *et al.*, 1993). Finally the cut-off should be established in the test population using appropriate validation techniques (see Chapter 1.1.5 *Principles and methods of validation of diagnostic assays for infectious diseases*).

The I-ELISAs that use sLPS or OPS as antigens are highly sensitive for the detection of anti-\(\text{Brucella}\) antibodies, but are not capable of fully resolving the problem of differentiating between antibodies resulting from S19 vaccination.

The problem with FPSR may be partly overcome by performing I-ELISAs using rough LPS (rLPS) or cytosol antigens. Most FPSR are a result of cross reaction with the OPS portion of the sLPS molecule, however, cross reaction among core regions of LPS are less frequent (Nielsen *et al.*, 2004; 2006).

For the screening I-ELISA, preparations rich in sLPS or OPS should be used as the optimal antigen. There are several protocols for preparing a suitable antigen.

Monoclonal, polyclonal antiglobulin or protein G or AG enzyme conjugates may be used depending on availability and performance requirements. An MAb specific for the heavy chain of bovine IgG\(_1\) may provide some improvement in specificity at the possible cost of some loss of sensitivity while a protein G or AG enzyme conjugate may provide a reagent useful for testing a variety of mammalian species (Munoz *et al.*, 2005; Nielsen *et al.*, 2004).

The test method described below is an example of a test that has been internationally validated and has been used extensively in internationally sponsored, technical cooperation and research collaboration projects world-wide.

The antigen-coating buffer is 0.05 M carbonate/bicarbonate buffer, pH 9.6, composed of sodium hydrogen carbonate (2.93 g) and sodium carbonate (1.59 g) (sodium azide [0.20 g/litre] is optional) in 1 litre of distilled water. The conjugate and test sera diluent buffer is 0.01 M PBS, pH 7.2, composed of disodium hydrogen orthophosphate (1.4 g), potassium dihydrogen phosphate (0.20 g), sodium chloride (8.50 g) and 0.05% Tween 20 dissolved in 1 litre of distilled water (PBST). This buffer is also used as wash buffer.

The conjugate used in this example is an MAb specific for the heavy chain of bovine IgG\(_1\), and conjugated to horseradish peroxidase (HRPO). The substrate stock solution is 3% hydrogen peroxide. The chromogen stock solution is 0.16 M 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in distilled water. Substrate buffer is citrate buffer, pH 4.5, composed of trisodium citrate dihydrate (7.6 g) and citric acid (4.8 g) dissolved in 1 litre of distilled water. The enzymatic reaction-stopping solution is 4% sodium dodecyl sulphate (SDS).

- **Antigen production (example)**

sLPS from \(B. \text{abortus}\) S1119-3 or S99 is extracted by heating 5 g dry weight (or 50 g wet weight) of cells suspended in 170 ml distilled water to 66°C followed by the addition of 190 ml of 90% (v/v) phenol at 66°C. The mixture is stirred continuously at 66°C for 15 minutes, cooled and centrifuged at 10,000 \(g\) for
Chapter 2.4.3. — Bovine brucellosis

15 minutes at 4°C. The brownish phenol in the bottom layer is removed with a long cannula and large cell debris may be removed by filtration (using a Whatman No. 1 filter) if necessary.

The sLPS is precipitated by the addition of 500 ml cold methanol containing 5 ml methanol saturated with sodium acetate. After 2 hours' incubation at 4°C, the precipitate is removed by centrifugation at 10,000 g for 10 minutes. The precipitate is stirred with 80 ml of distilled water for 18 hours and centrifuged at 10,000 g for 10 minutes. The supernatant solution is kept at 4°C. The precipitate is resuspended in 80 ml distilled water and stirred for an additional 2 hours at 4°C. The supernatant solution is recovered by centrifugation as above and pooled with the previously recovered supernatant.

Next, 8 g of trichloroacetic acid is added to the 160 ml of crude LPS. After stirring for 10 minutes, the precipitate is removed by centrifugation and the translucent supernatant solution is dialysed against distilled water (two changes of at least 4000 ml each) and then freeze dried.

The freeze-dried LPS is weighed and reconstituted to 1 mg/ml in 0.05 M carbonate buffer, pH 9.6, and sonicated in an ice bath using approximately 6 watts three times for 1 minute each. The LPS is then freeze dried in 1 ml amounts and stored at room temperature.

- **Test procedure (example)**
  1. The freeze-dried sLPS is reconstituted to 1 ml with distilled water and is further diluted 1/1000 (or to a dilution predetermined by titration against the OIE ELISA Standard Sera) in 0.05 M carbonate buffer, pH 9.6. To coat the microplates, 100 µl volumes of the diluted sLPS solution are added to all wells, and the plates are covered and incubated for 18 hours at 4°C. After incubation, the plates may be used or sealed, frozen and stored at –20°C for up to a year. Frozen plates are thawed for 30–45 minutes at 37°C before use.
  2. Unbound antigen is removed by washing all microplate wells with PBST four times. Volumes (100 µl) of serum diluted in the range of 1/50 to 1/200 in PBST, pH 6.3, containing 7.5 mM each of EDTA and ethylene glycol tetra-acetic acid (EGTA) (PBST/EDTA) are added to specified wells and incubated at ambient temperature for 30 minutes.
  3. Test sera are added to the plates and may be tested singly or in duplicate. The controls, calibrated against the OIE ELISA Standard Sera, are set up in duplicate wells and include a strong positive, a weak positive, a negative control serum, and a buffer control.
  4. Unbound serum is removed by washing four times with PBST (PBST containing EDTA/EGTA must not be used with HRPO as it inactivates the enzyme). Volumes (100 µl) of conjugate (MAb M23) specific for a heavy chain epitope of bovine IgG 1 conjugated with HRPO and diluted in PBST (predetermined by titration) are added to each well and the plates are incubated at ambient temperature for 30 minutes.
  5. Unbound conjugate is removed by four washing steps. Volumes (100 µl) of substrate/chromogen (1.0 mM H₂O₂ [100 µl/20 ml citrate buffer] and 4 mM ABTS [500 µl/20 ml citrate buffer]) are added to each well, the plate is shaken for 10 minutes and colour development is assessed in a spectrophotometer at 414 or 405 nm. If required, 100 µl volumes of 4% SDS may be added directly to all wells as a stopping reagent.
  6. The control wells containing the strong positive serum are considered to be 100% positive and all data are calculated from these absorbance readings (between 1.000 and 1.800) using the equation:

\[
\text{Per cent positivity (\%P)} = \frac{\text{absorbance (test sample)}}{\text{absorbance (strong positive control)}} \times 100
\]

The sLPS antigen, small amounts of the MAb specific for the heavy chain of bovine IgG₁, software for generation of data using particular spectrophotometers and a standard test protocol for the I-ELISA are available for research and standardisation purposes.

Using this or another similar I-ELISA calibrated against the OIE ELISA Standard Sera described above, the diagnostic sensitivity should be equal to or greater than that of the BBATs (RBT/BPAT) in the testing of infected cattle. However, like all other serological tests, it could give a positive result because of S19 vaccination or FPSR. Positive reactions should be investigated using suitable confirmatory and/or complementary strategies as for CFT.

- **Competitive ELISA**

The competitive ELISA (C-ELISA) using an MAb specific for one of the epitopes of the Brucella sp. OPS has been shown to have higher specificity but lower sensitivity than the I-ELISA (MacMillan et al., 1990; Munoz et al., 2005; Nielsen et al., 1995; Stack et al., 1999; Weynants et al., 1997). This is accomplished by

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3 Obtainable from the Animal Diseases Research Institute, 3851 Fallowfield Road, Nepean, Ontario K2H 8P9, Canada.
selecting an MAb that has higher affinity than cross-reacting antibody. However, it has been shown that the C-ELISA eliminates some but not all reactions (FPSR) due to cross-reacting bacteria (Munoz et al., 2005; Nielsen, 2002). The C-ELISA is also capable of eliminating most reactions due to residual antibody produced in response to vaccination with S19. The choice of MAB and its unique specificity and affinity will have a distinct influence on the diagnostic performance characteristics of the assay. As with any MAb-based assay, the universal availability of the MAb or the hybridoma must also be considered with respect to international acceptance and widespread use.

Several variations of the C-ELISA have been described including antigens prepared from different smooth Brucella strains. The C-ELISA is also commercially available. Some protocols are less sensitive than others, therefore results obtained from different assays are not always comparable. In the interests of international harmonisation, the three OIE ELISA Standard Sera should be used by national reference laboratories to check or calibrate the test method in question.

The assay should be calibrated such that the OD of the strong positive OIE ELISA Standard Serum should represent a point on the linear portion of a typical dose–response curve just above the plateau (i.e. close to maximal inhibition). The weak positive OIE ELISA Standard Serum should give a reaction that lies on the linear portion of the same dose–response curve just above the positive/negative threshold (i.e. moderate inhibition). The negative serum and the buffer/MAb control should give reactions that are always less than the positive/negative threshold (i.e. minimal inhibition). Moreover, the cut-off should be established in the test population with appropriate validation techniques (see chapter 1.1.5).

The test method described below is an example of a test, which has been internationally validated and has been used extensively in internationally sponsored, technical cooperation and research collaboration projects world-wide.

The buffer systems are the same as those described for the I-ELISA.

- **Antigen production (example)**
  sLPS from B. abortus S1119-3 is prepared and used as for the I-ELISA.

- **Test procedure**
  i) The freeze-dried sLPS is reconstituted to 1 ml with distilled water and further diluted 1/1000 with 0.05 M carbonate buffer, pH 9.6. To coat the microplates, 100 µl volumes of LPS solution are added to all wells and the plates are covered and incubated for 18 hours at 4°C. After incubation, the plates may be used or sealed, frozen and stored at –20°C for up to 1 year. Frozen plates are thawed for 30–45 minutes at 37°C before use.
  
  ii) Unbound antigen is removed by washing all microplate wells four times with PBST. Volumes (50 µl) of MAb (M84 in this example) diluted appropriately in PBST/EDTA are added to each well, followed immediately by 50 µl volumes of serum diluted 1/10 in PBST/EDTA. Plates are incubated for 30 minutes at ambient temperature with shaking for at least the initial 3 minutes.
  
  iii) Test sera are added to the plates and may be tested as singly or in duplicate. The controls, calibrated against the OIE ELISA Standard Sera, are set up in duplicate wells and include a strong positive, a weak positive, a negative control serum, and a buffer control.
  
  iv) Unbound serum and MAb are removed by washing the microplate four times with PBST. Volumes (100 µl) of commercial goat anti-mouse IgG (H and L chain) HRPO conjugate diluted in PBST (predetermined by titration) are added to each well and the plates are incubated at ambient temperature for 30 minutes.
  
  v) Unbound conjugate is removed by four washing steps. Volumes (100 µl) of substrate/chromogen (1.0 mM H$_2$O$_2$ and 4 mM ABTS) are added to each well, the plates are shaken for 10 minutes and colour development is assessed in a spectrophotometer at 414 or 405 nm. If required, 100 µl volumes of 4% SDS may be added directly as a stopping reagent.
  
  vi) The control wells containing MAb and buffer (no serum) are considered to give 0% inhibition and all data are calculated from these absorbance readings (between 1.000 and 1.800) using the equation:

  \[
  \text{Per cent inhibition (\%I)} = 100 - \frac{\text{absorbance [test sample]}}{\text{absorbance [buffer control]}} \times 100
  \]

The sLPS antigen, small amounts of the MAb, software for generation of data using particular spectrophotometers and a standard operating procedure for the C-ELISA are available for research and standardisation (see footnote 3 for address).
Using this or a similar C-ELISA protocol calibrated against the OIE ELISA Standard Sera, the diagnostic sensitivity could be equivalent to the BBATs and the I-ELISAs in the testing of infected cattle (Nielsen et al., 1995, 1996b; 2005). However, like all other serological tests, it could give a positive result because of S19 vaccination or FPSR. Positive reactions should be investigated using suitable confirmatory and/or complementary strategies as for CFT.

d) Fluorescence polarisation assay (a prescribed test for international trade)

The FPA is a simple technique for measuring antigen/antibody interaction and may be performed in a laboratory setting or in the field. It is a homogeneous assay in which analytes are not separated and it is therefore very rapid.

The mechanism of the assay is based on random rotation of molecules in solution. Molecular size is the main factor influencing the rate of rotation, which is inversely related. Thus a small molecule rotates faster than a large molecule. If a molecule is labelled with a fluorochrome, the time of rotation through an angle of 68.5° can be determined by measuring polarised light intensity in vertical and horizontal planes. A large molecule emits more light in a single plane (more polarised) than a small molecule rotating faster and emitting more depolarised light.

For most FPAs, an antigen of small molecular weight, less than 50 kD, is labelled with a fluorochrome and added to serum or other fluid to be tested for the presence of antibody. If antibody is present, attachment to the labelled antigen will cause its rotational rate to decrease and this decrease can be measured.

For the diagnosis of brucellosis, a small molecular weight fragment (average 22 kD) of the OPS of \textit{B. abortus} strain 1119-3 sLPS is labelled with fluorescein isothiocyanate (FITC) and used as the antigen. This antigen is added to diluted serum or whole blood and a measure of the antibody content is obtained in about 2 minutes (for serum) or 15 seconds (for blood) after the addition of antigen using a fluorescence polarisation analyser (Nielsen et al., 1996a; 2005).

The FPA can be performed in glass tubes or a 96-well plate format. The bovine serum is diluted 1/10 for the plate test or 1/100 for the tube test; if EDTA-treated blood is used the dilution for the tube test is 1/50 and 1/5 for the plate test (heparin-treated blood tends to increase assay variability). The diluent used is 0.01 M Tris (1.21 g), containing 0.15 M sodium chloride (8.5 g), 0.05% Igepal CA630 (500 µl) (formerly NP40), 10 mM EDTA (3.73 g) per litre of distilled water, pH 7.2 (Tris buffer). An initial reading to assess light scatter is obtained with the fluorescence polarisation analyser (FPM) after mixing. Suitably labelled titrated antigen (usually giving an intensity of 250,000–300,000) is added, mixed and a second reading is obtained in the FPM about 2 minutes later for serum and 15 seconds for blood. A reading in millipolarisation units, mP, over the established threshold level is indicative of a positive reaction. A typical threshold level is 90–100 mP units, however, the test should be calibrated locally against International Standard reference sera (the expected values are pending). Control sera of strong positive, weak positive and negative, as well as S19 vaccinate serum, should be included.

- **Antigen production (example)**

OPS from 5 g dry weight (or 50 g wet weight) of \textit{B. abortus} S1119-3 is prepared by adding 400 ml of 2% (v/v) acetic acid, autoclaving the suspension for 15 minutes at 121°C and removing the cellular debris by centrifugation at 10,000 g for 10 minutes at 4°C. The supernatant solution is then treated with 20 g of trichloroacetic acid to precipitate any proteins and nucleic acids. The precipitate is again removed by centrifugation at 10,000 g for 10 minutes at 4°C. The supernatant fluid is dialysed against at least 100 volumes of distilled water and freeze dried.

3 mg of OPS are dissolved in 0.6 ml of 0.1 M sodium hydroxide (4 g NaOH/litre) and incubated at 37°C for 1 hour, followed by the addition of 0.3 ml of FITC isomer 1 at a concentration of 100 mg/ml in dimethyl sulphoxide and a further incubation at 37°C for 1 hour. The conjugated OPS is applied to a 1 × 10 cm column packed with DEAE (diethylaminoethyl) Sephadex A 25 equilibrated in 0.01 M phosphate buffer, pH 7.4. The first fraction (after 10–15 ml of buffer) is bright green, after which the buffer is switched to 0.1 M phosphate, pH 7.4. This results in the elution of 10–15 ml of buffer followed by 25–40 ml of green fluorescent material. The latter material is the antigen used in the FPA. Antigen preparation may be scaled up proportionally.

The amount of antigen used per test is determined by diluting the material derived above until a total fluorescence intensity of 250,000–300,000 is achieved using the FPM.

The antigen can be stored as a liquid for several years at 4°C in a dark bottle or it may be freeze dried in dark bottles.

Small quantities of labelled antigen for research and standardisation purposes and standard operating procedures for antigen preparation and the FPA may be obtained (see footnote 3 for address).
Chapter 2.4.3. – Bovine brucellosis

- Test procedure

  i) 1 ml of Tris buffer is added to a 10 × 75 mm borosilicate glass tube followed by 10 µl of serum or 20 µl of EDTA-treated blood. For the 96-well format, 20 µl of serum is added to 180 µl of buffer. It is important to mix well. A reading is obtained on the FPM to determine light scatter.

  ii) A volume of antigen, which results in a total fluorescence intensity of 250–300 × 10^3, is added to the tube and mixed well. This volume will vary from batch to batch, but is generally in the range of about 10 µl. A second reading is obtained on the FPM after incubation at ambient temperature for approximately 2 minutes for serum and 15 seconds for EDTA-treated blood.

  iii) A reading above the predetermined threshold is indicative of a positive reaction.

  iv) The following are included in each batch of tests: a strong positive, a weak positive, a negative working standard serum (calibrated against the OIE ELISA Standard Sera).

The diagnostic sensitivity and specificity of the FPA for bovine brucellosis are almost identical to those of the C-ELISA. The diagnostic specificity for cattle recently vaccinated with S19 is over 99% (Nielsen et al., 1996a). However, the specificity of FPA in FPSR conditions is currently unknown. Like all other serological tests, positive reactions should be investigated using suitable confirmatory and/or complementary strategies. The diagnostic specificity and sensitivity of the FPA in FPSR conditions are currently unknown. The FPA should be standardised such that the OIE ELISA strong positive and weak positive sera consistently give positive results. Moreover, the cut-off should be established in the test population with appropriate validation techniques (see chapter 1.1.5).

3. Other tests

a) Brucellin skin test

An alternative immunological test is the brucellin skin test, which can be used for screening unvaccinated herds, provided that a purified (free of sLPS) and standardised antigen preparation (e.g. brucellin INRA) is used.

The diagnostic sensitivity and specificity of the FPA for bovine brucellosis are almost identical to those of the C-ELISA. The diagnostic specificity for cattle recently vaccinated with S19 is over 99% (Nielsen et al., 1996a). However, the specificity of FPA in FPSR conditions is currently unknown. Like all other serological tests, positive reactions should be investigated using suitable confirmatory and/or complementary strategies. The diagnostic sensitivity and specificity of the FPA in FPSR conditions are currently unknown. The FPA should be standardised such that the OIE ELISA strong positive and weak positive sera consistently give positive results. Moreover, the cut-off should be established in the test population with appropriate validation techniques (see chapter 1.1.5).

Not all infected animals react, therefore this test alone cannot be recommended as the sole diagnostic test or for the purposes of international trade.

It is essential to use a standardised, defined brucellin preparation that does not contain sLPS antigen, as this may provoke nonspecific inflammatory reactions or interfere with subsequent serological tests. One such preparation is brucellin INRA prepared from a rough strain of B. melitensis that is commercially available.

- Test procedure

  i) A volume of 0.1 ml of brucellin is injected intradermally into the caudal fold, the skin of the flank, or the side of the neck.

  ii) The test is read after 48–72 hours.

  iii) The skin thickness at the injection site is measured with vernier callipers before injection and at re-examination.

  iv) A strong positive reaction is easily recognised by local swelling and induration. However, borderline reactions require careful interpretation. Skin thickening of 1.5–2 mm would be considered as a positive reaction.

Although the brucellin intradermal test is one of the most specific tests in brucellosis (in unvaccinated animals), diagnosis should not be made solely on the basis of positive intradermal reactions given by a few animals in the herd, but should be supported by a reliable serological test. The intradermal inoculation of brucellin might induce a temporary anergy in the cellular immune response. Therefore an interval of 6 weeks is generally recommended between two tests on the same animal.

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4 Brucellergène OCB®, Synbiotics Europe, 2 rue Alexander Fleming, 69007 Lyon, France.
b) Serum agglutination test

While not recognised as a prescribed or alternative test, the SAT has been used with success for many years in surveillance and control programmes for bovine brucellosis. Its specificity is significantly improved with the addition of EDTA to the antigen (Garin et al., 1985; Lord et al., 1989; Nielsen et al., 1979).

The antigen represents a bacterial suspension in phenol saline (NaCl 0.85% [w/v] and phenol at 0.5% [v/v]). Formaldehyde must not be used. Antigens may be delivered in the concentrated state provided the dilution factor to be used is indicated on the bottle label. EDTA may be added to the antigen suspension to 5 mM final test dilution to reduce the level of false-positive results. Subsequently the pH of 7.2 must be readjusted in the antigen suspension.

The OIE ISS contains 1000 IU of agglutination. The antigen should be prepared without reference to the cell concentration, but its sensitivity must be standardised in relation to the OIE ISS in such a way that the antigen produces either 50% agglutination with a final serum dilution of 1/600 to 1/1000 or 75% aggregation with a final serum dilution of 1/500 to 1/750. It may also be advisable to compare the reactivity of new and previously standardised batches of antigen using a panel of defined sera.

The test is performed either in tubes or in microplates. The mixture of antigen and serum dilutions should be incubated for 16–24 hours at 37°C. If the test is carried out in microplates, the incubation time can be shortened to 6 hours. At least three dilutions must be prepared for each serum in order to refute prozone negative responders. Dilutions of suspect serum must be made in such a way that the reading of the reaction at the positivity limit is made in the median tube (or well for the microplate method).

Interpretation of results: The degree of Brucella agglutination in a serum must be expressed in IU per ml. A serum containing 30 or more IU per ml is considered to be positive.

c) Native hapten and cytosol protein-based tests

Native hapten tests are highly specific in S19 vaccination contexts, and have been used successfully in an eradication programme in combination with the RBT as a screening test (Asarta, 1989). The optimal sensitivity (close to that of CFT but lower than that of RBT and sLPS-based I-ELISAs) is obtained in a reverse radial immunodiffusion (RID) system in which the serum diffuses into a hypertonic gel containing the polysaccharide (Diaz et al., 1979; Jones et al., 1980). However, the double gel diffusion procedure is also useful (López-Gori et al., 2008; Lord & Cherwonogrodzky, 1992). Calves vaccinated subcutaneously with the standard dose of S19 at 3–5 months of age are negative 2 months after vaccination, and adult cattle vaccinated subcutaneously 4–5 months previously with the reduced dose of S19 do not give positive reactions unless the animals become infected and shed the vaccine in their milk (Jones et al., 1980). The conjunctival vaccination (both in young and adults) reduces the time to obtain a negative response in native hapten tests. A remarkable characteristic of the RID test is that a positive result correlates with Brucella shedding as shown in experimentally infected cattle and in naturally infected cattle undergoing antibiotic treatment (Joint FAO/WHO Expert Committee on Brucellosis, 1986). Precipitin tests using native hapten or Brucella cytosol proteins have also been shown to eliminate, in most cases, FPSR reactions caused by Yersinia enterocolitica O:9 and FPSR of unknown origin (Munoz et al., 2005).

d) Milk tests

An efficient means of screening dairy herds is by testing milk from the bulk tank. It should be borne in mind that in the last period of gestation, pregnant cows are dried and do not participate in the bulk tank sample. In contrast, these animals, if infected, are most likely to be positive by serological diagnosis. Therefore, immediately after parturition, bulk tank should be re-tested. Milk from these sources can be obtained cheaply and more frequently than blood samples and is often available centrally at dairies. When a positive test result is obtained, all cows contributing milk should be blood tested. The milk I-ELISA is a sensitive and specific test, and is particularly valuable for testing large herds. The milk ring test (MRT) is a suitable alternative if the ELISA is not available.

- Milk I-ELISA

As with the serum I-ELISA numerous variations of the milk I-ELISA are in use. Several commercial I-ELISAs are available that have been validated in extensive field trials and are in wide use. In the interests of international harmonisation, the three OIE ELISA Standard Sera should be used by national reference laboratories to check or calibrate the particular test method in question. The I-ELISA should be standardised such that the OIE ELISA strong positive standard when diluted 1/125 in negative serum and further diluted 1/10 in negative milk consistently tests positive. Bulk milk samples are generally tested at much lower dilutions than sera, i.e. undiluted to 1/2 to 1/10 in diluent buffer, with the remainder of the assay being similar.

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5 The detailed procedure can be obtained from the Brucellosis Laboratory, Centro de Investigacion y Tecnologia Agroalimentaria/Gobierno de Aragon, Avenida Montañana 930, 50059 Zaragoza, Spain.
to that described for serum. The C-ELISA should not be used to test whole milk but may be used with whey samples.

- **Milk ring test**

In lactating animals, the MRT can be used for screening herds for brucellosis. In large herds (> 100 lactating cows), the sensitivity of the test becomes less reliable. The MRT may be adjusted to compensate for the dilution factor from bulk milk samples from large herds. The samples are adjusted according to the following formula: herd size < 150 animals use 1 ml bulk milk, 150–450 use 2 ml milk sample, 451–700 use 3 ml milk sample. False-positive reactions may occur in cattle vaccinated less than 4 months prior to testing, in samples containing abnormal milk (such as colostrum) or in cases of mastitis. Therefore, it is not recommended to use this test in very small farms where these problems have a greater impact on the test results.

- **Antigen production**

MRT antigen is prepared from concentrated, killed *B. abortus* S99 or S1119-3 cell suspension, grown as described previously. It is centrifuged at, for example, 23,000 g for 10 minutes at 4°C, followed by resuspension in haematoxylin-staining solution. Various satisfactory methods are in use; one example is as follows: 100 ml of 4% (w/v) haematoxylin (Cl No. 75290) dissolved in 95% ethanol is added to a solution of ammonium aluminium sulphate (5 g) in 100 ml of distilled water and 48 ml of glycerol. 2 ml of freshly prepared 10% (w/v) sodium iodate is added to the solution. After standing for 30 minutes at room temperature, the deep purple solution is added to 940 ml of 10% (w/v) ammonium aluminium sulphate in distilled water. The pH of this mixture is adjusted to 3.1, and the solution must be aged by storage at room temperature in the dark for 45–90 days.

Before use, the staining solution is shaken and filtered through cotton wool. The packed cells are suspended in the staining solution at the rate of 1 g per 30 ml stain, and held at room temperature for 48 hours (some laboratories prefer to heat at 80°C for 10 minutes instead). The stained cells are then deposited by centrifugation, and washed three times in a solution of sodium chloride (6.4 g), 85% lactic acid (1.5 ml) and 10% sodium hydroxide (4.4 ml) in 1.6 litres of distilled water, final pH 3.0. The washed cells are resuspended at the rate of 1 g in 27 ml of a diluent consisting of 0.5% phenol saline, adjusted to pH 4.0 by the addition of 0.1 M citric acid (approximately 2.5 ml) and 0.5 M disodium hydrogen phosphate (approximately 1 ml) and maintained at 4°C for 24 hours. The mixture is filtered through cotton wool, the pH is checked, and the PCV is determined and adjusted to approximately 4%.

The sensitivity of the new batch should be compared with a previously standardised batch using a panel of samples of varying degrees of reaction prepared by diluting a positive serum in milk. The antigen should be standardised against the OIEISSL so that a 1/500 dilution is positive and 1/1000 dilution is negative. The antigen should be stored as recommended by the manufacturer but usually should be stored at 4°C.

The pH of the antigen should be between 3.3 and 3.7 and its colour should be dark blue. A little free stain in the supernatant of a centrifuged sample is permissible. When diluted in milk from a brucellosis-free animal, the antigen must produce a uniform coloration of the milk layer with no deposit and no coloration of the cream layer.

- **Test procedure**

The test is performed on bulk tank milk samples. If necessary, samples could be pretreated with preservative (0.1% formalin or 0.02% bronopol) for 2–3 days at 4°C prior to use.

i) Bring the milk samples and antigen to room temperature (20 ± 3°C); only sufficient antigen for the day’s tests should be removed from the refrigerator.

ii) Gently shake the antigen bottle well.

iii) The test is performed by adding 30–50 µl of antigen to a 1–2 ml volume of whole milk (the volume of milk may be increased for bulk samples from larger herds – see above "Milk ring test").

iv) The height of the milk column in the tube must be at least 25 mm. The milk samples must not have been frozen, heated, subjected to violent shaking or stored for more than 72 hours.

v) The milk/antigen mixtures are normally incubated at 37°C for 1 hour, together with positive and negative working standards. However, overnight incubation at 4°C increases the sensitivity of the test and allows for easier reading.

vi) A strongly positive reaction is indicated by formation of a dark blue ring above a white milk column. Any blue layer at the interface of milk and cream should be considered to be positive as it might be significant, especially in large herds.
vii) The test is considered to be negative if the colour of the underlying milk exceeds that of the cream layer.

viii) When the MRT is adjusted for large herd sizes (2 or 3 ml of milk used), 0.1 ml of pooled negative cream is added to the test tube and is followed by 30–50 µl of the ring test antigen. After mixing, the test is incubated and read in the same manner as the unadjusted MRT. The negative pooled cream is collected from the separation of composite, unpasteurised milk from a brucellosis negative herd of 25 or more cows.

e) Interferon gamma test

As the prevalence of brucellosis decreases, accuracy of serological tests becomes more important. False-positive reactions result in trace-backs and epidemiological investigations that are expensive and time consuming. Therefore, assays that eliminate FPSR will become more and more useful. In general, the interferon gamma test involves stimulation of lymphocytes in whole blood with a suitable antigen, in this case, Brucellin has been shown to work well and then measuring the resulting gamma interferon production by a capture ELISA (Kittelberger et al., 1997; Weynants et al., 1995; 1998). This test could be useful in the discrimination of FPSR but more specific antigens are needed and the protocol needs to be standardised.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

As mentioned previously, brucellosis is one of the most easily acquired laboratory infections, and strict safety precautions should be observed. Laboratory manipulation of live cultures of Brucella, including vaccine strains, is hazardous and must be done under containment level 3 or higher, as outlined in chapter 1.1.3, to minimise occupational exposure.

C1. Brucellin

Brucellin–INRA is an LPS-free extract from rough B. melitensis B115. This preparation does not provoke formation of antibodies reactive in BBAT, CFT or ELISA.

1. Seed management

a) Characteristics of the seed

Production of brucellin-INRA is based on a seed-lot system as described for antigens and vaccines. The original seed B. melitensis strain B115 for brucellin production should be propagated to produce a seed lot, which should be preserved by lyophilisation or freezing at liquid nitrogen temperature. It should conform to the properties of a pure culture of a rough strain of B. melitensis and must not produce smooth Brucella LPS. It should produce reasonable yields of a mixture of protein antigens reactive with antisera to smooth and rough Brucella strains.

b) Method of culture (Alton et al., 1988)

Brucella melitensis strain B115 is best grown in the liquid medium described above for fermenter culture. It may be grown by the batch or continuous method in a fermenter or in flasks agitated on a shaker. Purity checks should be made on each single harvest, and the organisms must be in the rough phase.

c) Validation as an in-vivo diagnostic reagent

Laboratory and field studies in France have confirmed that brucellin-INRA is safe, non-toxic and specific in action. The preparation contains 50–75% proteins, mainly of low molecular weight and 15–30% carbohydrate. It does not contain LPS antigens. Brucellin-INRA does not provoke inflammatory responses in unsensitised animals, and it is not in itself a sensitising agent. It does not provoke antibodies reactive in the standard serological tests for brucellosis. More than 90% of small ruminants infected with B. melitensis manifest delayed hypersensitivity to brucellin-NRA at some stage. The preparation is not recommended as a diagnostic agent for individual animals, but can be useful when used for screening herds. It is given to small ruminants in 100-µg doses by the intradermal route, and provokes a local delayed hypersensitivity reaction visible at 48–72 hours in sensitised animals. Positive reactions can be given by vaccinated as well as by infected animals (Pouillot et al., 1997; Saergerman et al., 1999).

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6 Obtainable from Institut National de la Recherche Agronomique (INRA), Laboratoire de Pathologie Infectieuse et Immunologie, 37380 Nouzilly, France.
2. Method of manufacture (Alton et al., 1988)

*Brucella melitensis* B115 cells are killed after culture by raising the temperature to 70°C for 90 minutes, cooled to 4°C, and harvested by centrifugation at 9000 g for 15 minutes at 4°C. The cells are washed in cold sterile distilled water and dehydrated by precipitating with three volumes of acetone at –20°C, and then allowed to stand at –20°C for 24-48 hours. After repeated washing in cold acetone, followed by a final rinse in diethyl ether, the cells are dried over calcium chloride and held at 4°C. The dried cells are subjected to a viability check. They are resuspended in sterile 2.5% sodium chloride to a final concentration of 5% (w/v) and agitated for 3 days at 4°C.

Bacterial cells are removed by centrifugation as above, and the supernatant is concentrated to one-fourth the volume by ultrafiltration on a Diaflo PM10 membrane (Amicon) and precipitated by the addition of three volumes of ice-cold ethanol. The mixture is held at 4°C for 24 hours and the precipitate is recovered by centrifugation, redissolved in sterile water, and dialysed to remove ethanol. After centrifugation at 105,000 g for 6 hours at 4°C, the supernatant material, comprising the unstandardised brucellin, is subjected to assays for protein and carbohydrate. It may be freeze-dried either as bulk material or after it has been dispensed into its final containers.

3. In-process control

The crude brucellin extract should be checked for sterility after acetone extraction, to ensure killing of *Brucella* cells, and again at the end of the process to check possible contamination. The pH and protein concentration should be determined, and identity tests should be performed on the bulk material before filling the final containers.

4. Batch control

a) Sterility

Allergen preparations should be checked for sterility as described in Chapter 1.1.7 *Tests for sterility and freedom from contamination of biological materials*.

b) Safety

Samples of brucellin from the final containers should be subjected to the standard sterility test. Brucellin preparations should also be checked for abnormal toxicity. Doses equivalent to 20 cattle doses (2 ml) should be injected intraperitoneally into a pair of normal guinea-pigs that have not been exposed previously to *Brucella* organisms or their antigens. Five normal mice are also inoculated subcutaneously with 0.5 ml of the brucellin to be examined. Animals are observed for 7 days, and there should be no local or generalised reaction to the injection.

Dermo-necrotic capacity is examined by intradermal inoculation of 0.1 ml of the product to be examined into the previously shaved and disinfected flank of three normal albino guinea-pigs that have not been exposed previously to *Brucella* organisms or their antigens. No cutaneous reaction should be observed. Absence of allergic and serological sensitisation is checked by intradermal inoculation of three normal albino guinea-pigs, three times every 5 days, with 0.1 ml of a 1/10 dilution of the preparation to be examined. A fourth similar injection is given, 15 days later, to the same three animals and to a control lot of three guinea-pigs of the same weight that have not been injected previously. The animals should not become seropositive to the standard tests for brucellosis (RBT, CFT) when sampled 24 hours after the last injection, and should not develop delayed hypersensitivity responses.

c) Potency

The potency of brucellin preparations is determined by intradermal injection of graded doses of brucellin into guinea-pigs that have been sensitised by subcutaneous inoculation of 0.5 ml of reference brucellin7 in Freund’s complete adjuvant from 1 to 6 months previously (the use of a live *Brucella* strain, for example Rev1 strain, is possible provided that it produces the same level of sensitisation). The erythematous reactions are read and measured at 24 hours and the titre is calculated by comparison with a reference brucellin8. This method is only valid for comparing brucellin preparations made according to the same protocol as the sensitising allergen. Initial standardisation of a batch of allergen and the sensitisation and titration in ruminants is described (Alton et al., 1988).

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7 A national French reference brucellin has been produced by INRA-PII (F-37380 Nouzilly, France) and is obtainable from the OIE Reference Laboratory for Brucellosis, Anses, 23 avenue du Général-de-Gaulle, 94706 Maisons-Alfort Cedex, France.

8 The statistical procedure can be obtained from the OIE Reference Laboratory for Brucellosis, Anses, 23 avenue du Général-de-Gaulle, 94706 Maisons-Alfort Cedex, France.
d) **Duration of sensitivity**

Duration of sensitivity is uncertain. Individual animals vary considerably in the degree of hypersensitivity manifested to brucellin. Animals in the very early stages of infection, or with long-standing infection, may not manifest hypersensitivity to intradermal injection.

e) **Stability**

The freeze-dried preparation retains full potency for several years. The liquid commercial preparation should retain potency for the recommended shelf-life.

f) **Preservatives**

The use of preservatives is not recommended when the preparation is freeze-dried. In the liquid form, sodium merthiolate (at most 0.1 mg/ml) may be used as a preservative. If freeze-dried, the preparation should not be reconstituted until immediately before use.

g) **Precautions (hazards)**

Brucellin is not toxic. Nevertheless it may provoke severe hypersensitivity reactions in sensitised individuals who are accidentally exposed to it. Care should be taken to avoid accidental injection or mucosal contamination. Used containers and injection equipment should be carefully decontaminated or disposed of by incineration in a suitable disposable container.

5. **Tests on final product**

a) **Safety**

A sterility test should be performed by the recommended method. The *in-vivo* safety tests are as those described for batch control (see Section C1.4.b). These tests on the batch may be omitted if the full test is performed on the final filling lots.

b) **Potency**

This is performed by injection of a single dose into guinea-pigs using the procedure described in Section C1.4.c.

C2. **Vaccines**

**Brucella abortus** strain 19 vaccine

The most widely used vaccine for the prevention of brucellosis in cattle is the *Brucella abortus* S19 vaccine, which remains the reference vaccine to which any other vaccines are compared. It is used as a live vaccine and is normally given to female calves aged between 3 and 6 months as a single subcutaneous dose of 5–8 × 10¹⁰ viable organisms. A reduced dose of from 3 × 10⁸ to 3 × 10⁹ organisms can be administered subcutaneously to adult cattle, but some animals will develop persistent antibody titres and may abort and excrete the vaccine strain in the milk (Stevens et al., 1994). Alternatively, it can be administered to cattle of any age as either one or two doses of 5 × 10⁹ viable organisms, given by the conjunctival route; this produces protection without a persistent antibody response and reduces the risks of abortion and excretion in milk when vaccinating adult cattle.

*Brucella abortus* S19 vaccine induces good immunity to moderate challenge by virulent organisms. The vaccine must be prepared from USDA-derived seed (see footnote 2 for address) and each batch must be checked for purity (absence of extraneous microorganisms), viability (live bacteria per dose) and smoothness (determination of dissociation phase). Seed lots for S19 vaccine production should be regularly tested for residual virulence and immunogenicity in mice.

Control procedures for this vaccine follow.

**Brucella abortus** strain RB51 vaccine

Since 1996, *B. abortus* strain RB51 has become the official vaccine for prevention of brucellosis in cattle in several countries (Schurig et al., 2002). However there is disagreement in regards to how the efficiency of strain RB51 compares to protection induced by S19 in cattle (Moriyon, 2002; Moriyon et al., 2004; Stevens et al., 1994; 1995; Uzal et al., 2000). Each country uses slightly different methods to administer the vaccine. In the USA, calves are vaccinated subcutaneously between the ages of 4 and 12 months with 1–3.4 × 10¹⁰ viable strain RB51
organisms. Vaccination of cattle over 12 months of age is carried out only under authorisation from the State or Federal Animal Health Officials, and the recommended dose is $1–3 \times 10^9$ viable strain RB51 organisms (Olsen, 2000; USDA, 2003). In other countries, it is recommended to vaccinate cattle as calves (4–12 months of age) with a $1–3.4 \times 10^{10}$ dose, with revaccination from 12 months of age onwards with a similar dose to elicit a booster effect and increase immunity (Samartino et al., 2000; Schurig et al., 2002).

It has been reported that full doses of RB51 when administered intravenously in cattle induce severe placentitis and placental infection in most vaccinated cattle (Palmer et al., 1996), and that there is excretion in milk in a relevant number of vaccinated animals. Field experience also indicates that it can induce abortion in some cases if applied to pregnant cattle. Due to these observations, vaccination of pregnant cattle should be avoided. One way to reduce the side effects of RB51 is to reduce the dose. When using the reduced dose of this vaccine ($1 \times 10^9$ colony-forming units [CFU]), on late pregnant cattle, no abortions or placental lesions are produced in subcutaneously vaccinated cattle (Palmer et al., 1997), but the vaccine strain can be shed by a significant proportion of vaccinated animals (Stevens et al., 1994). However, this reduced dose does not protect against B. abortus when used as a calfhood vaccination (Olsen, 2000), but does protect when used as an adult vaccine (Olsen, 2002).

It should be emphasised that RB51, as well as S19, can infect humans and cause undulant fever if not treated (Villarroel et al., 2000; WHO, 2004). There have been limited studies with RB51 in humans but it appears that the risk of developing undulant fever after exposure is low (Ashford et al., 2004; USDA, 2003; Villarroel et al., 2000). The diagnosis of the infection produced by RB51 requires special tests not available in most hospitals. Physicians making decisions on prophylactic treatment for accidental exposure to RB51 should be informed that this vaccine strain is highly resistant to rifampicin, one of the antibiotics of choice for treating human brucellosis.

Control procedures for this vaccine follow.

**Brucella melitensis strain Rev.1 vaccine**

It is not infrequent to isolate B. melitensis in cattle in countries with a high prevalence of this infection in small ruminants (Verger, 1985). There has been some debate on the protective efficacy of S19 against B. melitensis infection in cattle and it has been hypothesised that Rev.1 should be a more effective vaccine in these conditions. However there is very little information related to this issue (Joint FAO/WHO Expert Committee on Brucellosis, 1986; Van Drimmelen & Horwell, 1964). Evidence proving that S19 is able to control B. melitensis at the field level is also scanty (Jimenez et al., 1991). No experiments have been reported showing the efficacy of Rev.1 against B. melitensis infection in cattle. Moreover, the safety of this vaccine is practically unknown in cattle. Until the safety of Rev.1 in cattle of different physiological status and efficacy studies against B. melitensis under strictly controlled conditions are performed, this vaccine should not be recommended for cattle.

1. **Seed management**

   a) **Characteristics of the seed**

   Brucella abortus S19 original seed for vaccine production must be obtained from the USDA (see footnote 2 for address), and used to produce a seed lot that is preserved by lyophilisation or by freezing at liquid nitrogen temperature. The properties of this seed lot must conform to those of a pure culture of a CO₂-independent B. abortus biovar 1 that is also sensitive to benzylpenicillin, thionin blue and i-erythritol at recommended concentrations, and that displays minimal pathogenicity for guinea-pigs.

   Brucella abortus RB51 original seed for vaccine production is available commercially 9. These companies have legal rights to the vaccine.

   b) **Method of culture**

   Brucella abortus S19 for vaccine production is grown on medium free from serum or other animal products, under conditions similar to those described above for B. abortus S99 or S1119-3 (Alton et al., 1988).

   Brucella abortus strain RB51 follows similar culture methods.

   c) **Validation as a vaccine**

   Numerous independent studies have confirmed the value of S19 as a vaccine for protecting cattle from brucellosis. The organism behaves as an attenuated strain when given to sexually immature cattle. In rare

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9 Colorado Serum Company, 4950 York Street, P.O. Box 16428, Denver, Colorado 80216-0428, USA; or Veterinary Technologies Corporation, 1872 Pratt Drive, Suite 1100B, Blacksburg, Virginia 24060, USA.
cases, it may produce localised infection in the genital tract. Antibody responses persisting for 6 months or longer are likely to occur in a substantial proportion of cattle that have been vaccinated subcutaneously with the standard dose as adults. Some of the cattle vaccinated as calves may later develop arthropathy, particularly of the femor-o-tibial joints (Bracewell & Corbel, 1980; Corbel et al., 1989). The vaccine is safe for most animals if administered to calves between 3 and 6 months of age. It may also be used in adult animals at a reduced dose. It produces lasting immunity to moderate challenge with virulent \textit{B. abortus} strains, but the precise duration of this is unknown. The length of protection against \textit{B. melitensis} is unknown. The vaccine strain is stable and reversion to virulence is extremely rare. It has been associated with the emergence of i-erythritol-using strains when inadvertently administered to pregnant animals. The organism behaves as an attenuated strain in mice, and even large inocula are rapidly cleared from the tissues.

Reports from both experimental challenge studies and field studies remain controversial as far as the value of \textit{B. abortus} strain RB51 in protecting cattle from brucellosis is concerned (see above). The organism is attenuated in calves but not always in adults. \textit{Brucella abortus} strain RB51 contains minimally expressed OPS and there is no serological conversion in RBT and CFT in vaccinated animals. In addition, it has also been reported that RB51 does not induce detectable antibodies, using current testing procedures, to the OPS antigen (USDA, 2003). However, the presence of common core epitopes in both sLPS and OPS antigenic preparations does not allow the response to RB51 to be distinguished from the response to S strains, no matter which I-ELISA is used (Mainar-Jaime et al., 2008). RB51 produces immunity to moderate challenge with virulent strains, but the precise duration of this is unknown. The vaccine is very stable and no reversion to smoothness has been described \textit{in vivo} or \textit{in vitro}. The organism behaves as an attenuated strain in a variety of animals including mice where it is rapidly cleared from the tissues.

S19 and RB51 vaccines have some virulence for humans, and infections may follow accidental inoculation with the vaccine. Care should be taken in its preparation and handling, and a hazard warning should be included on the label of the final containers. In any case, accidental inoculations should be treated with appropriate antibiotics (see Section C2.4.g).

2. Method of manufacture

For production of S19 vaccine, the procedures described above can be used, except that the cells are collected in PBS, pH 6.3, and deposited by centrifugation or by the addition of sodium carboxymethyl cellulose at a final concentration of 1.5 g/litre. The yield from one fermenter run or the pooled cells from a batch of Roux flask cultures that have been inoculated at the same time from the same seed lot constitutes a single harvest. More than one single harvest may be pooled to form a final bulk, which is used to fill the final containers of a batch of vaccine. Before pooling, each single harvest must be checked for purity, cell concentration, dissociation and identity. A similar range of tests must be done on the final bulk, which should have a viable count of between 8 and 24 × 10^9 CFU/ml. Adjustments in concentration are made by the addition of PBS for vaccine to be dispensed in liquid form, or by the addition of stabiliser for lyophilised vaccine. If stabiliser is to be used, loss of viability on lyophilisation should be taken into account, and should not be in excess of 50%. The final dried product should not be exposed to a temperature exceeding 35°C during drying, and the residual moisture content should be 1–2%. The contents must be sealed under vacuum or dry nitrogen immediately after drying, and stored at 4°C.

The production process for \textit{B. abortus} strain RB51 is very similar to the one used for S19.

3. In-process control

\textit{Brucella abortus} S19 vaccine should be checked for purity and smoothness during preparation of the single harvests. The cell concentration of the bulks should also be checked. This can be done by opacity measurement, but a viable count must be performed on the final filling lots. The identity of these should also be checked by agglutination tests with antiserum to \textit{Brucella} A antigen. The viable count of the final containers should not be less than 50 × 10^6 per standard subcutaneous dose (5 × 10^6 for conjunctival dose) after lyophilisation, if this is to be done, and at least 95% of the cells must be in the smooth phase.

\textit{Brucella abortus} strain RB51 vaccine should be checked for purity and roughness during preparation of the single harvests. The cell concentration of the bulks should also be checked. A viable count must be performed on the final filling lots. The viable count of the final containers should be 1–3.4 × 10^{10} viable CFU of RB51 per dose (dose of 2 ml to be applied subcutaneously) and 100% of the cells must be in the rough phase. All colonies should be negative on dot-blot assays with MAbs specific for the OPS antigen.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.
b) Safety

The S19 vaccine is a virulent product per se, and it should keep a minimal virulence to be efficient (see Section C2.4.c). However a safety test is not routinely done. If desired, when a new manufacturing process is started and when a modification in the innocuousness of the vaccine preparation is expected, it may be performed on cattle. This control should be done as follows: the test uses 12 female calves, aged 4–6 months. Six young females are injected with one or three recommended doses. Each lot of six young females are kept separately. All animals are observed for 21 days. No significant local or systemic reaction should occur. If, for a given dose and route of administration, this test gives good results on a representative batch of the vaccine, it does not have to be repeated routinely on seed lots or vaccine lots prepared with the same original seed and with the same manufacturing process. A safety test on S19 vaccine may also be performed in guinea-pigs. Groups of at least ten animals are given intramuscular injections of doses of vaccine diluted in PBS, pH 7.2, to contain $5 \times 10^9$ viable organisms. The animals should show no obvious adverse effects and there must be no mortality.

If this safety test has been performed with good results on a representative seed lot or batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and using the same manufacturing process.

A safety test on B. abortus strain RB51 vaccine is not routinely done. If desired, 8–10-week-old female Balb/c mice can be injected intraperitoneally with $1 \times 10^8$ CFUs and the spleens cultured at 6 weeks post-inoculation. Spleens should be free from RB51 and the mice should not develop anti-OPS antibodies.

c) Potency

- S19 vaccine

An S19 vaccine is efficient if it possesses the characteristics of the S19 original strain, i.e. if it is satisfactory with respect to identity, smoothness, immunogenicity and residual virulence (Bosseray, 1993). Batches should also be checked for the number of viable organisms.

- Identity

The reconstituted S19 vaccine should not contain extraneous microorganisms. Brucella abortus present in the vaccine is identified by suitable morphological, serological and biochemical tests and by culture. Brucella abortus S19 has the normal properties of a biovar 1 strain of B. abortus, but does not require CO$_2$ for growth, does not grow in the presence of benzylpenicillin (3 µg/ml = 5 IU/ml), thionin blue (2 µg/ml), and i-erythritol (1 mg/ml) (all final concentrations).

- Smoothness (determination of dissociation phase)

The S19 vaccine reconstituted in distilled water is streaked across six agar plates (serum–dextrose agar or trypticase–soy agar (TSA) with added serum 5% [v/v] or yeast extract 0.1 % [w/v) in such a manner that the colonies will be close together in certain areas, while semi-separated and separated in others. Slight differences in appearance are more obvious in adjacent than widely separated colonies. Plates are incubated at 37°C for 5 days and examined by obliquely reflected light (Henry’s method) before and after staining (three plates) with crystal violet (White & Wilson’s staining method).

Appearance of colonies before staining: S colonies appear round, glistening and blue to blue-green in colour. R colonies have a dry, granular appearance and are dull yellowish-white in colour. Mucoid colonies (M) are transparent and greyish in colour and can be distinguished by their slimy consistency when touched with a loop. Intermediate colonies (I), which are the most difficult to classify, have an appearance intermediate between S and R forms: they are slightly opaque and more granular than S colonies.

Appearance of colonies after staining with crystal violet: S colonies do not take up the dye. Dissociated colonies (I, M, or R) are stained various shades of red and purple and the surface may show radial cracks. Sometimes a stained surface film slips off a dissociated colony and is seen adjacent to it.

The colony phase can be confirmed by the acriflavine agglutination test (Alton et al., 1988). S colonies remain in suspension, whereas R colonies are agglutinated immediately and, if mucoid, will form threads. Intermediate colonies may remain in suspension or a very fine agglutination may occur.

- Enumeration of live bacteria

Inoculate each of at least five plates of tryptose, serum–dextrose or other suitable agar medium with 0.1 ml of adequate dilutions of the vaccine spread with a sterile glass, wire or plastic spreader. CFU per vaccine volume unit are enumerated.
• Residual virulence (50% persistence time or 50% recovery time) (Bosseray, 1993; Diaz et al., 1979; Grillo et al., 2000; Pouillot et al., 2004)
  i) Prepare adequate suspensions of both the *B. abortus* S19 seed lot or batch to be tested (test vaccine) and the S19 original seed culture (as a reference strain). For this, harvest a 24–48 hours growth of each strain in sterile buffered saline solution (BSS: NaCl 8.5 g; KH₂PO₄ 1.0 g; K₂HPO₄ 2.0 g; distilled water 1000 ml; pH 6.8) and adjust the suspension in BSS to 10⁸ CFU/ml using a spectrophotometer (0.170 OD when read at 600 nm). The exact number of CFU/ml should be checked afterwards by plating serial tenfold dilutions on to adequate culture medium (blood agar base or TSA are recommended).

  ii) Inject subcutaneously 0.1 ml (10⁸ CFU/mouse) of the suspension containing the test vaccine into each of 32 female CD1 mice, aged 5–6 weeks. Carry out, in parallel, a similar inoculation in another 32 mice using the suspension containing the S19 reference strain. The original seed S19 strain, which has been shown satisfactory with respect to immunogenicity and/or residual virulence, can be obtained from USDA (see footnote 2 for address).

  iii) Kill the mice by cervical dislocation, in groups of eight selected at random 3, 6, 9 and 12 weeks later.

  iv) Remove the spleens and homogenise individually and aseptically with a glass grinder (or in adequate sterile bags with the Stomacher) in 1 ml of sterile BSS.

  v) Spread each whole spleen suspension *in toto* on to several plates containing a suitable culture medium and incubate in standard *Brucella* conditions for 5–7 days (lower limit of detection: 1 bacterium per spleen). An animal is considered infected when at least 1 CFU is isolated from the spleen.

  vi) Calculate the 50% persistence time or 50% recovery time (RT₅₀) by the SAS® statistical method specifically developed for RT₅₀ calculations (to obtain the specific SAS® file see footnote 5 for address). For this, determine the number of cured mice (no colonies isolated in the spleen) at each slaughtering point time (eight mice per point) and calculate the percentage of cured accumulated mice over time, by the Reed and Muench method (described in Bonet-Maury et al., 1954). The function of distribution of this percentage describes a sigmoid curve, which must be linearised for calculating the RT₅₀ values, using the computerised PROBIT procedure of the SAS® statistical package.

  vii) Compare statistically the parallelism (intercept and slope) between the distribution lines obtained for both tested and reference S19 strains using the SAS® file specifically designed for this purpose. Two RT₅₀ values can be statistically compared exclusively when they come from parallel distribution lines. If parallelism does not exist, the residual virulence of the tested strain should be considered inadequate, and discarded for vaccine production.

  viii) If the parallelism is confirmed, compare statistically the RT₅₀ values obtained for both tested and reference S19 strains using a SAS® file specifically designed for this purpose. To be accepted for vaccine production, the RT₅₀ obtained with the tested strain should not differ significantly from that obtained with the reference S19 strain (RT₅₀ and confidence limits are usually around 7.0 ± 1.3 weeks).

The underlying basis of the statistical procedure for performing the above residual virulence calculations have been recently described in detail (Bonet-Maury et al., 1954; Bosseray, 1992; 1993). Alternatively, the statistical calculations described in steps vi) to viii) can be avoided by an easy-to-use specific HTML-JAVA script program (Rev2) developed and available free at: http://www.anses.fr/RT50/REV-2 (Pouillot et al., 2004).

If this test has been done with good results on a representative seed lot or batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and using the same manufacturing process.

• Immunogenicity in mice (Bonet-Maury et al., 1954; Bosseray, 1992)

This test uses three groups of six female CD1 mice, aged 5–7 weeks, that have been selected at random.

  i) Prepare and adjust spectrophotometrically the vaccine suspensions as indicated above.

  ii) Inject subcutaneously a suspension containing 10⁵ CFU (in a volume of 0.1 ml/mouse) of the vaccine to be examined (test vaccine) into each of six mice of the first group.

  iii) Inject subcutaneously a suspension containing 10⁵ CFU of live bacteria of a reference S19 vaccine into each of six mice of the second group. The third group will serve as the unvaccinated control group and should be inoculated subcutaneously with 0.1 ml of BSS.
iv) The exact number of CFU inoculated should be checked afterwards by plating serial tenfold dilutions on to adequate culture medium (blood agar base or TSA are recommended).

v) All the mice are challenged 30 days after vaccination (and immediately following 16 hours’ starvation), intraperitoneally with a suspension (0.1 ml/mouse) containing 2 × 10^5 CFU of *B. abortus* strain 544 (*CO_2*-dependent), prepared, adjusted and retrospectively checked as above.

vi) Kill the mice by cervical dislocation 15 days later.

vii) Each spleen is excised aseptically, the fat is removed, and the spleen is weighed and homogenised. Alternatively, the spleens can be frozen and kept at –20°C for from 24 hours to 7 weeks.

viii) Each spleen is homogenised aseptically with a glass grinder (or in adequate sterile bags in Stomacher) in nine times its weight of BSS, pH 6.8 and three serial tenfold dilutions (1/10, 1/100 and 1/1000) of each homogenate made in the same diluent. Spread 0.2 ml of each dilution by quadruplicate in agar plates and incubate two of the plates in a 10% CO_2 atmosphere (allows the growth of both vaccine and challenge strains) and the other two plates in air (inhibits the growth of the *B. abortus* 544 *CO_2*-dependent challenge strain), both at 37°C for 5 days.

ix) Colonies of *Brucella* should be enumerated on the dilutions corresponding to plates showing fewer than 300 CFU. When no colony is seen in the plates corresponding to the 1/10 dilution, the spleen is considered to be infected with five bacteria. These numbers of *Brucella* per spleen are first recorded as *X* and expressed as *Y*, after the following transformation: *Y* = log (*X/log X*). Mean and standard deviation, which are the response of each group of six mice, are then calculated.

x) The conditions of the control experiment are satisfactory when: i) the response of unvaccinated mice (mean of *Y*) is at least of 4.5; ii) the response of mice vaccinated with the reference S19 vaccine is lower than 2.5; and iii) the standard deviation calculated on each lot of six mice is lower than 0.8.

xi) Carry out the statistical comparisons (the least significant differences [LSD] test is recommended) of the immunogenicity values obtained in mice vaccinated with the S19 strain to be tested with respect to those obtained in mice vaccinated with the reference vaccine and in the unvaccinated control group. The test vaccine would be satisfactory if the immunogenicity value obtained in mice vaccinated with this vaccine is significantly lower than that obtained in the unvaccinated controls and, moreover, does not differ significantly from that obtained in mice vaccinated with the reference vaccine. (For detailed information on this procedure, see footnote 5 for contact address.)

If this test has been done with good results on a representative batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and with the same manufacturing process.

- **RB51 vaccine**

As dosage (CFU) of the master seed was correlated to protection as part of licensure of RB51 for cattle in the USA, *in vivo* potency tests are not routinely conducted for serials of the RB51 vaccine. In the USA, plate counts of viable organisms have been approved and used as a measure of potency (this approach is identical to the potency test for S19 vaccine in the USA). A test in Balb/c female mice using 1 × 10^4 *B. abortus* strain 2308 organisms as the challenge strain has been proposed, but the correlation of this test to vaccine protection in cattle has not been completely determined. In the USA plate counts of viable organisms have been approved and used (Stevens *et al*., 1995). Rough vaccines for brucellosis have been discussed in some detail (Moriyon, 2002).

**d) Duration of immunity**

Vaccinating calves with a full dose of S19 vaccine is considered to give long-lasting immunity, and subsequent doses are not recommended. However, there is no proven evidence for this and revaccination could be advisable in endemic areas.

The duration of immunity induced by RB51 vaccine in cattle is unknown, whatever the dose applied and the age at vaccination.

**e) Stability**

*Brucella abortus* S19 vaccine prepared from seed stock from appropriate sources is stable in characteristics, provided that the in-process and batch control requirements described above are fulfilled, and shows no tendency to reversion to virulence. The lyophilised vaccine shows a gradual loss of viable count, but should retain its potency for the recommended shelf life. Allowance for this phenomenon is normally made by
ensuring that the viable count immediately following lyophilisation is well in excess of the minimum requirement. Maintenance of a cold chain during distribution of the vaccine will ensure its viability.

*Brucella abortus* strain RB51 has shown no tendency to revert to virulent smooth organisms after many passages *in vitro* or *in vivo*. This is probably due to the nature and place of the mutations found in this strain. *Brucella abortus* strain RB51, among other unknown mutations, has its wboA gene disrupted by an IS711 element impeding synthesis of OPS. Despite this, it has been reported that this strain accumulates low amounts of cytoplasmic M-like OPS (Cloeckaert et al., 2002b).

f) Preservatives

Antimicrobial preservatives must not be used in live S19 or *B. abortus* strain RB51 vaccines. For preparation of the lyophilised vaccine, a stabiliser containing 2.5% casein digest, e.g. Tryptone (Oxoid), 5% sucrose and 1% sodium glutamate, dissolved in distilled water and sterilised by filtration is recommended.

g) Precautions (hazards)

*Brucella abortus* S19 and RB51, although attenuated strains, are still capable of causing disease in humans. Accordingly cell cultures and suspensions must be handled under appropriate conditions of biohazard containment. Reconstitution and subsequent handling of the reconstituted vaccine should be done with care to avoid accidental injection or eye or skin contamination. Vaccine residues and injection equipment should be decontaminated with a suitable disinfectant (phenolic, iodophor or aldehyde formulation) at recommended concentration. Medical advice should be sought in the event of accidental exposure. The efficacy of the antibiotic treatment of infections caused by S19 and RB51 in humans has not been adequately established. If S19 contamination occurs, a combined treatment with doxycycline plus rifampicin could be recommended. In the case of contamination with RB51 (a rifampicin-resistant strain), the treatment with rifampicin should be avoided and a regimen of doxycycline plus streptomycin or gentamycin should be used except in pregnant women, which should be treated with trimethoprim and sulfa-methoxazole.

5. Tests of the final product

a) Safety

See Section C2.4.b. If this safety test has been performed with good results on a representative seed lot or batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and using the same manufacturing process.

b) Potency

Potency can also be determined on the final lyophilised product. The procedure is as described above in Section C2.4.c. If this potency test has been performed with good results on a representative seed lot or batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and using the same manufacturing process.

REFERENCES


Chapter 2.4.3. – Bovine brucellosis


Chapter 2.4.3. – Bovine brucellosis


Chapter 2.4.3. — Bovine brucellosis


Chapter 2.4.3. – Bovine brucellosis


* * *

NB: There are OIE Reference Laboratories for Bovine brucellosis
(see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list:
Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for bovine brucellosis.
CHAPTER 2.4.4.
BOVINE CYSTICERCOSIS

See Chapter 2.9.5. Cysticercosis

* * *
CHAPTER 2.4.5.

BOVINE GENITAL CAMPYLOBACTERIOSIS

SUMMARY

Definition of the disease: Bovine genital campylobacteriosis (BGC) is a venereal disease also known as bovine venereal campylobacteriosis (BVC). The causal agent of this sexually transmissible disease is Campylobacter fetus subsp. venerealis. The species is divided into two closely related subspecies: C. fetus subsp. venerealis and C. fetus subsp. fetus. By definition C. fetus subsp. venerealis is associated with BGC, causing fertility problems with considerable economic losses, particularly in endemic regions. Bovine infections with C. fetus subsp. fetus are associated with abortion and have a more sporadic occurrence.

Description of the disease: BGC is a venereal disease that is characterised by infertility, early embryonic death, and abortion. The disease is caused by C. fetus subsp. venerealis, a bacterium with pronounced tropism for the genital system of cattle. Transmission of the causal agent takes place mainly during natural mating, and the presence of C. fetus subsp. venerealis in the semen of bulls creates the risk of spread of the disease through artificial insemination.

Identification of the agent: Samples taken from bulls, cows or aborted fetuses can be analysed for the presence of the causal organism. The organism is a thin Gram-negative curved rod that may form S-shapes, seagull-shapes and spirals, and can be cultured at 37°C for at least 3 days in a microaerobic atmosphere. Confirmation of the isolate and discrimination between the subspecies of C. fetus can be performed by biochemical or molecular methods. Immunofluorescence may also be used to identify the organism, but it will not differentiate between different subspecies.

Serological tests: Enzyme-linked immunosorbent (ELISA) can be used for testing herd immunity, but is not suitable for diagnosis of the infection in individual animals. This test can not differentiate between infections caused by the two subspecies.

Requirements for vaccines and diagnostic biologicals: A vaccine may be prepared from C. fetus subsp. venerealis and/or C. fetus subsp. fetus that shares antigens with C. fetus subsp. venerealis. This vaccine is inactivated with formalin, and may be administered in an oil-emulsion adjuvant.

A. INTRODUCTION

1. Disease

Bovine genital campylobacteriosis (BGC, also known as bovine venereal campylobacteriosis [BVC]) is a venereal disease characterised by infertility, early embryonic death, and abortion in cattle. The causal agent of this sexually transmissible disease is Campylobacter fetus subsp. venerealis. It can be isolated from the genital tract of cattle (e.g. preputial smegma, vaginal mucus) or internal organs of aborted fetuses.

Campylobacter fetus is divided into the two closely related subspecies: C. fetus subsp. venerealis and C. fetus subsp. fetus (Véron & Chatelain, 1973). An intermediate biovar of C. fetus subsp. venerealis has been described. Whether this variant has specific clinical features is unclear. By definition C. fetus subsp. venerealis is associated with BGC, causing fertility problems with considerable economic losses particularly in endemic regions. Campylobacter fetus subsp. fetus can be recovered from the intestinal tract of cattle and other animal species (Garcia et al., 1983). Campylobacter fetus subsp. fetus can be isolated from aborted bovine fetuses showing its clinical relevance in cattle. However, C. fetus subsp. fetus is associated with sporadic cases of abortion in bovine whereas C. fetus subsp. venerealis is associated with endemic abortion and fertility problems in certain areas.
Although *C. fetus* is primarily recognised as a veterinary pathogen, *C. fetus* subsp. *fetus* is occasionally diagnosed as an opportunistic emerging pathogen in humans. Infections usually occur in pregnant or immunocompromised individuals and are often systemic with a variety of neurological and vascular complications (Thompson & Blaser, 2000).

### 2. Taxonomy

In 1991 a revision of the taxonomy and nomenclature of the genus *Campylobacter* was proposed. According to the Bergey’s Manual, the genus *Campylobacter* comprises sixteen species and six subspecies. More recently, two additional species have been proposed. Two subspecies of *C. fetus* have been recognised. Although the clinical signs of two subspecies overlap, they were originally defined by the differences in clinical presentation (Sebald & Véron, 1963; Véron & Chatelain, 1973). The two subspecies can be differentiated in the laboratory by one biochemical trait: glycine tolerance. Subspecies *venerealis* is considered as glycine sensitive and subspecies *fetus* as glycine tolerant. *Campylobacter fetus* subsp. *venerealis* biovar *intermedius* strains have been described (Salama *et al*., 1992), yet their taxonomic position needs to be clarified. On the basis of protein-banding patterns using polyacrylamide gel electrophoresis (PAGE) of whole cell proteins, no discrimination can be made between the two *C. fetus* subspecies (Vandamme *et al*., 1990). Studies of DNA–DNA hybridisation have failed to reveal any major difference between the *venerealis* and *fetus* subspecies (Harvey & Greenwood, 1983). However, several molecular methods have been shown to be able to differentiate the two subspecies, including polymerase chain reaction (PCR) (Hum *et al*., 1997; Tu *et al*., 2005; van Bergen *et al*., 2005c; Wang *et al*., 2002), PFGE (pulsed-field gel electrophoresis) (On & Harrington, 2001), multilocus sequence typing (MLST) (van Bergen *et al*., 2005a) and amplified fragment length polymorphism (AFLP) (Wagenaar *et al*., 2001) (see also Section B.1.h).

### B. DIAGNOSTIC TECHNIQUES

1. **Isolation and identification of the agent (the prescribed test for international trade)**

**a) Collection of samples**

i) **Male: preputial smegma and semen**

In bulls, smegma may be obtained by different methods: scraping (Tedesco *et al*., 1977), aspiration (Campero *et al*., 2003), and washing (Clarke & Dufty, 1978). Smegma is commonly collected by scraping and can be used for isolation of the bacteria, or is rinsed into a tube with approximately 5 ml of phosphate buffered saline (PBS) with 1% of formalin for immunofluorescence (IFAT) diagnosis. Smegma can also be collected from the artificial vagina after semen collection, by washing the artificial vagina with 20–30 ml of PBS.

For preputial washing, 20–30 ml of PBS is introduced into the preputial sac. After vigorous massage for 15–20 seconds, the infused liquid is collected.

Semen is collected under conditions that are as aseptic as possible. Semen samples must be diluted with PBS and are sown directly onto culture medium or transport and enrichment medium.

ii) **Female: (cervico) vaginal mucus (CVM)**

Samples may be obtained by aspiration, or washing the vaginal cavity.

For aspiration, the vulva region is cleaned with a tissue paper, and an artificial insemination (AI) pipette or Cassou pipette (blue sheath type) is inserted into the vaginal cavity so that the anterior reaches the cervix (Campero *et al*., 2003). Gentle suctioning is applied while moving the pipette gently backwards and forwards. The pipette is removed, and the collected mucus is sown directly onto culture medium or transport and enrichment medium.

CVM may also be collected by washing the vaginal cavity: 20–30 ml of PBS is infused into the cavity through a syringe attached to an AI pipette. The fluid is sucked out and re-infused four to five times before being collected and spread directly on to culture medium or added to transport and enrichment medium. Washing fluid in the vaginal cavity may also be collected by a tampon or gauze held inside the vagina for 5–10 minutes after PBS infusion. Samples of CVM obtained by suction may be diluted with PBS, or sown directly onto culture medium or transport and enrichment medium.

CVM is transferred into approximately 5 ml of PBS with 1% of formalin.

iii) **Aborted fetuses, placentas**

The placenta as well as the liver, lungs and stomach contents of the fetus provide the best samples for isolation of the causative bacteria. Samples are inoculated directly in transport and enrichment medium, or into PBS with 1% formalin for IFAT testing.
b) Transport of samples

The use of a transport medium is essential if the samples are not processed in the laboratory within the same day after collection. For dispatch to the laboratory, if the samples are not in transport medium, the samples must be placed in an insulated container (within the temperature range 4–10°C), and protected from light.

Various transport and enrichment media are available, such as Clark’s, Lander’s, SBL, Foley’s and Clark’s, Weybridge’s, Cary-Blair’s (Garcia et al., 1984; Hum et al., 1994; Monke et al., 2002).

Some of the transport and enrichment media mentioned above contain cycloheximide. Because of its potential toxicity, amphotericin B can be used as an alternative.

c) Treatment of samples

On arrival at the laboratory, samples should be inoculated directly onto culture medium, or processed further if required.

i) Genital tract samples

Preputial washings may be centrifuged (3500 g) to concentrate the sample. The final sample (reduced to 250 µl) may be inoculated onto the culture medium (directly and/or using the filter method).

If the CVM is not very viscous it can be inoculated directly or diluted with an equal volume of PBS. When the CVM is very viscous, it may be necessary to liquefy it by adding an equal volume of cysteine solution (aqueous solution of cysteine hydrochloride at 0.25 g/100 ml, pH 7.2, sterilised by membrane filtration). After 15–20 minutes, the diluted and liquefied mucus can then be inoculated onto isolation medium.

ii) Aborted fetuses, placentas

Fetal stomach contents are inoculated directly onto culture medium. Internal organs or pieces of organs are flamed to disinfect the surface, and are subsequently homogenised. The homogenate is inoculated onto culture medium.

After washing placental membranes with PBS to eliminate the majority of the surface contamination, the chorionic villi are scraped and the scrapings are transferred to culture medium.

d) Isolation of Campylobacter fetus

i) Culture media for isolation

Many media are currently in use for the bacteriological diagnosis of BGC. It should be noted that several media used for the isolation of Campylobacter spp. are not suitable for the isolation of C. fetus due to antimicrobials (e.g. cephalosporins) that may inhibit C. fetus growth (van Bergen et al., 2005b). Most culture media contain cycloheximide. Because of its potential toxicity, this antifungal agent can be replaced by amphotericin B. The recommended selective medium for isolation of C. fetus is Skirrow’s. Skirrow’s medium is a blood-based medium with 5–7% (lysed) defibrinated blood and contains the selective agents: polymyxin B sulphate (2.5 IU/ml), trimethoprim (5 µg/ml), vancomycin (10 µg/ml), and cycloheximide (50 µg/ml).

Alternatively, a non-selective blood-based (5–7% blood) medium in combination with filtration (0.65 µm) can be used; however, it may be less sensitive when compared with a selective medium.

Quality control of each batch of media should be performed using control strains.

ii) Incubation conditions

Plates are incubated at 37°C and under microaerobic atmosphere of 5–10% oxygen, 5–10% carbon dioxide and preferably 5–9% hydrogen for optimal growth (Vandamme, 2000). Appropriate microaerobic conditions may be produced by a variety of methods. In some laboratories the suitable atmosphere is created by a gas replacement in a jar. Gas generator kits are also available from commercial sources. Variable atmosphere incubators can also be used.

Conditions of culture and incubation are systematically verified by using control strains of C. fetus subsp. fetus and C. fetus subsp. venerealis. Such controls should be set up for each isolation attempt.
e) Identification of *Campylobacter* species

i) Colony morphology

Colonies of *C. fetus* usually appear on culture media after 2–5 days. To prevent overgrowth of specific colonies by contaminants, it is recommended that the media be evaluated daily and suspicious colonies be subcultured. After 3–5 days of incubation, colonies measure 1–3 mm in diameter. They are slightly grey-pink, round, convex, smooth and shiny, with a regular edge.

ii) Macroscopic morphology

*Campylobacter* is motile, a property that may disappear during sub-culturing. *Campylobacter* often takes the form of a thin, curved bacillus, 0.3–0.4 µm wide and 0.5–8.0 µm long. Short forms (comma-shaped), medium forms (S-shaped), and long forms (helical with several spirals) may be observed simultaneously in the living state. Old cultures may contain coccoid bacteria.

iii) Biochemical tests: see Table 1.

iv) Atmosphere: *Campylobacter* does not grow under aerobic conditions.

f) Immunological identification of *Campylobacter fetus*

The IFAT can be applied to identify the organism directly from samples or to confirm the identification of a strain after isolation. It can not differentiate between different subspecies.

i) Preparation of immune sera

*Campylobacter* strains, preferably standard strains from recognised culture collections (*C. fetus* subsp. *venerealis* or *C. fetus* subsp. *fetus*), are grown on blood-based medium at 37°C under microaerobic conditions for 3 days. The organisms are harvested into PBS, and washed twice by centrifugation. Rabbits aged 3 months are inoculated intramuscularly with 2 ml of 10^11 organisms/ml of a *C. fetus* subspecies resuspended in PBS and Freund's incomplete adjuvant. Inocula are administered at four sites, 0.5 ml at each site. The animals are bled before inoculation and at weekly intervals thereafter. When the serum titres reach high levels, as estimated by the immunofluorescence test or agglutination test, 0.1–1.0 ml of 10^10 viable organisms/ml are injected intravenously. The rabbits are bled for serum 7 days later. Heterologous sera are pooled. In a recent study, a conjugate prepared from chicken IgY was described as an alternative to rabbit antibodies. Monoclonal antibodies that can be used for immunodiagnostic detection of *C. fetus* have been described (Brooks *et al.*, 2002).

ii) Preparation of conjugates

Conjugates are prepared as described by Harlow & Lane (1988). The working dilution of the conjugate is determined by checkerboard titration against smears of a *C. fetus* culture using positive and negative control dilutions, and selecting twice the lowest concentration that produces brilliant fluorescence with *C. fetus* bacteria.

iii) Sample preparation

The genital fluid (fetal abomasal content, preputial smegma or CVM) samples are rinsed into approximately 5 ml PBS 1% formalin. Two centrifugation steps are carried out. First, samples are centrifuged at 600 g for 10 minutes at 4°C to remove debris. Subsequently, the supernatant is centrifuged at 8000 g for 30 minutes at 4°C. The pellet is dissolved in ~100 µl remaining supernatant.

iv) Immunofluorescence test (Mellick *et al.*, 1965)

The sample (20 µl) is applied in duplicate to microscopic slides. The material is air-dried and fixed in acetone at −20°C for 30 minutes or ethanol at 18–25°C for 30 minutes. Glass slides will be air-dried and the fluorescein isothiocyanate isomer (FITC)-conjugated antiserum is added at the appropriate dilution. Staining is carried out in a humid chamber at 37°C for 30 minutes in dark condition. Subsequently, the slides are washed three times for 10 minutes in PBS. The slides are mounted in buffered glycerol (90% glycerol: 10% PBS). The cover-slips are sealed to prevent drying, and the slides are examined under ultraviolet light in an epifluorescent microscope. Positive and negative control slides will be used each time the test is done. *Campylobacter fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* reference strains are used as positive controls, and another *Campylobacter* species are used as negative control. Samples that show fluorescent bacteria presenting the typical morphology of *C. fetus* is considered positive.

g) Biochemical identification of *Campylobacter fetus* subspecies

Tests described in Table 1 must be done on pure cultures.
### Table 1. Differential characteristics of Campylobacter species potentially isolated from the bovine genital tract and aborted fetuses (according to Bergey’s Manual 2nd edition, 2005)

<table>
<thead>
<tr>
<th></th>
<th>25°C</th>
<th>42°C</th>
<th>Oxidase</th>
<th>Catalase</th>
<th>NaCl 3.5%</th>
<th>Glycine 1%</th>
<th>H₂S(b)</th>
<th>Nalidixic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. fetus subsp. venerealis</td>
<td>V</td>
<td>–</td>
<td>+</td>
<td>V</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>V</td>
</tr>
<tr>
<td>C. fetus subsp. fetus</td>
<td>+</td>
<td>V(a)</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>R</td>
</tr>
<tr>
<td>C. jejuni</td>
<td>–</td>
<td>V(d)</td>
<td>+</td>
<td>V(d)</td>
<td>–</td>
<td>V</td>
<td>–</td>
<td>S(e)</td>
</tr>
<tr>
<td>C. hyointestinalis</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>V</td>
</tr>
<tr>
<td>C. sputorum</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>V</td>
</tr>
</tbody>
</table>

(a) = Although C. fetus does not belong to the thermophilic Campylobacters, a considerable number of strains of this species grows at 42°C;
(b) = On triple sugar iron agar medium;
(c) C. jejuni subsp. jejuni is positive, C. jejuni subsp. doylei is negative;
(d) C. jejuni subsp. jejuni is positive, C. jejuni subsp. doylei is variable;
(e) according to Bergey’s Manual strains are sensitive, however resistant strains have frequently been reported;
(+)= positive reaction or growth and (–)= negative reaction or absence of growth of the strain on an appropriate medium under specified conditions (see Section B.1.d ii); V = variable results; S = sensitive; R = resistant.

i) Growth at 25°C and 42°C
A cell-suspension (~McFarland no. 1) is inoculated onto two blood-based medium-plates. Each plate is incubated under the specified atmospheric conditions (see Section B.1.d ii) at 25°C and 42°C. Control strains are tested in parallel.

ii) Oxidase and catalase
Tests are performed according to a standard bacteriological protocol. Control strains are tested in parallel.

iii) Growth in the presence of sodium chloride
A cell-suspension is inoculated onto blood medium containing 3.5% NaCl (15 ml of blood medium + 2.04 ml of 5 M sodium chloride solution), and on to plain blood medium. Incubation is performed under the specified atmospheric conditions (see Section B.1.d ii). Control strains are tested in parallel.

iv) Growth in the presence of 1% glycine
A cell-suspension (~McFarland no. 1) is inoculated onto a glycine medium (15 ml of blood-based medium + 1.65 ml of 10% aqueous solution of filter sterilised glycine), and onto the same medium without glycine. Incubation is performed under the specified atmospheric conditions (see Section B.1.d ii). Two control strains (of subspecies venerealis and fetus) are tested in parallel. As all strains are fastidious, small changes in media can be important, and lack of growth in the presence of glycine should be considered to be a presumptive test for C. fetus subsp. venerealis. The reproducibility of the assay is poor and intermediate strains have been described (Salama et al., 1992).

v) Hydrogen sulphide (H₂S) production in TSI medium
This hydrogen sulphide (H₂S) test is done on triple sugar iron agar (TSI) under the specified growth conditions (see Section B.1.d ii). The medium contains peptone (20 g/litre), meat extract (2.5 g/litre), yeast extract (3 g/litre), sodium chloride (5 g/litre), ferric citrate (0.5 g/litre), sodium thiosulphate (Na₂S₂O₃) (0.5 g/litre), lactose (10 g/litre), sucrose (10 g/litre), glucose (1 g/litre), phenol red (0.024 g/litre), agar (11 g/litre), and distilled water (to 1 litre). This medium is sterilised after distribution into tubes by autoclaving at 115°C for 15 minutes and are solidified to obtain a slope. A cell-suspension (~McFarland no. 1) is inoculated onto the slope and into the medium by a loop. A colour change from red to black indicates H₂S production. Control strains are tested in parallel.

vi) Hydrogen sulphide production (H₂S) in cysteine medium (not listed in the Table 1)
The H₂S test is done in a Brucella broth medium containing 0.02% cysteine. H₂S production is detected by a lead-acetate strip that is attached inside the top of the tube. A cell suspension (~McFarland no. 1) is inoculated into the medium. Blackening of the lead acetate strip is considered as a positive reaction. Control strains are tested in parallel.
vii) Sensitivity to cephalothin and nalidixic acid

Sensitivity to cephalothin (CN) and nalidixic acid (NA) is tested by the disks containing CN (30 µg) or NA (30 µg).

For the test, 72-hour cultures are suspended in PBS at a concentration of $10^9$ bacteria/ml. The culture medium is dried before the culture is deposited on the surface. Using the suspension, 100 µl are spread onto the basic blood medium. The sensitivity disks are then placed on top. These plates are incubated at 37°C in the specified atmosphere (see Section B.1.d.ii), and examined after 48 hours and 72 hours. A zone of inhibition of at least 3 mm around a disk indicates that the strain is sensitive to this antibiotic. All *C. fetus* subsp. *fetus* strains and most of the *C. fetus* subsp. *venerealis* strains are resistant to NA (On, 1996). All *C. fetus* are sensitive to CN (On, 1996).

h) Molecular identification of *Campylobacter fetus* subspecies

Several molecular methods for the identification of *C. fetus* subspecies have been described, including 16S sequencing (Gorkiewicz et al., 2003; On & Harrington, 2001), PFGE (On & Harrington, 2001), AFLP (Wagenaar et al., 2001), and MLST (van Bergen et al., 2005a). However, most of these methods are time consuming and/or require expensive apparatus and knowledge. Routine diagnostic laboratories would be served best by a simple PCR. Several PCRs have been claimed to be subspecies specific including those developed by Hum et al. (1997), Wang et al. (2002), and more recently by Tu et al. (2005) and Van Bergen et al. (2005c).

The multiplex PCR described by Hum et al. (1997) is currently the most cited PCR. It enables the amplification of a *C. fetus*-specific DNA fragment (approximately 200 bp smaller than the 960 bp described in the original publication), as well as a *C. fetus* subsp. *venerealis*-specific fragment. Thus, performance of this multiplex PCR allows differentiation of the two subspecies (*C. fetus* = one amplification product vs *C. fetus* subsp. *venerealis* = two amplification products). *Campylobacter fetus* subsp. *venerealis* biovar intermidii strains have not been evaluated in Hum’s study, but isolates identified as belonging to biovar intermidii with AFLP, classify in the PCR of Hum as either *C. fetus* subsp. *fetus* or *C. fetus* subsp. *venerealis* (van Bergen et al., 2005a). Comparison of this PCR against AFLP and MLST (van Bergen et al., 2005a) and against the glycine test (Willoughby et al., 2005) confirms that PCR can give false positive and negative reactions.

The PCR described by Wang et al. (2002) reveals only a *C. fetus* subsp. *fetus*-specific product. These results were obtained only for a very limited number of strains. Recent evaluations of its value for subspecies differentiation using larger sets of strains yielded both false positive and negative reactions (van Bergen et al., 2005c).

The random amplification of polymorphic DNA (RAPD)-PCRs described by Tu et al. (2005) are published only recently, and are apparently evaluated with a very limited number of *C. fetus* subsp. *venerealis* strains. Their value should be evaluated more extensively with a larger group of strains.

The recently described PCR by Van Bergen et al. (2005c) showed full consistency with the *C. fetus* subsp. *venerealis* as defined by AFLP and is therefore considered as the best PCR for detection method of *C. fetus* subsp. *venerealis* currently available. However, *C. fetus* subsp. *venerealis* biovar intermidii as defined by AFLP is not identified by this PCR.

2. Serological tests/antibody detection

An ELISA is available to detect antigen-specific secretory IgA antibodies in the vaginal mucus following abortion due to *C. fetus* subsp. *venerealis*. These antibodies are long lasting, and their concentration remains constant in the vaginal mucus for several months (Hum et al., 1991).

Initial sampling can be done after the early involution period (usually 1 week after abortion) when mucus becomes clear.

An ELISA for the detection of the serum humoral IgG response after vaccination is described.

a) Antigen preparation and coating

Cultures are transferred to PBS with 0.5% formalin for 1 hour, centrifuged at 17,000 g, washed twice with PBS, and then resuspended in 0.05 M carbonate buffer, pH 9.6. The final absorbance is adjusted to $\text{OD}_{610} = 0.21$. Flat-bottomed polystyrene microtitre plates coated with 10 µl of antigen are left overnight at 4°C, and then stored at -20°C. Before use, the plates are rinsed twice with distilled water and then tapped gently to remove moisture.
Chapter 2.4.5. – Bovine genital campylobacteriosis

- **Test procedure**
  i) Diluted vaginal mucus (100 µl) is added to each well, and the plate is incubated at 37°C for 2 hours. The plates are then washed as before, and 100 µl of rabbit anti-bovine IgA is added. After 2 hours incubation at 37°C, the plates are washed, and 100 µl of substrate is added (0.8 mg/µl 5 amino-salicylic acid; pH 6.0), immediately activated by the addition of 2% 1 M hydrogen peroxide). The plates are left at room temperature for 30 minutes and the reaction is stopped by the addition of 50 µl of 3 M sodium hydroxide. The absorbance is measured on an ELISA reader at 450 nm. Each sample is tested in duplicate, and positive and negative controls are included in each plate. The absorbance measurements yielded by the test sample are corrected for the absorbance measurement of positive and negative controls according to the formula:

\[
\text{Result} = \frac{\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{negative control}}}{\text{Absorbance}_{\text{positive control}} - \text{Absorbance}_{\text{negative control}}} \times 100
\]

The test is considered to be positive if the result is above 40. Vaccinated animals will not react to IgA ELISA as their vaginal mucus contains only IgG isotype antibodies.

**C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS**

Two groupings of antigens of *C. fetus* are recognised: the thermolabile 'H' flagellar antigens and the thermostable 'O' somatic antigens. In addition, a capsular 'K' antigen should be present. The K antigen is easily destroyed under *in vitro* conditions. The vaccine must incorporate these different antigens. Other vaccine preparations have also been described (Clarke et al., 1972). Experimental *C. fetus* subsp. *fetus* vaccine confers immunity against *C. fetus* subsp. *venerealis* because both strains share common antigens (Bouters et al., 1973), however, the addition of a second strain of *C. fetus* subsp. *venerealis* to the biological product is widely practised and strongly suggested. The presence of four to five heat-labile glycoprotein immunogens, shared by many *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* strains, is critical. The presence of such immunogens should be confirmed. The vaccine concentration (dry weight) should be around 40 mg protein per dose in order to have a good protection level.

In infected herds, all breeding animals (bulls, cows and heifers) will be vaccinated twice prior to the breeding season. In most of the cases, the vaccine reduces the length of the infection and carrier-cows can keep the infection from one season to the next. Bulls require two vaccine doses annually, because the vaccine may not always be effective in terminating established infections. The next year’s bulls and replacement heifers are vaccinated, and from the third year, bulls are vaccinated annually.

In non-infected herds, only the bulls are vaccinated annually, and this will be done twice a year (two doses with 21 days interval; 2 weeks before the start of the breeding season).

1. **Seed management**
   a) **Characteristics of the seed**

   The seed consists of a large, homogeneous batch of a culture of *C. fetus* subsp. *fetus* or *C. fetus* subsp. *venerealis* that has been thoroughly characterised as to identity and purity, preserved in small aliquots.

   b) **Method of culture**

   The initial growth of the seed is accomplished in semisolid medium. This consists of basal medium with the addition of 0.16% agar. Basal medium is composed of 2.8% *Brucella* broth, 0.5% yeast extract, 1.2% sodium succinate, and 0.001% calcium chloride. The initial culture is maintained for 3 days at 37°C under specified conditions (see Section B.1.d.ii). The growth is transferred to additional tubes with semisolid medium and incubated for 48 hours. The resulting growth is used for vaccine production.

   This culture should be stored at 4°C.

   c) **Validation as a vaccine**

   The seed must be free from contaminating organisms. The purity of the seed must be checked by a suitable culture method.
It is not practicable to test efficacy under laboratory conditions. It is determined in the field on the basis of epidemiological observations.

2. Method of manufacture

The working seed material is seeded into broth medium consisting of basal medium with the addition of 0.025% sodium thioglycollate. These cultures are incubated at 37°C for 24 hours while being shaken at a rate of 80 rpm. The fluids are harvested, and formaldehyde is added to a final concentration of 0.2% (0.74 g/litre).

The vaccine is mixed with an oil-emulsion adjuvant.

3. In-process control

The identity of the organism should be checked by culture and identification, as well as the absence of contaminating organisms.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological material may be found in chapter 1.1.7.

b) Safety

The inactivation process must be complete and the method to insure inactivation should be validated before it can safely be used. Inactivation is checked by inoculating the equivalent of one dose on to the same medium under the same conditions as those used in the production process. This culture is incubated under the same conditions for 72 hours, after which there should be no evidence of bacterial growth. The final product must also be shown to be free from viable bacterial and fungal contaminants, using suitable culture methods.

Two guinea-pigs are inoculated with 2 ml of the product, either intramuscularly or subcutaneously. They must not have an adverse reaction attributable to the vaccine during a 7-day observation period following inoculation.

c) Potency

Potency of the vaccine may be measured by seroconversion in rabbits. Their serum titres are measured by immunofluorescence or by the tube agglutination test. Five rabbits, serologically negative at 1/100 serum dilution, are vaccinated twice subcutaneously with half the dose used in cattle, at an interval of 14 days. Serum from at least four of the five rabbits, collected 14 days after the second vaccination, must show at least a four-fold increase in titre.

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

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REFERENCES

Chapter 2.4.5. — Bovine genital campylobacteriosis


**Chapter 2.4.5. – Bovine genital campylobacteriosis**

**NB:** There is an OIE Reference Laboratory for bovine genital campylobacteriosis (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)).

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for bovine genital campylobacteriosis.
CHAPTER 2.4.6.

BOVINE SPONGIFORM ENCEPHALOPATHY

SUMMARY

Bovine spongiform encephalopathy (BSE) is a fatal neurological disease of adult cattle that was first recognised in Great Britain (GB). It is a transmissible prion disease. The archetype for this group of diseases is scrapie of sheep and goats (see Chapter 2.7.12 Scrapie).

The epizootic of BSE can be explained by oral exposure to a scrapie-like agent in the ruminant-derived protein of meat-and-bone meal included in animal feedstuffs. Initial cases of BSE in some countries were considered to be the result of exports from GB of infected cattle or contaminated meat-and-bone meal, although exports from other countries are now implicated. In others, initial cases have no clear link with imported meat-and-bone meal, suggesting that earlier, undetected indigenous and possibly spontaneous cases may have occurred. As a result of control measures, the epizootics are in decline. Cases of BSE have been detected in most European countries, in North America and in a few Asian countries.

Experimental transmissibility of BSE to cattle has been demonstrated. The BSE agent is also believed to be the common source, via dietary routes, of transmissible spongiform encephalopathies (TSEs) in other ruminant species and felidae. There is evidence of a causal link between the BSE agent and the variant Creutzfeldt-Jakob disease (vCJD) in humans. Recommendations for handling BSE-infected material assume that BSE is a zoonosis and a containment category 3 (with derogation) has been ascribed.

Identification of the agent: Clinical BSE has a peak incidence in cattle aged between 4 and 5 years. The clinical course is variable but can extend to several months. Overt clinical signs are distinctive, if differential diagnoses can be eliminated. Early clinical signs may be subtle and mostly behavioural, and may lead to disposal of affected animals before suspicion of BSE is triggered. In countries with a statutory policy toward the disease, clinically suspect cases must be killed, the brain examined and the carcass destroyed. Now, in most countries, active surveillance at abattoirs identifies infected cattle slaughtered prior to the onset of clinical signs, and the screening of fallen stock identifies cases in which there may have been unrecognised clinical signs. No diagnostic test is currently available for live animals. The nature of the agents causing the TSE is unclear. A disease-specific partially protease-resistant, misfolded isoform of a membrane protein PrP^C, originally designated PrP^Sc, has a critical importance in the pathogenesis of these diseases and according to the prion hypothesis is the principal or sole component of the infectious agent. Confirmation of the diagnosis, formerly by histopathological examination of the brain, is now, therefore, by the application of immunohistochemical (IHC) and/or immunochromical methods to brain tissue for the detection of PrP^Sc. PrP^Sc can be detected in specific neuroanatomical loci in the CNS of affected cattle by IHC methods in formalin-fixed material, or by immunoblotting and other enzyme immunoassay methods using unfixed brain extracts.

Transmission from infected brain tissue is the only method currently available for detection of infectivity and is important for characterisation of agent strains. Variant or atypical forms of BSE have been detected across all continents that have experienced classical BSE. While in the majority of instances atypical phenotypes have been based on Western immunoblot banding pattern, bioassay characterisation supports the concept of strain diversity in BSE.

Commercial diagnostic kits for BSE are available and are used for diagnosis of BSE in many countries; similarly a number of anti-PrP antibodies form the basis of many diagnostic methods. Some are available commercially, or from OIE Reference Laboratories or other laboratories with active TSE programmes.
Serological tests: Specific immune responses have not been detected in TSEs.
Requirements for vaccines: There are no vaccines available currently.

A. INTRODUCTION

Bovine spongiform encephalopathy (BSE), a fatal prion disease of cattle, may present clinically with apprehension, hyper-reactivity and ataxia. Without an isolatable causal agent, cases can only be conclusively confirmed post-mortem by the accumulation in the central nervous system (CNS) of prion protein (PrPSc, PrPd or PrPres), a partially protease-resistant isoform of a host-encoded protein (PrPC). The prion hypothesis proposes that the agent is composed entirely of PrPSc, which is capable of inducing conversion of PrPC. The function of PrPC remains unclear. Alternative hypotheses, such as viral or bacterial origins or the involvement of cofactors such as mineral imbalances, remain unproven. The basis for strain variation is still unclear, but within the prion hypothesis, ‘strains’ are encoded in different conformations of the protein.

Pathological and bioassay characterisation showed that the epidemic was sustained by a single strain, and consistently distinctive neuropathology and PrPSc molecular profiles in clinically affected animals were the basis for the case definition of BSE. Since 2003, reports of variant pathology (Casalone et al., 2004) and/or molecular characteristics in aged cattle from several countries have indicated possible agent strain variation (for examples, see refs Jacob et al., 2007 and Yamakawa et al., 2003). Most cases were identified through active surveillance of non-suspect populations using rapid PrP immunodetection methods. So far, approximately 50 BSE cases have now been recognised that differ in their molecular profiles by Western immunoblotting from those typically found in the epidemic. Because of the detection of most of these cases by active surveillance, correlation of laboratory diagnostic data with clinical histories is lacking, and most focus only on Western immunoblotting data. An interesting common feature is that most of these variant characteristics originate from older cattle. Initial bioassay data support the hypothesis that these isolates are biologically distinct from classical BSE (Beringue et al., 2006; Lombardi et al., 2008). It is not known whether atypical cases have any relevance to forms of human prion diseases. At present, these atypical cases appear as two distinct types classified by the molecular mass of the unglycosylated PrPres protein band relative to that of classical BSE. One type is of a higher molecular mass (H-type) and the other shows a lower molecular mass (L-type).

Epidemiological studies established that BSE occurred as an extended common source epizootic, through feed-borne exposure (Anderson et al., 1996). BSE occurred in many countries at a lower incidence than in GB. Many cases are most likely to have resulted directly or indirectly from the export of infected cattle or infected meat-and-bone meal, with subsequent local propagation through contaminated feedstuffs. However, in some countries, cases reflected indigenous exposure (OIE: World animal health situation – Bovine spongiform encephalopathy [see OIE website at www.oie.int]).

There is no evidence of horizontal transmission and little data to support maternal transmission (Prince et al., 2003). Studies have not revealed evidence of risk from semen, milk or embryos (Prince et al., 2003).

The epizootics of BSE have declined, and show the success of controls in the form of changes in age-specific incidence. Interpretation of the status of epizootics has been enhanced by active surveillance detecting infected animals that were not clinically suspect.

The emergence of vCJD in humans has been causally linked to ingestion of BSE (Bruce et al., 1997). Recommended safety precautions for handling the agent are based on the assumption that BSE is zoonotic. Biocontainment for necropsies and tissue handling should be risk-based and compliant with relevant national regulations; any procedure creating aerosols must be conducted under containment level 3 (see Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities), and the laboratory must comply with national biocontainment and biosafety regulations to protect staff from exposure to the pathogen. Recommended decontamination procedures may not be completely effective when dealing with high-titre material or when the agent is protected within dried organic matter. Recommended physical inactivation is by porous load autoclaving at 134°C–138°C for 16 minutes at 30 lb/in² (208 kPa or 2.2 bar). However, temperatures at the higher end of the range may be less effective than those at the lower end and total inactivation may not be achieved under certain conditions, such as when the test material is in the form of a macerate. Recommended decontamination procedures may not be completely effective under some circumstances. Disinfection of potential fomites is carried out using sodium hypochlorite containing 2% available chlorine, or 2 N sodium hydroxide, applied for more than 1 hour at 20°C for surfaces, or overnight for equipment.
B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

There is no method capable of confirming the presence of BSE in the live animal. Identification of the ‘agent’ begins with clinical suspicion of disease, or the post-mortem demonstration of PrPSc accumulation in a non-suspect animal through active surveillance. The nature of the ‘agent’ itself remains hypothetical, and it cannot therefore be isolated for diagnostic purposes. However, PrPSc is widely accepted as a consistent disease marker, and, with the exception of clinical examination and histopathology, all current diagnostic methods are based on the demonstration of this protein.

Clinical ‘classical’ BSE occurs in adult cattle (ranging between 20 months and 22 years in the UK). During the main epidemic most cases were observed in dairy cattle aged 4–6 years. Subsequently the impact of effective controls has been reflected in an increasing age at onset of clinical disease. BSE has an insidious onset and usually a slowly progressive course (Konold et al., 2004; Wilesmith et al., 1992). The onset of clinical signs is not associated with season or stage of breeding cycle. Occasionally, a case will present with acute signs and then deteriorate rapidly, although frequency of observation is a significant factor in determining early clinical signs. Presenting signs, though variable, usually include changes in behaviour and temperament, hyper-reactivity and incoordination. Affected cows may be reluctant to enter the milking parlour or may kick vigorously during milking, which is often the first observed sign. In dry cows, especially, hind-limb incoordination and weakness can be the first clinical features to be noticed. The most commonly reported nervous signs have been apprehension, pelvic limb ataxia, and hypeaesthesia to touch and sound. Startle responses to external stimuli, which are repeatable, are frequent and usually used to support the clinical diagnosis in suspect BSE cases (Konold et al., 2004). The intense pruritus characteristic of most sheep with scrapie is not prominent in cattle with BSE. Affected cows will sometimes stand with low head carriage and the neck extended, an arched back or wide-based hind limbs. A tremor of the head may also be visible. Abnormalities of gait, such as incoordination and hypermetria, are usually confined to the hind limbs and are most readily appreciated when cattle are observed at pasture. With advancing severity of locomotor signs, generalised weakness, resulting in falling and recumbency, can dominate the clinical picture. General clinical features of loss of bodily condition and reduction in milk yield often accompany nervous signs as the disease progresses. There has been no change in the clinical picture of BSE over the course of the epizootic in the UK (Konold et al., 2004; Wilesmith et al., 1992). Clinical signs are essentially similar in other countries where BSE has occurred. The protracted clinical course, extending usually over a period of weeks or months, would eventually require slaughter on welfare considerations. However, a statutory policy to determine the BSE status of a country requires compulsory notification and diagnostic investigation of clinically suspect cases, their slaughter and post-mortem examination of the brain. Early in the disease course, the signs may be subtle, variable and nonspecific, and thus may prevent clinical diagnosis on an initial examination. Continued observation of such equivocal cases, together with appropriate clinical pathology procedures to eliminate differential diagnoses, especially metabolic disorders, will establish the essential progression of signs. Some early clinical signs of BSE may show similarities with features of nervous ketosis, hypomagnesaemia, encephalic listeriosis and other central nervous system diseases. Subtle signs may sometimes be exacerbated following stress, such as that caused by transport. Video clips of cattle affected by BSE may be downloaded from the web site of the OIE Reference Laboratory for BSE in the UK1, which also provides DVD or videotape footage upon request, or the OIE Reference Laboratory in Switzerland2.

The small number of variant forms of TSE in cattle, operationally defined as BASE (bovine amyloidotic spongiform encephalopathy) or L-type and H-type BSE based on the mass of the unglycosylated PrP fragment in Western blots (Casalone et al., 2004; Jacob et al., 2007) have yielded little clinical information as most have been identified in apparently healthy cattle or fallen stock.

The laboratory diagnosis of BSE has evolved with increasing knowledge of the disease pathogenesis and technical advances (Gavier-Widen et al., 2005). In the absence of in-vitro methods for isolation of the causative agent, the historical basis of disease confirmation was the demonstration of the morphological features of spongiform encephalopathy by histopathological examination, at several different levels of the brain. Histopathology remains the only method by which this characteristic TSE-specific vacuolation can be identified. The original diagnosis of BSE was based on the identification of histopathological features consistent with a scrapie-like spongiform encephalopathy. This was supported by the electron microscopic visualisation of fibrils, termed scrapie-associated fibrils (SAF), which are composed largely of PrPSc, in detergent extracts of affected brain. The material examined was invariably from suspect clinical cases. During the rapidly increasing epizootic in the late 1980s, histopathological diagnosis based on examination of a single section of medulla oblongata (at the level of the obex) was validated against more extensive examination of the brainstem. This simplified approach enabled modification of sampling of the fresh brain; instead of whole brain removal, the required section was

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1 The laboratory is also the European Commission (EC) Transmissible Spongiform Encephalopathies (TSEs) Community Reference Laboratory. All its Web Resources can be found at: http://www.vla.gov.uk/science/sci_tse_rl_tests.htm
2 www.neurocenter-bern.ch
taken from brainstem removed via the foramen magnum, using customised instrumentation. As the diagnostic specificity of PrPSc was established, immunochemical methods of disease-specific PrP detection, including IHC techniques and Western blotting/SAF-immunoblotting, were used, in addition to histopathology, to confirm the diagnosis and improve diagnostic sensitivity in early or autolysed cases. The introduction of more rapidly performed in-vitro methods, mostly enzyme-linked immunosorbent assay (ELISA)-based, for the detection of PrPSc has led to a variety of ‘rapid’ tests, which are now the principal screening tools for active surveillance. Such tests provide a preliminary diagnosis from which positive or inconclusive results are subject to examinations by IHC or Western blot confirmatory methods. Rapid tests are currently the main approach by which cases are detected and their wider use as part of the confirmatory process has been agreed (OIE Reference Laboratory in the UK). All currently recognised forms of BSE are detectable by these methods although a full evaluation for atypical forms (H and L types) has not been carried out.

The choice of any particular method will depend on the context of its use. Contexts will extend from confirmation of clinical suspects to the screening of healthy populations for evidence of covert or preclinical disease. The case definition adopted will also differ according to whether the diagnostic method is to be applied for confirmation or for screening. Care should be taken in the interpretation of diagnostic data using methodologies that do not enable careful cross-referencing with the standards for confirmatory diagnosis that are defined here. Without appropriate comparison with previously published criteria defining the BSE phenotype, and in the absence of transmission studies, diagnostic results that claim the identification of a new strain may be premature. Quality control (QC) and quality assurance (QA) are essential parts of the testing procedures and advice can be supplied by the OIE Reference Laboratories. Whether BSE-infected animals are identified by passive or active surveillance, it is good practice to detect and confirm disease by a combination of at least two test methods. The primary test can be one of the confirmatory test methods described below or a rapid test, but it is important to apply a secondary test to confirm a positive or inconclusive primary test result. Where there is a conflict between primary and secondary test results, further tests using immunohistochemistry or scrapie-associated fibrils (SAF)-immunoblot (or approved alternative) should be applied or samples should be submitted to an OIE Reference Laboratory for resolution.

a) Sample preparation

The BSE status of a country, the relative implementation of passive and active surveillance programmes and the diagnostic methods applied, will all influence sampling strategy.

In all circumstances of passive surveillance of neurological disease in adult cattle where the occurrence of BSE within a country or state has not been established or is of low incidence, it is recommended that clinically suspect cases are subjected to a standard neuropathological approach in which the whole brain is sampled, and a range of representative areas examined. Cattle suspected of having the disease should be killed with an intravenous injection of a concentrated barbiturate solution preceded, if necessary, by sedation. The brain should be removed as soon as possible after death by standard methods. There are no gross lesions associated with BSE, so if any are observed when the brain is removed, these should be specifically sampled to facilitate differential diagnosis.

Care must be taken to preserve suitable fixed and fresh brain samples for the immunohistochemical and immunochemical detection of PrPSc. Departure from this approach of collecting and retaining the entire brain may prevent appropriate characterisation of the case, to confirm whether or not it is typical of BSE.

Histopathology and IHC examinations are carried out initially on a single block (0.5–1.0 cm in width) cut at the obex of the medulla oblongata (Fig. 1a and b, level A–A representing the centre of the block for examination), which should be fixed for a minimum of 3–5 days (dependent on block thickness) in 4% formaldehyde solution (i.e. 10% formal saline or 10% normal buffered formalin [NBF]). Subsequent histological processing should be by conventional paraffin wax embedding methods for neural tissue. (An example of appropriate processing methods can be obtained from the OIE Reference Laboratory in the UK.

Fresh material for the immuno-detection of PrPSc should be taken initially as a hemisection of the medulla to the level of the obex, or as a complete coronal section (2–4 g), immediately rostral, or caudal, to the obex block taken for fixation. All other brain areas should be subdivided by a sagittal paramedian cut (3–5 mm off the median). The smaller portion is reserved for the PrPSc detection by immunochemical methods (e.g. immunoblot) and is stored frozen prior to testing (if testing is not done immediately after sampling). After sampling of the obex region for fixation and sampling of fresh tissue, the larger portion of the brain tissue is placed, intact, in approximately 4–6 litres of 10% formalin fixative, which should be changed twice weekly. After fixation for 2 weeks, if further investigation is necessary, the brain can be cut into coronal slices. The fixation time may be shortened by cutting the fresh brainstem (detached from the rest of the brain) into smaller coronal pieces, similarly to the initial removal of the obex region, but leaving intact the remaining diagnostically important cross-sectional areas at the levels of the cerebellar peduncles and the rostral colliculi (Figure 1a and b, levels B–B and C–C, respectively). Depending on some other factors
(temperature, agitation, thickness of tissue block, use of microwave) the fixation time for these smaller pieces of brain may be reduced. However, evaluation of the effects of these kinds of accelerated fixation processes on subsequent IHC protocols needs to satisfy proficiency testing standards. The other formol-fixed parts of the brain may be used for differential diagnosis after completing the standard 2 weeks' fixation.

Revised levels at which sections should be taken:

A–A = medulla, at the obex; B–B = medulla through caudal cerebellar peduncles; C–C = midbrain through rostral colliculi.

When the occurrence of BSE in a particular country has been established in the indigenous cattle population, and there is evidence that the distribution of lesions, and other aspects of disease phenotype are consistent with those described for classical BSE, it is adequate, although not ideal, for disease confirmation and monitoring purposes, to remove the brainstem alone.

This can be achieved via the foramen magnum without removal of the calvarium (Fig. 2). This will reduce the amount of fixative required and the time and equipment needed, thereby lowering costs and improving safety. The major target areas for histological examination can still be maintained. This method allows for collecting and examining a large number of samples for passive surveillance or for an active surveillance programme in abattoirs. The brainstem is dissected through the foramen magnum without opening the skull by means of a specially designed spoon-shaped instrument with sharp edges around the shallow bowl. Such instruments are available commercially. It is possible that variations in technique, including orientation, are required with different forms of the instrument, and it is important to train operators once there is agreement on equipment to be used. This training should include information on the cross-sectional distribution of PrPSc in the brainstem, and the need for the accurate sampling and preservation of the diagnostic target areas (see below). Under abattoir conditions it has also been shown possible to obtain expulsion of intact brainstem via the foramen magnum, providing histologically good material, by application of pressure (air or water) (Hejazi & Danyluk, 2005) through the entry wound in the skull when penetrative stunning has been used in slaughtering. Clearly the feasibility and efficacy of this method will be dependent on the slaughter method and before implementation for routine use requires local risk assessment.

Where the index case is identified through active surveillance, the necessary brain areas for full phenotypic characterisation are unlikely to be available. In most countries, brainstem alone is collected, even before the first confirmation of BSE. Provision should be made for heads that have been sampled in the course of active surveillance to be retained until the outcome of initial testing is available. This would enable comprehensive sampling of the brain of positive animals in retrospect for the characterisation of cases. This is particularly important if in-house tests that are not subject to external quality assurance are used and where, in the absence of direct comparison with the methods described here, claims are made that new phenotypes have been identified. Where rapid immunoassays are used as the primary surveillance tool it is necessary to make material available for further morphological (including immunohistochemistry) and molecular examination that would allow identification of disease phenotype in the absence of a diagnosis of BSE having ever been made in that country.
Fig. 2. After the head has been removed from the body by cutting between the atlas vertebra and the occipital condyles of the skull, it is placed on a support, ventral surface uppermost (A), with the caudal end of the brainstem (medulla oblongata) visible at the foramen magnum (see B, expanded drawing of cranium).

The instrument (C) is inserted through the foramen magnum between the dura mater and the ventral/dorsal aspect (depending upon the specific approach) of the medulla and advanced rostrally, keeping the convexity of the bowl of the instrument applied to the bone of the skull and moving with a side-to-side rotational action. This severs the cranial nerve roots without damaging the brain tissue. The instrument is passed rostrally for approximately 7 cm in this way and then angled sharply (i.e. toward the dorsal/ventral aspect of the brainstem, depending on the approach) to cut and separate the brainstem (with some fragments of cerebellum) from the rest of the brain. The instrument, kept in the angled position, is then withdrawn from the skull to eject the tissue through the foramen magnum.

- Sampling of brainstem in active surveillance with use of rapid tests

The sampling and processing of brain tissue for use in any rapid test should be carried out precisely as specified by the supplier or manufacturer of the test method or kit. Details of this procedure vary from method to method and should not be changed without supportive validation data from the manufacturer for the variant methodology. The preferred sample for immunocassay should be at, or within 1.0 cm rostral, or caudal to, the obex, based on the caudo-rostral extent of the key target sites (Fig. 3) for demonstration of PrPSc accumulations and the evaluation of sampling for rapid tests. The choice of target site has to take into account the subsequent method of confirmation. At least a hemi-section of the medulla at the level of the obex should be kept intact for fixation for immunohistochemistry/histology (as described above) should a positive result require confirmation. Sampling the medulla rostral or caudal to the obex for rapid testing does not compromise examination by histological or IHC means. However, to obtain comparable samples for rapid and confirmatory testing, sampling by hemi-section of the medulla at the level of the obex is preferable. While there is resultant loss of the ability to assess the symmetry of any histopathological lesions (notably vacuolation), this approach is less likely to compromise the more important IHC examination. If hemisectioning is adopted however, it becomes critical to ensure that the target sites are not compromised in either sample. For example, the nucleus of the solitary tract and the dorsal motor nucleus of the vagus nerve (target areas for lesions in cattle with BSE) are small, and lie relatively close to the midline (Fig. 3). If sampled tissue is autolysed to the point that anatomical orientation is not possible, an unidentified aliquot can still be taken and tested. A positive result in such cases is still a valid result, but a negative test result cannot be taken to indicate a negative animal, and it should be interpreted with caution and reported with appropriate qualification.
Fig 3. Cross section of the bovine brainstem at the level of the obex identifying the key target sites for diagnosis by histopathology and immunohistochemistry in BSE. These are principally the nucleus of the solitary tract [1] and the nucleus of the spinal tract of the trigeminal nerve [2]; but also the dorsal motor nucleus of the vagus nerve [3]. It follows that material taken for application of a rapid test must also include representation of these areas.

Inaccurate hemi-sectioning could result in the complete loss of a target area for confirmatory testing, and could compromise a surveillance programme. Failure to accurately sample target areas may also arise through inappropriate placement of proprietary sampling tools. Such approaches therefore need to be implemented with a very clear policy and monitoring programme for training and quality assurance of sampling procedures, including anatomical positioning, and not just sample weight. Because of the specifically targeted distribution of PrPSc, sample size and location should be as described in the diagnostic kit or, if not specified, at least 0.5 g taken from the diagnostic target areas for all confirmatory tests as detailed in Fig 3. Performance characteristics of the tests may be compromised by autolysis, particularly due to loss of the ability to ensure inclusion of target areas in the sample taken from the diagnostic target areas detailed in Fig 3.

b) Diagnostic examination

i) Histological examination

Histopathology is no longer the diagnostic method of choice for investigation of suspect animals, or the screening of healthy populations. However, an awareness of the histopathological changes is important, to facilitate detection of cases when conducting routine diagnostic histological examinations of cattle brains sampled for reasons other than BSE testing. For differential diagnosis, sections of medulla–obex are cut at 5 µm thickness and stained with haematoxylin and eosin (H&E). If tissue quality permits, the histopathological examination of H&E sections allows confirmation of the characteristic neuropathological changes of BSE (Simmons et al., 1996; Wells & Wilesmith, 1995) by which the disease was first detected as a spongiform encephalopathy. These changes comprise mainly spongiform change and neuronal vacuolation and are closely similar to those of all other animal TSEs, but in BSE the frequent occurrence of neuroparenchymal vacuolation in certain anatomic nuclei of the medulla oblongata at the level of the obex can provide a satisfactory means of establishing a histopathological diagnosis on a single section of the medulla. As in other species, vacuolar changes in the brains of cattle, particularly vacuoles within neuronal perikarya of the red and oculomotor nuclei of the midbrain are an incidental finding (Gavier-Widen et al., 2001). The histopathological diagnosis of BSE must therefore not rely on the presence of vacuolated neurons alone, particularly in these anatomical locations.

Irrespective of the histopathological diagnosis, immunohistochemistry is routinely employed in addition, as unpublished evidence suggests that as many as 5% of clinical suspects (which are negative on H&E section examination for vacuolar changes at the obex) can be diagnosed by IHC examination. Clearly, examination of the medulla–obex does not allow a full neuropathological examination for differential diagnoses, nor does it allow a comprehensive phenotypic characterisation of any TSE. It is for this reason that it is recommended to remove whole brains from all clinical suspects. There are still insufficient data available to describe specific histopathological features of H or L type BSE. There are some histological data from Italian researchers on BASE (L-type BSE) (Casalone et al., 2004). Few atypical BSE cases have been found in
passive surveillance and it is not possible to obtain whole brains in active surveillance to increase our knowledge in this respect. The poor condition of the brain of fallen stock, where most atypical cases have been identified, also rules out a complete histological examination because of the effects of autolysis. Experimental transmission studies of L- and H-type BSE into calves and adults are underway in some countries and this should enable histological comparisons with the accumulated data already obtained for classical BSE.

ii) Detection of disease-specific forms of PrP

The universal use of PrP detection methods provides a disease specific means of diagnosis independent of the morphological changes defined by histopathology. Many laboratories now supplement or have replaced histopathological examination by IHC and/or other PrP-detection methods. The detection of accumulations of PrPSc is the approach of choice for surveillance programmes and confirmatory diagnosis. It is possible (but not desirable) to undertake immunohistochemistry for PrP on material that has been frozen prior to fixation (Debeer et al., 2002). Freezing prior to fixation will not compromise the immunoreactivity of a sample, but it may compromise the proper identification of target sites. A positive case will have disease-specific immunolabelling (Casalone et al., 2006) in at least one of the diagnostic target areas. For a case to be diagnosed as negative it must be possible to identify the presence of the target areas and to demonstrate that the IHC ‘run’ was technically successful through appropriate controls. If there is no disease-specific immunolabelling, and target areas cannot be identified, the case should be classified as 'unconfirmed' as opposed to negative. Both H and L-type variants demonstrate accumulation of PrPSc in the medulla at the level of the obex (Casalone et al., 2006; Gavier-Widen et al., 2008; and the OIE Reference Laboratory in the UK). The range and morphological appearance of immunolabelling throughout the neuraxis differ, particularly in L-type, where PrPSc is more abundant in the frontal cortical areas, and tends to occur as multiple small plaque-like deposits.

- Immunohistochemical (IHC) methods

IHC examination for PrPSc accumulation is performed on the same formalin-fixed paraffin-embedded material used for the histopathological diagnosis. Different protocols have been applied successfully to the IHC detection of PrPSc for the diagnosis of BSE and although a standardised IHC method would seem desirable, it might be more important to recognise robust methods that achieve a standardised output, as monitored by participation in proficiency testing exercises, and by comparison with the results of a standardised model method in a Reference Laboratory. The generic technique established for histopathology still applies and it works well in autolysed tissues in which morphological evaluation is no longer possible (Monleon et al., 2003). However, it is imperative to recognise the anatomy of the sample to determine whether or not target areas are represented. This is essential for a negative diagnosis, and may also be pivotal in accurately interpreting equivocal immunolabelling. IHC detection of PrPSc accumulations approximates to the sensitivity of the Western blotting approach for detection of PrPSc (Schaller et al., 1999). In combination with good histological preparations, immunohistochemistry allows detection of PrPSc accumulations and, as this, like the vacuolar pathology, exhibits a typical distribution pattern and appearance. This provides simultaneous evaluation or confirmation of this aspect of the disease phenotype. Current methods are available by reference to the OIE Reference Laboratories.

In contrast to the diagnosis of scrapie of sheep, the limited detection of PrPSc in lymphoid tissues in BSE does not provide any scope for utilising such tissues for preclinical diagnosis by biopsy techniques.

- Western blot methods

Immunoblotting techniques, are carried out on fresh (unfixed) tissue, and can be applied successfully even when tissue is autolysed (Hayashi et al., 2004). The SAF-immunoblot (OIE Reference Laboratory in the UK) was the first such method for use in BSE diagnosis. It has similar diagnostic sensitivity to the IHC techniques, and remains the method of choice, along with immunohistochemistry, for the confirmation of BSE. It is a highly sensitive method using a large mass (ideally 2–4 g) of starting material and several steps to concentrate PrPSc. Alternative less time-consuming and less costly methods are now available. These use less material and are more practical, but in cases where the amount of PrPSc is at very low concentrations, they can be less sensitive. Most of these techniques use a precipitation of PrPSc using phosphotungstic acid (PTA) or other chemicals, and some are commercially available. A range of Western immunoblotting methods are available on the website of the OIE Reference Laboratory in the UK or from the other OIE BSE Reference Laboratories.

While Western blot methodology is now in general use around the world, analytical sensitivity when used to detect PrPSc varies significantly between methods and laboratories. Where in-house methods are preferred to published methods for confirmatory purposes, it is important that they are evaluated as being fit for purpose and validated in consultation with an OIE Reference Laboratory.
• Rapid test methods

Rapid Western blot, lateral flow device (LFD) and ELISA techniques have been developed that allow screening of large numbers of brain samples and are commercially available. Such techniques can be performed in a few hours (see EC evaluations of rapid tests for the detection of BSE on known IHC positive and negative sample groups [OIE Reference Laboratory in the UK]). Tests that have been evaluated and approved for BSE surveillance within the EU are listed in Annex C Chapter X of the TSE Regulation (EC 999/2001 and subsequent amendments). An algorithm of how these tests may be used is available on the website of the OIE Reference Laboratory in the UK.

While many countries, and an OIE ad hoc Group on BSE tests, accept EU approval as an indicator of test performance, others have established their own evaluation mechanisms, most notably the United States of America, Canada and Japan (OIE Reference Laboratory in the UK; Canada’s protocols for BSE surveillance5; National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, Outline of Regulation System of Veterinary Drugs in Japan). The OIE also has an approval process and protocols for such evaluations are posted on the OIE web site: Validation and certification of Diagnostic Assays5), and the EU approval process has been accepted as the gold standard for future evaluations in terms of acceptable sensitivity and specificity.

The relative sensitivities of rapid tests, immunohistochemistry and other confirmatory methods have not been fully determined as, by definition, the tests cannot all be applied to identical samples, and the PrPSc distribution is anatomically variable. (As a compromise, tissue homogenates or mixtures of finely chopped tissue may be used and provide some information, for certain types of tests.) Acknowledgement of this is particularly important in evaluating rapid test performance when testing animals that are not presenting clinical signs of BSE and are likely to have lower depositions of PrPSc than cattle with more advanced disease. Initial evaluations completed in the EU were restricted to a comparison of the examination of a sample of brains of cattle identified as suspect clinical animals with histopathological changes characteristic of BSE and a sample of brains of cattle from New Zealand that were unexposed to BSE and histopathologically negative. Later studies have involved field trial evaluation of potential new tests compared with methods that are already approved. Any discrepant results were resolved by a variety of confirmatory approaches in an OIE Reference Laboratory. Rapid tests provide a means of initial screening for animals in the last few months of the incubation period (Arnold et al., 2007), for example in surveys of post-mortem material collected from routinely slaughtered cattle. In countries conducting surveillance for the detection of the novel occurrence of BSE and in those countries in which a means, independent of the system of notification of suspect cases, of assessing the prevalence of BSE is considered necessary, these screening tests offer an efficient approach. Since their introduction for active screening in Europe from January 2001, such tests have been responsible for the identification of the majority of BSE-infected animals and are the preferred primary test. However, confirmation of a diagnosis of BSE ideally requires either the examination of fixed brain by immunohistochemistry or the application of an appropriate Western blot protocol. In 2006, the OIE accepted that through their use in active surveillance programmes, commercial rapid tests have proved themselves to be very effective and consistent, provided they are performed by appropriately trained personnel. Indeed, at times they may out-perform the acknowledged standard of comparison if training and experience in the latter are deficient. Under such circumstances, it is now considered acceptable for diagnosis, even if not ideal for characterisation, for rapid tests to be used in combination for both primary screening in active or passive surveillance programmes and subsequent confirmation. It is essential however to ensure that the choice of primary and secondary test are compatible, and do not present a danger of generating false positive results through shared reagents. Consequently, an algorithm of preferred test combinations will be maintained on the website of the OIE Reference Laboratory in the UK to assist those who wish to use this approach instead of histopathology and immunohistochemistry, or SAF immunoblot for confirmation. The combination of tests should include a Western blot method to generate useful complementary data that will assist in phenotypic characterisation of the sample in the absence of examination of fixed tissue. The confirmation should be carried out in a National Reference Laboratory.

The combination of the two rapid tests can only be used for the confirmation of a BSE case. A negative result by the secondary test is insufficient to define a case as negative following a primary positive result. BSE suspect cases with discordant rapid test results must therefore be investigated further using either the SAF-immunoblot (or approved alternative) or IHC for the demonstration of PrPSc, or if these methods are not available, by histopathology. If histopathology is unable to confirm the initial reactive result, samples should be submitted to an OIE Reference Laboratory for further examination.

5 http://www.oie.int/en/our-scientific-expertise/certification-of-diagnostic-tests/background-information/
Although the test evaluation programmes conducted in Europe were in support of legislation on surveillance for BSE, the consequences are of relevance to other countries. The consequences of false-positive or false-negative results are so great that the introduction of new tests should be supported by thorough evaluation of test performance. Claims by test manufacturers should always be supported by data, ideally evaluated independently. It must be stressed that the process of full validation of all of these diagnostic methods for BSE has been restrained by the lack of a true gold standard and the consequent need to apply standards of comparison based on relatively small studies. There is therefore a continuing need for the publication of larger scale studies of assay performance, and none of the data published so far equate with recognised procedures for test validation for other diseases.

d) Other diagnostic tests

The demonstration of characteristic fibrils, the bovine counterpart of SAF (see Chapter 2.7.12 Scrapie), by negative-stain electron microscopy in detergent extracts of fresh or frozen brain or spinal cord tissue has been used as an additional diagnostic method for BSE and has been particularly useful when histopathological approaches were precluded by the occurrence of post-mortem decomposition. With modification, the method may be applied successfully to formalin-fixed tissue (Chaplin et al., 1998).

Detection of fibrils has been shown to correlate well with the histopathological diagnosis of BSE, but does not offer the sensitivity available from IHC or immunoblotting methods. BSE infectivity can be shown by inoculating mice with brain tissue from terminally affected cattle, but bioassay is impractical for routine diagnosis because of the long incubation period. It is, however, the nearest approach to a ‘gold standard’ for the characterisation of isolates, which has to be based on secondary biological properties in a standardised host, in the absence of an isolatable physical agent. Transgenic mice, such as those over-expressing the bovine PrP gene, offer bioassays with reduced incubation periods for BSE, but none as yet represent practical diagnostic tools.

There remains the need for a live animal test for BSE with a sensitivity capable of detecting PrPSc at low levels early in the incubation of the disease. As yet, the effectiveness of potential approaches has not been shown. The EC remains committed to the evaluation of in-vivo tests, and sets out protocols for the evaluation of such tests (European Food Safety Authority (EFSA). Opinions of the Scientific Panel on Biological Hazards (http://www.efsa.europa.eu/efsajournals/topic-1178620753812_1178620776473.htm).

The detection of certain protein markers of neurodegeneration, including apolipoprotein E (Apo E), the 14-3-3 protein and S-100 proteins in cerebrospinal fluid have not proved useful for diagnosis of preclinical cases of BSE. The diagnostic potential of the observation of IgG light chains as a surrogate marker for prion infection in the urine of scrapie-infected hamsters, has not been investigated for the diagnosis of BSE.

e) Availability of diagnostic reagents and kits

As discussed previously, diagnostic kits have been licensed for use in many countries and reagents are available commercially and from OIE reference and other laboratories with a TSE programme.

2. Serological tests

The infectious agents of prion diseases cannot easily be grown in vitro and do not induce a significant immune response in the host.

C. REQUIREMENTS FOR VACCINES

There are no vaccines available currently.

REFERENCES


* * *

NB: There are OIE Reference Laboratories for Bovine spongiform encephalopathy (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ ). Please contact the OIE Reference Laboratories for any further information on diagnostic tests and reagents for bovine spongiform encephalopathy.
**CHAPTER 2.4.7.**

**BOVINE TUBERCULOSIS**

**SUMMARY**

Bovine tuberculosis is a chronic bacterial disease of animals and humans caused by Mycobacterium bovis. In a large number of countries bovine tuberculosis is a major infectious disease among cattle, other domesticated animals, and certain wildlife populations. Transmission to humans constitutes a public health problem.

Aerosol exposure to *M. bovis* is considered to be the most frequent route of infection of cattle, but infection by ingestion of contaminated material also occurs. After infection, nonvascular nodular granulomas known as tubercles may develop. Characteristic tuberculous lesions occur most frequently in the lungs and the retropharyngeal, bronchial and mediastinal lymph nodes. Lesions can also be found in the mesenteric lymph nodes, liver, spleen, on serous membranes, and in other organs.

Bovine tuberculosis infection in cattle is usually diagnosed in the live animal on the basis of delayed hypersensitivity reactions. Infection is often subclinical; when present, clinical signs are not specifically distinctive and can include weakness, anorexia, emaciation, dyspnoea, enlargement of lymph nodes, and cough, particularly with advanced tuberculosis. After death, infection is diagnosed by necropsy and histopathological and bacteriological techniques. Rapid nucleic acid methodologies, such as the polymerase chain reaction (PCR), may also be used although these are demanding techniques and should only be used when appropriately validated. Traditional mycobacterial culture remains the gold standard method for routine confirmation of infection.

**Identification of the agent:** Bacteriological examinations may consist of the demonstration of acid-fast bacilli by microscopic examination, which provides presumptive confirmation. The isolation of mycobacteria on selective culture media and their subsequent identification by cultural and biochemical tests or DNA techniques, such as PCR, confirms infection. Animal inoculation, which has been used in the past for confirming infection with *M. bovis*, is now rarely used because of animal welfare considerations.

**Delayed hypersensitivity test:** This test is the standard method for detection of bovine tuberculosis. It involves measuring skin thickness, injecting bovine tuberculin intradermally into the measured area and measuring any subsequent swelling at the site of injection 72 hours later.

The comparative intradermal tuberculin test with bovine and avian tuberculin is used mainly to differentiate between animals infected with *M. bovis* and those sensitised to tuberculin due to exposure to other mycobacteria or related genera.

The decision to use the single or comparative test generally depends on the prevalence of tuberculosis infection and on the level of environmental exposure to the other sensitising organisms.

Due to their higher specificity and easier standardisation, purified protein derivative (PPD) products have replaced heat-concentrated synthetic medium tuberculins. The recommended dose of bovine PPD in cattle is at least 2000 International Units (IU) and in the comparative tuberculin test, the doses should be no lower than 2000 IU each. The reactions are interpreted on the basis of the test method used.

**Blood-based laboratory tests:** Diagnostic blood tests are now available, e.g. the gamma-interferon assay, which uses an enzyme-linked immunosorbent assay (ELISA) as the detection method for interferon, the lymphocyte proliferation assay, which detects cell-mediated immune responses, and the indirect ELISA, which detects antibody responses. The logistics and laboratory
execution of some of these tests may be a limiting factor. The use of blood-based assays can be advantageous, especially with intractable cattle, zoo animals and wildlife, although interpretation of the test may be hampered by lack of data for some species. Information on the use of various diagnostic tests in animal species other than bovine is provided in a recent review by Cousins & Florisson (2005).

**Requirements for vaccines and diagnostic biologicals:** Vaccines are being developed and evaluated for use in bovine and wildlife species, but at this time are not routinely administered as they may compromise the use of the tuberculin skin test and other immunological tests to detect infected animals. There are standard methods for the production of bovine PPD tuberculins. PPD, used for performing the tests specified, should be prepared in accordance with the World Health Organization requirements and should conform to these requirements with respect to source materials, production methods and precautions, added substances, freedom from contamination, identity, safety, potency, specificity and freedom from sensitising effect. The bioassays for biological activity are of particular importance, and the potency should be expressed in IUs.

**A. INTRODUCTION**

*Mycobacterium bovis* is a zoonotic organism and should be treated as a risk/hazard group III organism with appropriate precautions to prevent human infection occurring.

Bovine tuberculosis is an infectious disease caused by *M. bovis* that affects cattle, other domesticated animals and certain free or captive wildlife species. It is usually characterised by formation of nodular granulomas known as tubercles. Although commonly defined as a chronic debilitating disease, bovine tuberculosis can occasionally assume a more progressive course. Any body tissue can be affected, but lesions are most frequently observed in the lymph nodes (particularly of the head and thorax), lungs, intestines, liver, spleen, pleura, and peritoneum.

It should be noted that other members of the *M. tuberculosis* complex, previously considered to be *M. bovis*, have been accepted as new species despite identical 16s RNA sequences and over 99.9% identity of their genome sequences. These include *M. caprae* (Aranaz et al., 2003) (in some countries considered to be a primary pathogen of goats) and *M. pinnipedii* (Cousins et al., 2003), a pathogen of fur seals and sea lions. These two new species are known to be zoonotic. In central Europe, *M. caprae* has been identified as a common cause of bovine tuberculosis (Prodinger et al., 2005). Disease caused by *M. caprae* is not considered to be substantially different from that caused by *M. bovis* and the same tests can be used for its diagnosis. In countries with tuberculosis eradication programmes, clinical evidence of tuberculosis in cattle is seldom encountered because the intradermal tuberculin test enables presumptive diagnosis and elimination of infected animals before signs appear. Prior to the national tuberculosis eradication campaigns, however, clinical signs associated with tuberculosis were commonly observed (Cousins et al., 2001).

In many cases, the course of the infection is chronic and signs may be lacking, even in advanced cases when many organs may be involved. When present, clinical signs vary: lung involvement may be manifested by a cough, which can be induced by changes in temperature or manual pressure on the trachea. Dyspnoea and other signs of low-grade pneumonia are also evidence of lung involvement.

In advanced cases, lymph nodes are often greatly enlarged and may obstruct air passages, the alimentary tract, or blood vessels. Lymph nodes of the head and neck may become visibly affected and sometimes rupture and drain. Involvement of the digestive tract is manifested by intermittent diarrhoea and constipation in some instances. Extreme emaciation and acute respiratory distress may occur during the terminal stages of tuberculosis. Lesions involving the female genitalia may occur. Male genitalia are seldom involved.

At necropsy, tubercles are most frequently seen in bronchial, mediastinal, retropharyngeal and portal lymph nodes and may be the only tissue affected. In addition, the lung, liver, spleen and the surfaces of body cavities are commonly affected. Early nodular pulmonary lesions can often be detected by palpation. The lesions are usually non-odoriferous. Other anatomical sites can be infected and should be examined.

Macroscopically, a tuberculous granuloma usually has a yellowish appearance and is caseous, caseo-calcareous, or calcified in consistency. Occasionally, its appearance may be purulent. The appearance may be more purulent in cervids and camels. Some nontuberculous granulomas may be indistinguishable macroscopically from tuberculous granulomas.

The caseous centre is usually dry, firm, and covered with a fibrous connective capsule of varying thickness. Lesion size ranges from small enough to be missed by the unaided eye, to involvement of the greater part of an organ. Serial sectioning of organs and tissues may be required to detect the small lesions contained within the...
tissue. Histologically, lesions caused by *M. bovis* are often paucibacillary (having few organisms) and the absence of acid-fast organisms does not exclude tuberculosis in lymphadenitis of unknown aetiology. In cervidae and some exotic species, tuberculosis should be considered when thin-walled purulent abscesses are observed in the absence of specific aetiology.

*Mycobacterium bovis* has been identified in humans in most countries where isolates of mycobacteria from human patients have been fully characterised. The incidence of pulmonary tuberculosis caused by *M. bovis* is higher in farm and slaughterhouse workers than in urban inhabitants. The transmission of *M. bovis* to humans via milk and its products is eliminated by the pasteurisation of milk. One of the results of bovine tuberculosis eradication programmes has been a reduction in disease and death caused by bovine tuberculosis in the human population.

Although cattle are considered to be the true hosts of *M. bovis*, the disease has been reported in many domesticated and nondomesticated animals. Isolations have been made from buffaloes, bison, sheep, goats, equines, camels, pigs, wild boars, deer, antelopes, dogs, cats, foxes, mink, badgers, ferrets, rats, primates, South American camelids, kudus, elands, tapirs, elk, elephants, sitatungas, oryxes, addax, rhinoceroses, possums, ground squirrels, otters, seals, hares, moles, raccoons, coyotes and several predatory felines including lions, tigers, leopards and lynx (De Lisle *et al.*, 2001; O’Reilly & Daborn, 1995).

Bovine tuberculosis in wildlife was first reported in 1929 in greater kudu (*Tragelaphus strepsiceros*) and common duiker (*Sylvicapra grimmii*) in South Africa and by the 1940s, the disease was found to be endemic in greater kudu. In 1982 in Uganda, a prevalence of 10% in African buffalo and 9% in warthog (*Phacochoerus africanus*) was found, and in Zambia, *M. bovis* infection has been reported in Kafue lechwe (*Kobus leche kafuensis*) and in a single eland (*Taurotragus oryx*). An outbreak of tuberculosis in wild olive baboons (*Papio cynocephalus anubis*) was reported in Kenya. *Mycobacterium bovis* infection has also been diagnosed in African buffalo in the Kruger National Park in South Africa (Bengis *et al.*, 1996), and more recently spill over to other species such as chacma baboon (*Papio ursinus*), lion (*Panthera leo*) and cheetah (*Acynonyx jubatus*) as well as greater kudu has occurred.

The rigorous application of tuberculin testing and culling of reactor cattle has eliminated *M. bovis* infection from farmed bovine populations in some countries, but this strategy has not been universally successful. Extensive investigations of sporadic *M. bovis* reoccurrence have shown that wildlife reservoirs exist in some countries and can act as a source of infection for cattle, deer and other livestock. The risk that these reservoirs of infection constitute for domestic animals and humans is quite variable depending on the specific epidemiological situation for the species and the environment (Comer *et al.*, 2006; Morris *et al.*, 1994). The detection of infection in a wildlife population requires bacteriological investigation or the use of a valid testing method for the species involved (the tuberculin test is not effective in all species) together with epidemiological analysis of information. The badger (*Meles meles*) in the United Kingdom (Wilesmith, 1991) and the Republic of Ireland (O’Reilly & Daborn, 1995), wild boar (*Sus scrofa*) in Spain (Naranjo *et al.*, 2008), the brush-tail possum (*Trichosurus vulpecula*) in New Zealand (Animal Health Division, 1986), and several wild living species in Africa have been shown to be capable of maintaining *M. bovis* infection. Control of transmission from the wildlife population to farmed species is complex and, to date has relied on the reduction or eradication of the infected wildlife population. The use of vaccination to control the disease in some species continues to be investigated.

*Mycobacterium bovis* has been isolated from farmed and free-living cervidae. The disease may be subacute or chronic, with a variable rate of progression. A small number of animals may become severely affected within a few months of infection, while others may take several years to develop clinical signs, which are related to lesions in the animal. The lesions produced may resemble those found in cattle (caseating granulomatous inflammation that is often mineralised). The lesions may take the form of thin-walled abscesses with little calcification and containing purulent material. Thin-walled abscesses have also been observed in llamas. In cervids, tuberculosis should be considered when abscess-like lesions of unknown aetiology are observed. The lymph nodes affected are usually those of the head and thorax. The mesenteric lymph nodes may be affected – large abscesses may be found at this site. The distribution of lesions will depend on the infecting dose, route of infection and the incubation period before examination.

The tuberculin test can be used in farmed deer. The test must be carried out on the side of the neck. To obtain valid results that are valid, the hair should be clipped around the injection site, there should be accurate intradermal injection of the tuberculin and careful pre- and post-inoculation skin thickness measurement should be made using callipers (Clifton-Hadley & Wilesmith, 1991).

**B. DIAGNOSTIC TECHNIQUES**

When diagnostic techniques are used within official TB control or eradication programmes, it is recommended the Veterinary Administration authorises:
• The diagnostic test(s);
• Laboratories performing the tests; and
• Those persons applying diagnostic techniques to animals, i.e. skin tests.

1. Identification of the agent

In cattle, clinical evidence of tuberculosis is usually lacking until very extensive lesions have developed. For this reason, its diagnosis in individual animals and an eradication programme were not possible prior to the development of tuberculin by Koch in 1890. Tuberculin, a concentrated sterile culture filtrate of tubercle bacilli grown on glycerinated beef broth and, more recently, on synthetic media, provides a means of detecting the disease.

Immunological responses to *M. bovis* infections in cattle continue to be studied in attempts to develop improved or alternative diagnostic methods, as skin testing sometimes has practical drawbacks. The gamma interferon test is increasingly being used as a diagnostic blood test for tuberculosis in cattle and for other animals (e.g. goat, buffalo) and is available commercially. The lymphocyte proliferation test and the IgG1 enzyme-linked immunosorbent assay (ELISA) have proven to be useful as ancillary serial (to enhance specificity) and parallel (to enhance sensitivity) tests in farmed red deer.

The presence of *M. bovis* in clinical and post-mortem specimens may be demonstrated by examination of stained smears or tissue sections and confirmed by cultivation of the organism on primary isolation medium. Collection containers should be clean and preferably sterile (use of sample containers that are contaminated by environmental mycobacteria may result in the failure to identify *M. bovis* infection due to the rapid growth of the environmental mycobacteria); where feasible, single-use plastic, disposable containers, 50 ml in capacity, may be used for a variety of specimen types. Specimens that are to be sent to the laboratory must be cushioned and sealed to prevent leakage, and properly packaged to withstand breakage or crushing in transit. The International Air Transport Association (IATA), Dangerous Goods Regulations (DGR) for shipping specimens from a suspected zoonotic disease must be followed. The requirements are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens. Prompt delivery of specimens to the laboratory greatly enhances the chances of cultural recovery of *M. bovis*. If delays in delivery are anticipated, specimens should be refrigerated or frozen to retard the growth of contaminants and to preserve the mycobacteria. In warm ambient conditions, when refrigeration is not possible, boric acid may be added (0.5% [w/v] final concentration) as a bacteriostatic agent, but only for limited periods, no longer than 1 week.

Precautions should be taken to prevent infection of laboratory personnel (see Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities). All procedures involving culture should be performed in a biological safety cabinet.

a) Microscopic examination

*Mycobacterium bovis* can be demonstrated microscopically on direct smears from clinical samples and on prepared tissue materials. The acid fastness of *M. bovis* is normally demonstrated with the classic Ziehl–Neelsen stain, but a fluorescent acid-fast stain may also be used. Immunoperoxidase techniques may also give satisfactory results. The presumptive diagnosis of mycobacteriosis can be made if the tissue has characteristic histological lesions (caseous necrosis, mineralisation, epithelioid cells, multinucleated giant cells and macrophages). As lesions are often paucibacillary, the presence of acid-fast organisms in histological sections may not be detected, although *M. bovis* can be isolated in culture. However, large numbers of acid-fast organisms are seen in lesions in primates, felids, mustelids (badgers) and marsupials (brush-tailed possums).

b) Culture

To process specimens for culture, the tissue is first homogenised using a mortar and pestle, stomacher or blender, followed by decontamination with either detergent (such as 0.375–0.75% hexadecylpyridinium-chloride [HPC]), an alkali (2–4% sodium hydroxide) or an acid (5% oxalic acid). The alkali or acid mixture is shaken for 10–15 minutes at room temperature and then neutralised. Neutralisation is not required when using HPC. The suspension is centrifuged, the supernatant is discarded, and the sediment is used for culture and microscopic examination. It is recommended that, as a minimum, pooled lymph node samples from the head and thorax be cultured when no visible lesions are detected in tuberculin or interferon test positive animals at post-mortem examination.

For primary isolation, the sediment is usually inoculated on to a set of solid egg-based media, such as Lowenstein–Jensen, Coletos base or Stonebrinks; these media should contain either pyruvate or pyruvate and glycerol. An agar-based medium such as Middlebrook 7H10 or 7H11 or blood based agar medium (Cousins *et al.*, 1989) may also be used.
Cultures are incubated for a minimum of 8 weeks (and preferably for 10–12 weeks) at 37°C with or without CO₂. The media should be in tightly closed tubes to avoid desiccation. Slopes are examined for macroscopic growth at intervals during the incubation period. When growth is visible, smears are prepared and stained by the Ziehl–Neelsen technique. Growth of *M. bovis* generally occurs within 3–6 weeks of incubation depending on the media used.

Liquid culture systems are used routinely in some hospital and veterinary laboratories; in these systems growth is measured by radiometric or fluorometric means.

If gross contamination of culture media occurs, the culture process should be repeated using the retained inocula with an alternative decontaminating agent. The limiting factor in isolation is often the poor quality of the samples submitted and every effort should be made to insure that the laboratory receives good quality samples.

Characteristic growth patterns and colonial morphology can provide a presumptive diagnosis of *M. bovis*; however every isolate needs to be confirmed. It is necessary to distinguish *M. bovis* from the other members of the *tuberculosis complex*, i.e. *M. tuberculosis* (the primary cause of tuberculosis in humans), *M. africanum* (occupies an intermediate phenotypic position between *M. tuberculosis* and *M. bovis*), *M. microti* (the ‘vole bacillus’, a rarely encountered organism), *M. pinnipedii* and *M. caprae*.

*Mycobacterium tuberculosis* may infect cattle and sensitise cattle to bovine tuberculin without causing typical lesions. Sometimes *M. avium* or other environmental mycobacteria may be isolated from tuberculosis-like lesions in cattle. In such cases, a careful identification is needed, and a mixed infection with *M. bovis* should be excluded.

Isolates can be identified by determining traditional cultural and biochemical properties. On a suitable pyruvate-based solid medium, colonies of *M. bovis* are smooth and off-white (buff) in colour. The organism grows slowly at 37°C, but does not grow at 22°C or 45°C. *Mycobacterium bovis* is sensitive to thiophen-2-carboxylic acid hydrazide (TCH) and to isonicotinic acid hydrazide (INH). This can be tested for by growth on 7H10/7H11 Middlebrook agar medium or on egg-containing media. The egg medium should be prepared without pyruvate because it inhibits INH and could have a similar effect on TCH (which is an analogue of INH) and thus give false-positive (resistant) results. *Mycobacterium bovis* strains are also sensitive to para-amino salicylic acid and streptomycin. Effective drug concentrations are different for egg-based and agar-based media. Results for niacin production and nitrate reduction are negative in *M. bovis*. In the amidase test, *M. bovis* is positive for urease and negative for nicotinamidase and pyrazinamidase. It is a microaerophilic and nonchromogenic bacterium.

c) **Nucleic acid recognition methods**

Rapid identification of isolates to the level of *M. tuberculosis* complex can be made by Gen Probe TB complex DNA probe or polymerase chain reaction (PCR) targeting 16S–23S rRNA, the insertion sequences IS6110 and IS1081, and genes coding for *M.-tuberculosis*-complex-specific proteins, such as MP870 and the 38 kDa antigen b have been used. Specific identification of an isolate as *M. bovis* can be made using PCR targeting a mutation at nucleotide positions 285 in the oxyR gene, 169 in the pncA gene, 675/756/1311/1410 and 1450 of the gyrB gene and presence/absence of RDs (Regions of Difference) (Espinosa de los Monteros et al., 1998; Huard et al., 2003; Niemann et al., 2000; Parsons et al., 2002). Alternatively molecular typing techniques, such as spoligotyping will identify *M. bovis* isolates and provide some molecular-typing information on the isolate that is of epidemiological value (Kamerbeek et al., 1997).

PCR has been widely evaluated for the detection of *M. tuberculosis* complex in clinical samples (mainly sputum) in human patients and has recently been used for the diagnosis of tuberculosis in animals. A number of commercially available kits and various ‘in-house’ methods have been evaluated for the detection of the *M. tuberculosis* complex in fresh and fixed tissues. Various primers have been used, as described above. Amplification products have been analysed by hybridisation with probes or by gel electrophoresis. Commercial kits and the in-house methods, in fresh, frozen or boric acid-preserved tissues, have shown variable and less than satisfactory results in interlaboratory comparisons (Noredhoek et al., 1996). False-positive and false-negative results, particularly in specimens containing low numbers of bacilli, have reduced the reliability of this test. Variability in results has been attributed to the low copy number of the target sequence per bacillus combined with a low number of bacilli. Variability has also been attributed to decontamination methods, DNA extraction procedures, techniques for the elimination of polymerase enzyme inhibitors, internal and external controls and procedures for the prevention of cross-contamination. Improvement in the reliability of PCR as a practical test for the detection of *M. tuberculosis* complex in fresh clinical specimens will require the development of standardised and robust procedures. Cross contamination is the greatest problem with this type of application and this is why proper controls have to be set up with each amplification. However, PCR is now being used on a routine basis in some laboratories to detect the *M. tuberculosis* group in paraffin-embedded tissues (Miller et al., 1997; 2002). Although direct PCR can
produce a rapid result, it is recommended that culture be used in parallel to confirm a viable *M. bovis* infection.

A variety of DNA-fingerprinting techniques has been developed to distinguish the *M. tuberculosis* complex isolates for epidemiological purposes. These methods can distinguish between different strains of *M. bovis* and will enable patterns of origin, transmission and spread of *M. bovis* to be described (Durr et al., 2000a 2000b). The most widely used method is spoligotyping (from ‘spacer oligotyping’), which allows the differentiation of strains inside each species belonging to the *M. tuberculosis* complex, including *M. bovis*, and can also distinguish *M. bovis* from *M. tuberculosis* (Heifets & Jenkins, 1998; Kamerbeek et al., 1997). The use of a standard nomenclature for the spoligotypes according to the database Mbovis.org (http://www.mbovis.org) is encouraged to allow international comparison of profiles.

Other techniques include restriction endonuclease analysis (REA) and restriction fragment length polymorphism (RFLP) using IS6110 probe (especially where there are >3–4 copies of IS6110 in the isolate), the direct repeat (DR) region probe, the PGRS (polymorphic GC repeat sequence) probe (Skuce et al., 1996) and the pUCD probes (O’Brian et al., 2000). The mycobacterial interspersed repetitive units (MIRU)-variable number tandem repeat (VNTR) typing has also been developed to increase the discrimination of the *M. tuberculosis* complex species (Frothingham & Meeker-O’Connell, 1998; Supply et al., 2000). Often a combination of techniques may be used to gain the maximum discrimination between strains (Cousins et al., 1998).

The genome of *M. bovis* has been sequenced (Garnier et al., 2003) and this information has contributed to improved methods of genetic fingerprinting and to the development of PCR assays that define the subspecies of the *M. tuberculosis* complex.

2. Delayed hypersensitivity test

   • **The tuberculin test (the prescribed test for international trade)**

   The standard method for detection of bovine tuberculosis is the tuberculin test, which involves the intradermal injection of bovine tuberculin purified protein derivative (PPD) and the subsequent detection of swelling (delayed hypersensitivity) at the site of injection 72 hours later. This may be performed using bovine tuberculin alone or as a comparative test using avian and bovine tuberculins. The tuberculin test is usually performed on the mid-neck, but the test can also be performed in the caudal fold of the tail. The skin of the neck is more sensitive to tuberculin than the skin of the caudal fold. To compensate for this difference, higher doses of tuberculin may be used in the caudal fold.

   Delayed hypersensitivity may not develop for a period of 3–6 weeks following infection. Thus, if a herd/animal is suspected to have been in contact very recently with infected animals, delaying testing should be considered in order to reduce the probability of false-negatives. As the sensitivity of the test is less than 100%, it is unlikely that eradication of tuberculosis from a herd will be achieved with only a single tuberculin test. It should be recognised that when used in chronically infected animals with severe pathology, the tuberculin test may be unresponsive. The tuberculin test has not been well validated in most non-bovid and non-cervid species.

   The comparative intradermal tuberculin test is used to differentiate between animals infected with *M. bovis* and those responding to bovine tuberculin as a result of exposure to other mycobacteria. This sensitisation can be attributed to the antigenic cross-reactivity among mycobacterial species and related genera. The test involves the intradermal injection of bovine tuberculin and avian tuberculin into different sites, usually on the same side of the neck, and measuring the response 3 days later.

   The potency of tuberculins must be estimated by biological methods, based on comparison with standard tuberculins, and potency is expressed in International Units (IU). In several countries, bovine tuberculin is considered to be of acceptable potency if its estimated potency guarantees per bovine dose at least 2000 IU (±25%) in cattle. In cattle with diminished allergic sensitivity; a higher dose of bovine tuberculin is needed, and in national eradication campaigns, doses of up to 5000 IU are recommended. The volume of each injection dose must not exceed 0.2 ml.

   • **Test procedure**

   i) A correct injection technique is important. The injection sites must be clipped and cleaned. A fold of skin within each clipped area is measured with callipers and the site marked prior to injection. A short needle, bevel edge outwards and graduated syringe charged with tuberculin attached, is inserted obliquely into the deeper layers of the skin. The dose of tuberculin is then injected. A multi-dose syringe or multiple injection gun may be used provided that delivery of the volume and safety are assured. The dose of tuberculin injected must be no lower than 2000 International Units (IU) of bovine or avian tuberculin. A correct injection is confirmed by palpating a small pea-like swelling at each site of injection.
injection. The distance between the two injections should be approximately 12–15 cm. In young animals in which there is no room to separate the sites sufficiently on one side of the neck, one injection must be made on each side of the neck at identical sites in the centre of the middle third of the neck. The skin-fold thickness of each injection site is re-measured 72 hours after injection. The same person should measure the skin before the injection and when the test is read.

ii) A number of alternative methods of interpreting the skin test responses have been adopted, recognising that false-positive reactions may be caused by sensitisation by other mycobacteria and by local inflammation. It is important to recognise that there is a balance between sensitivity and specificity and achieving high concurrent values may not be possible. Appropriate policies need to be in place depending on disease prevalence and according to risk (e.g. where a wildlife reservoir is present). The interpretation is based on observation and the recorded increases in skin-fold thickness. In the single intradermal test (which requires a single injection of bovine tuberculin), the reaction is commonly considered to be negative if only limited swelling is observed, with an increase of no more than 2 mm and without clinical signs, such as diffuse or extensive oedema, exudation, necrosis, pain or inflammation of the lymphatic ducts in that region or of the lymph nodes. The reaction is considered to be inconclusive if none of these clinical signs is observed and if the increase in skin-fold thickness is more than 2 mm and less than 4 mm. The reaction is considered to be positive if clinical signs, as mentioned above, are observed or if there is an increase of 4 mm or more in skin-fold thickness. Moreover, in *M.-bovis*-infected herds, any palpable or visible swelling should be considered to be positive. Sometimes a more stringent interpretation is used, particularly in a high risk population or in contact animals. Animals that are inconclusive by the single intradermal test should be subjected to another test after an interval of 42 days to allow desensitisation to wane (in some areas 60 days for cattle and 120 days for deer are used). Animals that are not negative to this second test should be deemed to be positive to the test. Animals that are positive to the single intradermal test may be subjected to a comparative intradermal test or blood test. Any retest should be performed in accordance with the local or national control programmes standard.

iii) In the interpretation of the intradermal comparative test, a reaction is usually considered to be positive if the increase in skin thickness at the bovine site of injection is more than 4 mm greater than the reaction shown at the site of the avian injection. The reaction is considered to be inconclusive if the increase in skin thickness at the bovine site of injection is greater than the avian reaction with a difference of less than 4 mm. The reaction is considered to be negative if the increase in skin thickness at the bovine site of injection is less than or equal to the increase in the skin reaction at the avian site of injection. This interpretation scheme is used in European Union (EU) countries and is recommended in Council Directive 64/432/EEC (EU, 1980). Sometimes a more stringent interpretation is used.

iv) In the caudal fold test, a short needle, bevel edge outwards, is inserted obliquely into the deeper layers of the skin on the lateral aspect of the caudal fold, midway along the fold and midway between the hairline and the ventral aspect of the fold. The standard interpretation is that any palpable or visible change is deemed to be a reaction. A modified interpretation is also in use: a positive test is any palpable or visible swelling at the site of the injection that has a caudal fold thickness difference of 4 mm when compared with the thickness of the opposite caudal fold. If an animal has only one caudal fold, it is considered to be test positive if the caudal fold thickness is 8 mm or more.

3. Blood-based laboratory tests

Besides the classical intradermal tuberculin test, a number of blood tests have been used (Haagsma, 1993). Due to the cost and the more complex nature of laboratory-based assays, they are usually used as ancillary tests to maximise the detection of infected animals (parallel testing), or to confirm or negate the results of an intradermal skin test (serial testing). There is also evidence that when an infected animal is skin tested, an enhanced blood test can occur during the following week. This allows for better separation of *in-vitro* blood test responses leading to greater test accuracy. The gamma-interferon assay and the lymphocyte proliferation assay measure cellular immunity, while the ELISA measures humoral immunity.

a) Gamma-interferon assay (the alternative test for international trade)

In this test, the release of a lymphokine gamma interferon (IFN-γ) is measured in a whole-blood culture system. The assay is based on the release of IFN-γ from sensitised lymphocytes during a 16–24-hour incubation period with specific antigen (PPD-tuberculin) (Wood et al., 1990). The test makes use of the comparison of IFN-γ production following stimulation with avian and bovine PPD. The detection of bovine IFN-γ is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine gamma-interferon. It is recommended that the blood samples be transported to the laboratory and the assay set up as soon as practical, but not later than the day after blood collection (Coad et al., 2007; Ryan et al., 2000). In some areas, especially where ‘nonspecificity’ is prevalent, some concerns about the accuracy have been expressed. However, because of the IFN-γ test capability of detecting early infections, the use of both tests in parallel allows detection of a greater number of infected animals before they become a source of infection for other animals as well as a source of contamination of the environment (Gormley et al., 2006). The use of
defined mycobacterial antigens such as ESAT 6 and CFP-10 shows promise for improved specificity (Buddle et al., 2001), and these antigens are employed in a number of countries such as the United Kingdom and New Zealand for serial testing. The use of such antigens may also offer the ability to differentiate BCG-vaccinated from unvaccinated animals. In animals that are difficult or dangerous to handle, such as excitable cattle or other bovidae, the advantage of the IFN-γ test over the skin test is that the animals need be captured only once. The IFN-γ test has been approved for use in a number of national programmes including in the European Union (EU), USA, New Zealand, and Australia. In New Zealand and the United Kingdom for example, the IFN-γ test is used for serial testing (to enhance specificity) and parallel testing (to enhance sensitivity). The test is available as commercial kits for bovine species and primates; however it has been validated in only a few species of these taxa.

b) Lymphocyte proliferation assay

This type of in-vitro assay compares the reactivity of peripheral blood lymphocytes to tuberculin PPD (PPD-B) and a PPD from Mycobacterium avium (PPD-A). The assay can be performed on whole blood (Buddle et al., 2001) or purified lymphocytes from peripheral blood samples (Griffin et al., 1994). These tests endeavour to increase the specificity of the assay by removing the response of lymphocytes to ‘nonspecific’ or cross-reactive antigens associated with non-pathogenic species of mycobacteria to which the animal may have been exposed. Results are usually analysed as the value obtained in response to PPD-B minus the value obtained in response to PPD-A. The B–A value must then be above a cut-off point that can be altered in order to maximise either specificity or sensitivity of the diagnosis. The assay has scientific value, but is not used for routine diagnosis because the test is time-consuming and the logistics and laboratory execution are complicated (it requires long incubation times and the use of radio-active nucleotides). As with the IFN-γ test, the lymphocyte proliferation assay should be performed shortly after blood is collected. The test may be useful in wildlife and zoo animals. A blood test comprising lymphocyte transformation assays and ELISA has been reported to have a high sensitivity and specificity in diagnosis of M. bovis infection in deer (Griffin et al., 1994). The test is relatively expensive and has not yet been subject to inter-laboratory comparisons.

c) Enzyme-linked immunosorbent assay

There have been numerous unsuccessful attempts to develop clinically useful serodiagnostic tests for tuberculosis. The ELISA appears to be the most suitable of the antibody-detection tests and can be a complement, rather than an alternative, to tests based on cellular immunity. It may be helpful in anergic cattle and deer. An advantage of the ELISA is its simplicity, but sensitivity is limited mostly because of the late and irregular development of the humoral immune response in cattle during the course of the disease. Specificity is also poor in cattle when complex antigens such as tuberculin or M. bovis culture filtrates are used. However, a comparison of antibody levels to PPD-B and PPD-A has been shown to be useful in increasing specificity in the ELISA (Griffin et al., 1993). The antibody response in deer however seems to develop earlier and more predictably and the sensitivity of a comparative ELISA has been reported to be as high as 85% in this species (Griffin et al., 1993). Improvement may be possible by using a combination of different antigens, including proteins (e.g. MPB70 and MPB83, which are specific but lack sensitivity). Moreover, in M. bovis-infected animals, an anamestic rise has been described, resulting in better ELISA results 2–8 weeks after a routine tuberculin skin test (Lyashchenko et al., 2004). The ELISA may also be useful for detecting M. bovis infections in wildlife. In New Zealand, the ELISA is approved as an ancillary parallel test for farmed deer, carried out 13–33 days after the mid-cervical skin test (Griffin et al., 1994). Alternative serum test formats have also been developed. For example, a lateral flow-based rapid test (TB StatPak) has been shown to be useful for detecting tuberculous animals, particularly in some domestic animals, wildlife (Lyashchenko et al., 2008) and zoo animals such as South American camelds, badgers (Greenwald et al., 2003), nonhuman primates or elephants (Greenwald et al., 2009) where no cellular immunity tests like the gamma-interferon tests are available and where skin testing has been proven unreliable. However, its sensitivity in cattle is relatively low. This test is now licensed in the USA by the USDA for species such as elephants and nonhuman primates and is approved for use in the United Kingdom for badgers.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

At present the only available vaccine against M. bovis infections is bacille-Calmette-Guerin (BCG), which is a live attenuated strain of M. bovis. This has shown variable efficacy in cattle trials, which may be attributable to various factors including vaccine formulation, route of vaccination, and the degree of exposure to environmental mycobacteria (Skinner et al., 2001). Trials have been conducted on a number of other vaccines, but none has been shown to induce a superior protection to BCG. The efficacy of BCG has been shown to vary in a similar manner to that reported for humans. A number of new candidate vaccines are currently being tested. The genetic make-up of the tuberculosis organism is now being studied in detail and entire genome sequences of M. tuberculosis, M. bovis and BCG (Pasteur) have been published (Brosch et al., 2002; Cole et al., 1998; Garnier et al., 2003). This may be particularly useful in identifying genes associated with virulence and in advancing towards a subunit vaccine. In infected countries where there is no test and slaughter control scheme,
BCG vaccination may be used to reduce the spread of infection in cattle; however, there is no solid knowledge of long-term reduction in prevalence and safety for human beings and the environment. Before embarking on a vaccination programme, the vaccination schedule must be optimised for local conditions. Typical dosage would be from $10^4$ to $10^6$ colony-forming units given subcutaneously. Vaccine should be based on the standard reference strain, BCG Pasteur or Danish (WHO/FAO/OIE, 1994). It is important to recognise that use of vaccine will compromise tuberculin skin tests or other immunological tests relying on the use of tuberculin as diagnostic antigen. Cattle vaccination should not therefore be used in countries where control or trade measures based on such testing are in operation. However, significant progress has been made in the development of so-called DIVA antigens that allow the differentiation of BCG vaccinated from \textit{M. bovis} infected animals, particularly when used in the gamma-interferon test (Buddle et al., 1999; Cockle et al., 2006; Sidders et al., 2008; Vordermeier et al., 2001). Such antigens are based on the use of antigens that are encoded on \textit{M. bovis} gene regions that are deleted in BCG (such as ESAT-6 and CFP-10 [Buddle et al., 1999; Vordermeier et al., 2001]), that are under-expressed in some BCG strains (such as MPB83), or are not secreted by BCG (such as Rv3615c [Sidders et al., 2008]). It can therefore be envisaged that BCG vaccination could be applied in combination with such DIVA tests once these reagents have been fully validated and the legal framework amended accordingly. BCG vaccines may also be used to reduce spread of \textit{M. bovis} in wildlife reservoirs of infection. Prior to using the vaccine, it is essential to validate the delivery system for the particular wildlife species. The environmental impact of the vaccine on other wildlife species must also be considered.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 \textit{Principles of veterinary vaccine production}. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

Tuberculin preparations were originally made from the heat-treated products of growth and lysis of \textit{M. tuberculosis} or \textit{M. bovis} (known as human and bovine tuberculins, respectively) grown in glycerol broth. In the 1940s, the ‘heat-concentrated synthetic medium tuberculins’ or HCSM tuberculins, prepared from cultures in a synthetic liquid medium, replaced the ‘old’ tuberculins. The old and HSCM tuberculins have been replaced, almost world-wide, with the purified protein derivatives or PPDs. Bovine PPDs prepared with the \textit{M. bovis} production strain AN5 are more specific for detecting bovine tuberculosis than human PPDs prepared with \textit{M. tuberculosis}.

- \textbf{Production of tuberculin}

1. \textbf{Seed management}
   
   a) \textbf{Characteristics of the seed}

   Strains of \textit{M. bovis} used to prepare seed cultures must be identified to species level by appropriate tests. A record must be kept of their origins and subsequent history. Seed cultures must not be passaged more than five times. The production strains \textit{M. bovis} AN5 or Vallee are the most commonly used.

   b) \textbf{Method of culture}

   If the source culture was grown on solid medium, it is necessary to adapt the organism to grow as a floating culture (e.g. by incorporating a sterile piece of potato in the culture flasks of liquid media, such as Watson Reid’s medium). When the culture has been adapted to liquid medium, it may be used to produce the master seed lot, which is preserved in freeze-dried form. This is used to inoculate media for the production of the secondary seed lots, which must not be more than four culture passages from the master seed. The secondary seed is used to inoculate production cultures (Angus, 1978; Haagsma & Angus, 1994).

   The production culture substrate must be shown to be capable of producing a product that conforms to recognised international standards (WHO, European Pharmacopoeia or other recognised control authorities). It must be free from ingredients known to cause toxic or allergic reactions.

   c) \textbf{Validation}

   The strains of \textit{M. bovis} used as seed cultures must be shown to be free from contaminating organisms.

   Seed lots must be shown to be efficacious in producing tuberculin with sufficient potency. The necessary tests are described in Section C.4 below.

2. \textbf{Method of manufacture}

   The organism is cultured in a synthetic medium, the protein in the filtrate is precipitated chemically (ammonium sulphate or trichloroacetic acid [TCA] are used), then washed and resuspended. PPD tuberculin is recommended as it can be standardised more precisely.
Chapter 2.4.7. — Bovine tuberculosis

An antimicrobial preservative that does not give rise to false-positive reactions, such as phenol (not more than 0.5% [w/v]), may be added. Glycerol (not more than 10% [w/v]) or glucose (2.2% [w/v]) may be added as a stabiliser. Mercurial derivatives should not be used. The product is also dispensed aseptically into sterile, neutral glass containers, which are sealed so as to preclude contamination. The product may be freeze-dried.

3. In-process control

The production flasks, inoculated from suitable seed cultures, are incubated for the appropriate time period. Any flasks showing contamination or grossly abnormal growth should be discarded after autoclaving.

As incubation proceeds, the surface growth of many cultures becomes moist and may sink into the medium or to the bottom of the flask.

In PPD tuberculins, the pH of the dissolved precipitate (the so-called concentrated tuberculin) should be 6.6–6.7.

The protein level of the PPD concentrate is determined by the Kjeldahl or other suitable method. Total nitrogen and TCA precipitable nitrogen are usually compared.

The final product should be bioassayed in guinea-pigs. Potency and specificity assays are carried out in comparison with a reference tuberculin (PPD). Further dilutions are made with a buffer according to the protein content and the required final concentration, usually 1.0 mg/ml (Angus, 1978; Haagsma & Angus, 1994).

4. Batch control

Samples should comply with the officially recognised standards for the production of tuberculin as set out in the European Pharmacopoeia or equivalent regulatory standards.

a) Sterility

Sterility testing is generally performed according to international guidelines (see also Chapter 1.1.7 Tests for sterility and freedom from contamination of biological materials).

b) Safety

Two guinea-pigs, each weighing not less than 250 g and that have not been treated previously with any material that will interfere with the test, are injected subcutaneously with 0.5 ml of the tuberculin under test. No abnormal effects should occur within 7 days.

Tests on tuberculin for living mycobacteria may be performed either on the tuberculin immediately before it is dispensed into final containers or on samples taken from the final containers themselves. A sample of at least 10 ml must be taken and this must be injected intraperitoneally into at least two guinea-pigs, dividing the dose between them. It is desirable to take a larger sample, such as 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea-pigs are observed for at least 42 days and are then examined macroscopically at post-mortem. Any lesions found are examined microscopically and by culture.

c) Sensitising effect

To test the sensitising effect, three guinea-pigs that have not been treated previously with any material that could interfere with the test are injected intradermally on each of three occasions with the equivalent of 500 IU of the preparation under test in a 0.1 ml volume. Each guinea-pig, together with each of three control guinea-pigs that have not been injected previously, is injected intradermally 15–21 days after the third injection with the same dose of the same tuberculin. The reactions of the two groups of guinea-pigs should not be significantly different when measured 24–28 hours later.

d) Potency

Potency is determined by comparison with a reference preparation of bovine tuberculin in guinea-pigs sensitised with M. bovis.

As early as the 1960s, the European Economic Community (EEC, now the EU) recognised an EEC standard for bovine PPD, which was given a potency of 50,000 provisional Community tuberculin units per mg of PPD, and was dispensed in the lyophilised state. Unfortunately, the number of freeze-dried ampoules was not sufficient for the WHO’s requirements and therefore it was decided to produce a new bovine PPD preparation that could be designated by the WHO as the new international standard for bovine PPD tuberculins.
This new bovine PPD standard had to be calibrated against the existing EEC standard. Based on international collaborative assays, both in guinea-pigs and cattle, it was found that the new bovine standard had a relative potency of 65% against the EEC standard. Therefore, in 1986, the WHO officially gave the international standard for bovine PPD tuberculins a unitage of 32,500 IU/mg. This means that the provisional Community tuberculin units are equipotent with the IUs. The European Pharmacopoeia has also recognised the WHO international standard for bovine PPD.

In order to save the stock of the actual international standard, it is desirable that the countries where bovine PPD tuberculins is produced, establish their own national reference preparations for bovine PPD as working standards. These national reference preparations must have been calibrated against the official international standard for bovine PPD, both in guinea-pigs and cattle (Maxlid et al., 1976; Schneider et al., 1979; WHO, 1987).

- Standardisation in guinea-pigs

The guinea-pigs are sensitised with a low dose (e.g. 0.001 or 0.0001 mg wet weight) of live bacilli of a virulent strain of M. bovis 5–7 weeks prior to the assay. The bacilli are suspended in physiological saline, and a deep intramuscular injection of 1 ml is made on the medial side of the thigh. At the time of the assay, the guinea-pigs infected with the low dose of M. bovis should still be in good health and the results of numerous post-mortem examinations carried out shortly after the standardisation assays should show that the guinea-pigs do not suffer from open tuberculosis and thus are not excreting tubercle bacilli.

An alternative potency test can be used that does not use live pathogenic mycobacteria and is more suitable for laboratories that do not have isolation areas for safe housing of infected guinea-pigs. In addition, this option is more convenient in terms of experimental animal welfare. This tuberculin potency test is performed as follows: the PPD tuberculin is bioassayed in homologously sensitised guinea-pigs against the standard for bovine PPD tuberculin by an eight-point assay comprising four dilutions corresponding to about 20, 10, 5 or 2.5 IU. The injection volume is 0.1 ml. In this assay, two test tuberculins are compared with standard tuberculin in eight guinea-pigs, applying eight intradermal injections per animal and employing a Latin square design. The guinea-pigs are sensitised with inactivated bacilli of M. bovis, 5–7 weeks before the assay. The bacilli are suspended in buffer and made into an emulsion with Freund’s incomplete adjuvant. A deep intramuscular injection is made on the medial side of the thigh, using a dose of 0.5 ml.

A suitable assay for potency is as follows: The produced PPD tuberculins are bioassayed in homologously sensitised guinea-pigs against the standard for bovine PPD tuberculin by a six-point assay comprising three dilutions at five-fold intervals of each tuberculin. The dilutions of the tuberculin preparations are made in isotonic buffer solution containing 0.0005% (w/v) polysorbate 80 (TWEEN 80). Volumes of 0.001, 0.0002 and 0.00004 mg tuberculoprotein corresponding to the international standard for PPD of 32, 6.4 and 1.28 IU, respectively, are chosen because these amounts give good readable skin reactions with acceptable limits. The injection volume is 0.2 ml. In one assay, two test tuberculins are compared with the standard tuberculin in nine guinea-pigs, applying eight intradermal injections per animal and employing a balanced incomplete Latin square design (Finney, 1964).

Normally, the reading of the assays is done 24 hours after the injection of the tuberculins, but a second additional reading can be performed after 48 hours. The different diameters of erythema are measured with callipers in millimetres and recorded on assay sheets. The results are statistically evaluated using standard statistical methods for parallel-line assays according to Finney (1964). The relative potencies of the two test tuberculins are calculated with their 95% confidence limits, the slopes of the log dose–response curves for each preparation (increase in mean reaction per unit increase in log dose) and the F ratios for deviations from parallelism.

According to the European Pharmacopoeia, the estimated potency for bovine tuberculins must be less than 66% and not more than 150% of the potency stated on the label.

- Standardisation of bovine tuberculin in cattle

According to WHO Technical Report Series No. 384, potency testing should be performed in the animal species and under the conditions in which the tuberculins will be used in practice (WHO, 1987). This means that bovine tuberculins should be assayed in naturally infected tuberculous cattle. As this requirement is difficult to accomplish, routine potency testing is conducted in guinea-pigs. However, periodic testing in tuberculous cattle is necessary and standard preparations always require calibration in cattle. The frequency of testing in cattle can be reduced if it is certain that the standard preparations are representative of the routine issue tuberculins and that the production procedures guarantee consistency.

A suitable potency assay for bovine tuberculins in cattle is as follows: The test tuberculins are assayed against a standard for bovine PPD tuberculin by a four-point assay using two dilutions at five-fold intervals of each tuberculin. For the standard, 0.1 and 0.02 mg of tuberculoprotein are injected as these volumes
correspond with about 3250 and 650 IU if the international standard for bovine PPD tuberculin is used. The test tuberculins are diluted in such a way that the same weights of protein are applied. The injection volume is 0.1 ml, and the distance between the middle cervical area injection sites is 15–20 cm. In one assay, three test tuberculins are compared with the standard tuberculin in eight tuberculous cattle, applying eight intradermal injections per animal in both sides of the neck, and employing a balanced complete Latin square design. The thickness of the skin at the site of each injection is measured with callipers in tenths of a millimetre, as accurately as possible before and 72 hours after injection (Haagsma et al., 1984).

The results are statistically evaluated using the same standard methods for parallel-line assays as employed in the potency tests in guinea-pigs.

e) Specificity

A suitable assay for specificity is as follows: three bovine test tuberculins are assayed against the standard for avian PPD tuberculin (or three avian test tuberculins against the standard for bovine PPD tuberculin) by a four-point assay in heterologously sensitised guinea-pigs, comprising two dilutions at 25-fold intervals of each tuberculin. Quantities of 0.03 mg and 0.0012 mg of test tuberculoprotein, corresponding to approximately 1500 and 60 IU, are chosen because these doses give good readable skin reactions. The injection doses of the standard are lower, namely 0.001 mg and 0.0004 mg. In one assay, three test tuberculins are compared with the standard tuberculin in eight guinea-pigs by applying eight intradermal injections per animal and employing a balanced complete Latin square design. The reading of the results and the statistical evaluation are identical with the potency test.

f) Stability

Provided the tuberculins comply with the legislative standards required for production and are stored at a temperature of between 2°C and 8°C and protected from light, they may be used up to the expiry date as specified in the licence for production of tuberculin. For long-term storage, it is recommended to keep the PPD in a concentrated form rather than the diluted form and the concentrate should also be stored in the dark.

g) pH control

The pH should be between pH 6.5 and 7.5.

h) Protein content

The protein content is determined as indicated in Section C.3 In-process control.

i) Storage

During storage, liquid bovine tuberculin should be protected from light and held at a temperature of 5±3°C. Freezing of the liquid product may compromise the quality. However, freeze-dried preparations can be prepared and they may be stored at higher temperatures (but not exceeding 25°C); they should be and protected from light. Periods of exposure to higher temperatures or to direct sunlight should be kept to a minimum.

j) Preservatives

Antimicrobial preservatives or other substances that may be added to a tuberculin must have been shown not to impair the safety and effectiveness of the product.

The maximum permitted concentration for phenol is 0.5% (w/v), and for glycerol it is 10% (v/v).

k) Precautions (hazards)

Experience both in humans and animals led to the observation that appropriately diluted tuberculin, injected intradermally, results in a localised reaction at the injection site without generalised manifestations. Even in very sensitive individuals, severe, generalised reactions are extremely rare and limited. But experience has shown that a hypersensitive operator can acquire severe generalised signs after accidental intradermal contact (needle stab-wound) with bovine tuberculin. These individuals should be advised not to carry out the tuberculin skin test with the high dose of 2000–5000 IU tuberculin, which is about 1000 times the normal human dose of 5 IU.

5. Tests on the final product

a) Safety

A test for the absence of toxic or irritant properties must be carried out (see Section C.4.b).
b) Potency

The potency of tuberculins must be estimated by biological methods. These methods must be used for HCSM and PPD tuberculins; they are based on comparison of the tuberculins to be tested with a standard reference preparation of tuberculin of the same type (see also Section C.4.d).

REFERENCES


Chapter 2.4.7. – Bovine tuberculosis


Chapter 2.4.7. — Bovine tuberculosis


* * *

NB: There are OIE Reference Laboratories for Bovine tuberculosis
(see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for bovine tuberculosis.
CHAPTER 2.4.8.

BOVINE VIRAL DIARRHOEA

SUMMARY

Cattle of all ages are susceptible to infection with bovine viral diarrhoea virus (BVDV). Distribution of the virus is world-wide. The clinical signs range from subclinical to the fulminating fatal condition called mucosal disease. Acute infections may result in transient diarrhoea or pneumonia, usually in the form of group outbreaks. Acute forms of the disease associated with high mortality have also been described, often, but not always, associated with a haemorrhagic syndrome. However, most infections in the young calf are mild and go unrecognised clinically. The virus spreads mainly by contact between cattle. Vertical transmission plays an important role in its epidemiology and pathogenesis.

Infections of the bovine fetus may result in abortions, stillbirths, teratogenic effects or persistent infection in the neonatal calf. Persistently viraemic animals may be born as weak, unthrifty calves or may appear as normal healthy calves and be unrecognised clinically. Some of these animals may later develop mucosal disease with anorexia, gastrointestinal erosions, and profuse diarrhoea, leading invariably to death. Mucosal disease can arise only in persistently infected animals.

It is important to avoid the trade of viraemic animals. It is generally considered that serologically positive, nonviraemic cattle are ‘safe’, providing that they are not pregnant. Antibody-positive pregnant cattle carrying persistently infected fetuses are important transmitters of the virus between herds. About 15% of persistently viraemic animals have antibody to the NS/2 protein and a lower percentage to the E2 glycoprotein. Therefore, seropositivity cannot be equated with ‘safety’. Latent infections are not generally thought to occur following recovery from acute infection, though semen from acutevially infected animals and, very rarely, recovered animals may be suspect.

Identification of the agent: BVDV is a pestivirus in the Flaviviridae and is closely related to classical swine fever and ovine Border disease viruses. BVDV occurs in two forms: noncytopathogenic and cytopathogenic. There are two antigenically distinct genotypes (types 1 and 2), and virus isolates within these groups exhibit considerable biological and antigenic diversity.

Persistently viraemic healthy animals resulting from congenital infection can be readily identified by isolation of noncytopathogenic virus in cell cultures from blood or serum. It is necessary to use an immune-labelling method to detect the growth of virus in the cultures. Alternative methods based on direct detection of viral antigen or viral RNA in leukocytes are also available. Persistence of virus should be confirmed by resampling after an interval of at least 3 weeks. These animals will usually have no or low levels of antibodies to BVDV.

Viraemia in acute cases is transient and can be difficult to detect. In fatal cases of haemorrhagic disease, virus can be isolated from tissues post-mortem. Confirmation of mucosal disease can be made by isolation of the cytopathogenic biotype of BVDV, particularly from intestinal tissues. Noncytopathogenic virus may also be detected, especially in blood.

Serological tests: Acute infection with BVDV is best confirmed by demonstrating seroconversion using sequential paired samples from several animals in the group. The testing of paired (acute and convalescent samples) should be done a minimum of 21 days apart and samples should be tested side by side. The enzyme-linked immunosorbent assay for antibody and the virus neutralisation test are the most widely used.

Requirements for vaccines and diagnostic biologicals: There is no standard vaccine for BVD, but a number of commercial preparations are available. Modified live virus vaccine should not be administered to pregnant cattle (or to their sucking calves) due to the risk of transplacental
infection. There is also a risk of inducing mucosal disease in persistently infected animals. Killed virus vaccines generally require booster vaccinations. An ideal vaccine should be able to prevent transplacental infection in pregnant cows.

BVDV is a particularly important hazard to embryo transfer and the manufacture of biological products for other diseases due to the high frequency of contamination of batches of fetal calf serum used as a culture medium supplement dams subject to embryo transfer, which makes use of this material, may be at risk of infection.

A. INTRODUCTION

Bovine viral diarrhoea virus (BVDV) is a pestivirus in the family Flaviviridae and is closely related to classical swine fever and ovine Border disease viruses (Donis, 1995). Two antigenically distinct genotypes of BVDV exist, types 1 and 2, with further subdivisions discernable by genetic analysis (Vilcek et al., 2001). The two genotypes may be differentiated from each other, and from other pestiviruses, by monoclonal antibodies (MAbs) directed against the E2 and ERNS major glycoproteins, or by genetic analysis (Pellerin et al., 1994; Ridpath et al., 1994). Multiplex polymerase chain reaction (PCR) enables virus typing direct from blood samples (Gilbert et al., 1999).

Type 1 virus is generally more common although the prevalence of type 2 is reported to be almost as high as type 1 in North America. BVDV of both genotypes may occur in noncytopathogenic and cytopathogenic forms (biotypes), classified according to whether or not it produces visible change in cell cultures. Usually, it is the noncytopathogenic biotype that circulates in cattle populations. Each biotype has a specific role in a variety of clinical syndromes – acute, congenital and chronic infections (Bolin, 1995; Brownlie, 1985). Type 2 viruses are usually noncytopathogenic and have been associated with outbreaks of severe acute infection and a haemorrhagic syndrome (Carman et al., 1998). However recent type 2 viruses isolated in the United Kingdom have been associated with a disease indistinguishable from that seen with the more frequently isolated type 1 viruses. Some type 1 isolates have been associated with particularly severe and fatal disease outbreaks in adult cattle (David et al., 1994) clinically mild and inapparent infections are common with both genotypes.

Although ubiquitous, control of BVDV can be achieved at the herd level, and even at the national level, as evidenced by the progress towards eradication made in many European countries (Moennig et al., 2005).

B. DIAGNOSTIC TECHNIQUES

a) Acute infections

Acute infections of cattle occur particularly in young animals, and may be clinically inapparent or associated with diarrhoea (Baker 1995). Affected animals may be predisposed to secondary infections, for example those leading to shipping disease, perhaps due to an immunosuppressive effect of the virus. Bulls may suffer a temporary depression of fertility and can show transient shedding of virus in the semen (Paton et al., 1989). Cows may also suffer from infertility, likely associated with changes in ovarian function (Grooms et al., 1998) and secretions of gonadotrophin and progesterone (Fray et al., 2002). During acute infections, a brief viraemia may be detectable and nasal shedding of virus may occur. There may also be a transient leukopenia, thrombocytopenia or temperature response, but these can vary greatly among animals. A serological response is the most certain means of diagnosing a previous infection. The clinical picture is generally one of high morbidity and low mortality, though more severe disease is sometimes seen (Brownlie, 1990). In particular, outbreaks of a severe form of acute disease with haemorrhagic lesions, thrombocytopenia and high mortality have been reported sporadically from some countries (Baker, 1995; Bolin & Ridpath, 1992) and infection with Type 2 viruses in particular has been demonstrated to cause altered platelet function (Walz et al., 2001). Other acute outbreaks may show fever, pneumonia, diarrhoea and sudden death in any age group, with haemorrhagic signs (Carman et al., 1998).

b) Congenital infection

If noncytopathogenic virus infects the bovine fetus, this may result in abortion, stillbirth, teratogenic effects or a congenital infection that persists in the neonatal calf (Baker, 1995; Brownlie, 1985; Duffell & Harkness, 1985; Moennig & Liess, 1995). Confirmation that an abortion is caused by BVDV is often difficult to establish (Ruth, 1987), but virus may be isolated from fetal tissue in some cases, or viral antigen or genome may be demonstrated. An attempt should also be made to detect specific antibody in samples of fetal fluids or serum, or in the supernatant fluid from a tissue suspension. Stillbirths or teratogenic effects may be associated with an active fetal immune response to the virus during mid-to-late gestation. The dam will often have high antibody titres (>1/2000) to BVDV, which is suggestive of fetal infection and is probably due to the fetus providing the dam an extended challenge of virus (Lindberg et al., 2001).
Although congenital infection with BVDV often leads to abortion, it is not always recognised in the field. Infection during the first third of the gestation period can result in the abortion of a conceptus that is small and goes unnoticed by the farmer. The cow would return to service and the failure to maintain pregnancy would be classified as an example of early embryonic death. Another possible outcome of infection is the death and subsequent resorption of fluids from the fetus that results in mummification. It is frequently observed that aborted fetuses have subcutaneous oedema and copious pleural and peritoneal effusions. There may also be congenital abdominal abnormalities that result in growth retardation and in selective central nervous system (CNS) defects, such as cerebellar hypoplasia and dysmyelination (Trautwein et al., 1986), and eye defects, such as cataracts and retinal atrophy. Sometimes there are skeletal defects, the most advanced of which is arthrogryposis.

Stillborn calves has been reported to be sequel to congenital infection before 150 days of gestation and the calves usually appear to be fully developed at parturition, but fail to survive. However, it has been reported, that in many cases, BVD virus cannot be isolated from these animals and they are PCR negative. If infection occurs after day 150 of pregnancy, the immune system of the fetus will be developed and infection of the fetus will usually result in an antibody response and the birth of a normal calf.

c) Persistent infection

When infections of the fetus occur before approximately 110 days of gestation and before immunocompetence, the calf may be born with a persistently infected. Identification of these animals is readily made by detection of noncytopathogenic BVDV in blood. The virus can also be identified in the skin by immunohistochemistry. Furthermore, animals with a persistent infection will also lack specific antibody, but diagnosis in the young calf, up to approximately 3 months of age, may be confused by the presence of maternal antibody to BVDV. Maternal antibody may also interfere with virus isolation. In older animals with persistent viraemia, low levels of antibody may be present due to their ability to seroconvert to strains of BVDV (including vaccines) ‘heterologous’ (antigenically different) from the persisting virus (Brownlie, 1990). To confirm a diagnosis of persistent infection, animals should be retested after an interval of at least 3 weeks.

There are no pathognomonic lesions in the viraemic calf. Depending on the gestational age at infection, lesions may be mediated entirely by the effects of the virus on the differentiating cells of the fetus, they may be mediated by the maturing immune system of the developing fetus, or both. The clinical signs vary from the apparently normal healthy animal to the weak, unthrifty calf that has difficulty in standing and sucking. These latter calves can show CNS defects, such as muscular tremors, incoordination and blindness. They often die within days of birth, thus contributing to the ‘weak calf syndrome’.

Approximately 1–2% of cattle within a population are persistently infected, with many viraemic animals surviving to sexual maturity and retained for breeding. Calves born to these infected dams are always persistently viraemic, and are often weak at birth and fail to thrive. Persistently viraemic animals are a continual source of infective virus to other cattle, and thus their rapid identification and removal from the herd are required. Animals being traded should first be screened for the absence of persistent BVD viremia.

Bulls that are persistently infected usually have poor quality, highly infective semen and, as a result, reduced fertility (Kirkland et al., 1994; Revell et al., 1988). All bulls used for natural or artificial insemination should be screened for persistent BVD infection. A rare event, possibly brought about by acute infection during pubescence, can result in persistent infection of the testes and thus strongly seropositive bulls (Niskanen et al., 2002; Voges et al., 1998). This phenomenon has also been observed following vaccination with an attenuated virus (Givens et al., 2007). Female cattle used as embryo recipients should always test negative for BVD viremia before first use. Donor cows that are persistently infected with BVDV also represent a potential source of infection, as oocysts without an intact zona pellucida are shown to be susceptible to infection in vitro (Vanroose et al., 1998). However, a limited study of two persistently infected animals revealed that the majority of oocysts were BVDV-negative (Tsuboi & Imada, 1998). Embryos may also become contaminated following acute infection of the donor (Bielanski et al., 1998). Biological materials used for in-vitro fertilisation techniques (bovine serum, bovine cell cultures) have a high risk of contamination and should be screened for BVDV (Booth et al., 1995). Recent incidents of apparent introduction of virus via such techniques (Drew et al., 2002; Lindberg et al., 2000) have highlighted this risk. It is considered essential that serum supplements used in media should be sterilised as outlined in Section B.1.a of this chapter. Importing countries may consider requesting additional tests to confirm sterilisation.

d) Mucosal disease

It is well established that persistently viraemic animals may later succumb to mucosal disease (Brownlie, 1985); however, cases are rare. This syndrome has been shown to be associated with the presence of the cytopathogenic biotype, which can arise either through superinfection (Bolin, 1995; Brownlie et al., 1984), recombination between noncytopathogenic biotypes, or mutation of the persistent biotype (Loehr et al., 1998). Consequently, confirmatory diagnosis of mucosal disease should include the isolation of...
cytopathogenic virus from affected cattle. This biotype may sometimes be isolated from blood, but it can be recovered more consistently from a variety of other tissues, in particular intestinal and Peyer’s patch tissue (Clarke et al., 1987). Virus isolation is also readily accomplished from spleen. This is easy to collect and is seldom toxic for cell culture after preparation for viral isolation. Isolation from gut samples may be difficult if autolysis has occurred; in this case suspensions from lymph nodes or tonsil should then be tested. Noncytopathogenic virus can also be detected, particularly from blood or blood-associated organs. Cryostat tissue sections from mucosal disease cases can be stained for viral antigen by immunofluorescence or immunoperoxidase labelling.

Mucosal disease is invariably fatal. Its onset may be so rapid that the first signs seen are dead or moribund animals. However, it is more common for animals to become anorexic over a period of several days, to be disinclined to move and to show signs of abdominal pain. They can develop a profuse diarrhoea and rapidly lose bodily condition. Erosions can often be seen in the mouth, particularly along the gingival margin. Lacrimation and excessive salivation occur. Generally, cases of mucosal disease are sporadic and rare.

Post-mortem examination reveals erosions in the mucosa at various sites along the gastrointestinal tract. The most noticeable are those overlying the lymphoid Peyer’s patches in the small intestine and in the ileocaecal lymph nodes. On histological examination, there is a clear demonstration of destruction of the lymphoid tissue within the gut-associated lymphoid tissue. Most of the Peyer’s patch lymphoid cells have been lysed and replaced by inflammatory cells, debris and cells from the overlying collapsed epithelium.

Severe acute BVD infection can be clinically similar to mucosal disease and confusion can arise, particularly when a number of animals are so affected. Mucosal disease can occur among cohorts of persistently infected animals when oestrus synchronisation has been carried out. Differentiation requires a careful examination of case histories and testing for antibody as well as antigen or virus among infected and any recovered animals. Seroconversion among recovered animals is indicative of acute infection, whereas two antigen or virus positive results on samples from an affected animal, taken 3 weeks apart, is diagnostic of mucosal disease. Generally, animals with mucosal disease are antibody negative, though low levels of antibody can sometimes be detected.

1. Identification of the agent (the prescribed test for international trade)

All test methods must be validated by testing on known noninfected and infected populations of cattle, including animals with low- and high-titre viraemias. Methods based on MAb-binding assays or on nucleic acid recognition must be shown to detect the full range of antigenic and genetic diversity found among BVD viruses. There are two designated OIE Reference Laboratories for BVD (see Table given in Part 4 of this Terrestrial Manual); the reference laboratories for classical swine fever could also be approached to offer advice.

a) Virus isolation

The virus may be isolated in a number of bovine monolayer cell cultures (e.g. kidney, lung, testis or turbinate). Growth of both biotypes is usually satisfactory. Noncytopathogenic BVDV is a common contaminant of fresh bovine tissue, and cell cultures must be checked for freedom from adventitious virus by regular testing (Bolin et al., 1994; Edwards, 1993). Primary or secondary cultures can be frozen as cell suspensions in liquid nitrogen. These can then be tested over a series of passages, or seeded to other susceptible cells and checked before routine use. Such problems may be overcome by the use of continuous cell lines, which can be obtained BVD-free (Bolin et al., 1994).

The fetal bovine serum that is selected for use in cell culture must also be free not only from virus, but also and of equal importance, from BVDV neutralising antibody (Edwards, 1993). Heat treatment (56°C for 30–45 minutes) is inadequate for the destruction of BVDV in contaminated serum; irradiation at 25 kiloGrays (2.5 Mrad) is more certain. Commercial batches of fetal bovine serum mostly test positive by PCR even after the virus has been inactivated by irradiation. Where appropriate, horse serum can be substituted for bovine fetal serum, although it is often found to have poorer cell-growth-promoting characteristics.

Buffy coat cells, whole blood, washed leukocytes or serum are suitable for isolation of the virus from live animals. Maternal antibody may interfere with isolation from serum in young calves. Tissue suspensions from post-mortem cases should be prepared by standard methods. Semen can also be examined, but a blood sample from the donor bull is preferable if it can be obtained. There is a report of an atypical persistent shedding of BVDV in semen from a bull that was not viraemic (Voges et al., 1998). Raw semen is cytotoxic and must be diluted in culture medium. Extended semen can usually be inoculated directly on to cell monolayers, but may occasionally cause cytotoxicity. For these reasons, it is important to monitor the health of the cells by microscopic examination at intervals during the incubation.

There are many variations of procedure in use for virus isolation. All should be optimised to give maximum sensitivity of detection of a standard virus preparation. This may include one or more in-vitro passage(s).
Conventional methods for virus isolation are used, with the addition of a final immune-labelling step (fluorescence or enzymatic) to detect growth of noncytopathogenic virus. Thus tube cultures should include flying cover-slips, while plate cultures can be fixed and labelled directly in the plate. Examples are given below.

- **Microplate immunoperoxidase method for mass screening for virus detection in serum samples** (MEYLING, 1984)
  - i) 10 µl of the serum sample is placed into each of four wells of a 96-well tissue-culture grade microplate. This is repeated for each sample. Known positive and negative controls are included.
  - ii) 100 µl of a cell suspension of 150,000 cells/ml in medium without fetal calf serum (FCS) is added to all wells. NB: the sample itself acts as the cell-growth supplement. If testing samples other than serum, use medium with 10% FCS that is free of antibodies to ruminant pestiviruses.
  - iii) The plate is incubated at 37°C for 4 days, either in a 5% CO₂ atmosphere or with the plate sealed.
  - iv) Each well is examined microscopically for evidence of cytopathic effect (CPE), or signs of cytotoxicity.
  - v) The plate is emptied by gentle inversion and rinsed in phosphate buffered saline (PBS).
  - vi) The plate is fixed as follows: the plate is dipped into a bath of 20% acetone in PBS, emptied immediately, and then transferred to a fresh bath of 20% acetone in PBS for 10 minutes. The plate is drained thoroughly and as much fluid as possible is removed by tapping and blotting. The plate is dried thoroughly for at least 3 hours at a temperature of 25–30°C (e.g. using radiant heat from a bench lamp). NB: the drying is part of the fixation process.
    
    **Alternative fixation methods** include paraformaldehyde or heat (see Chapter 2.8.3 Classical swine fever, Section B.2.b.viii).
  - vii) The fixed cells are rinsed by adding PBS to all wells.
  - viii) The wells are drained and the BVD antibody (50 µl) is added to all wells at a predetermined dilution in PBS containing 1% Tween 80 (PBST) and 5% horse serum. (Horse serum may be added to reduce nonspecific staining.) The plate is incubated at 37°C for 15 minutes.
  - ix) The plate is emptied and washed three times in PBST.
  - x) The plate is then drained and appropriate antispecies serum conjugated to peroxidase is added at a predetermined dilution in PBST (50 µl per well) for 15 minutes at 37°C.
  - xi) The plate is emptied and washed three times in PBST.
  - xii) The plate is rinsed in distilled water. All fluid is tapped out from the plate.
  - xiii) Freshly prepared hydrogen peroxide substrate with a suitable chromogen, e.g. 3-amino-9-ethyl carbazole (AEC) is added. The stock solution is: AEC (0.1 g) dissolved in dimethyl formamide (15 ml). For use, the stock (0.3 ml) is added to 0.05 M acetate buffer (5 ml, pH 5.0), and then 30% H₂O₂ (5 µl is added). An alternative substrate can be made, consisting of 9 mg diaminobenzidine tetrahydrochloride and 6 mg sodium perborate tetrahydrate dissolved in 15 ml of PBS. Though the staining is not quite so intense, these chemicals have the advantage that they can be shipped by air.
  - xiv) The plate is examined microscopically. Virus-positive cells show red-brown cytoplasmic staining.

- **Tube method for tissue or buffy coat suspensions, or semen samples**
  
  **NB:** this method can also be conveniently adapted to 24-well plastic dishes.
  - i) Tissue samples are ground up and a 10% suspension in culture medium is made. This is then centrifuged to remove the debris. Raw semen is diluted 1/10 in culture medium.
  - ii) Test tube cultures (with cover-slips) with newly confluent or subconfluent monolayers of susceptible bovine cells are inoculated with 0.1 ml of the sample. The culture is left to adsorb for 1 hour at 37°C.
  - iii) The culture is washed with 1 ml of medium; this is then discarded and 1 ml of culture maintenance medium is added.
  - iv) The culture is incubated for 4–5 days at 37°C, and examined microscopically for evidence of CPE or signs of cytotoxicity.
  - v) Culture may then either be frozen and thawed for passage to fresh cultures, or the cover-slip may be removed, fixed in acetone and stained with direct immunofluorescent conjugate to BVDV. In this case, examine under a fluorescent microscope for diffuse, cytoplasmic fluorescence characteristic of pestiviruses.
Alternatively, cultures may be freeze/thaw harvested and passaged on to microtitre plates for culture and staining by the immunoperoxidase method (see section on microplate immunoperoxidase method for mass screening of serum samples above) or by the immunofluorescent method described here.

b) Enzyme-linked immunosorbent assay for antigen detection

Several methods for the enzyme-linked immunosorbent assay (ELISA) for antigen detection have been published (e.g. Entrican et al., 1995) and a number of commercial kits are available. Most are based on the sandwich ELISA principle, with a capture antibody bound to the solid phase, and a detector antibody conjugated to a signal system, such as peroxidase. Both monoclonal- and polyclonal-based systems are described. The test is suitable for detection of persistently infected animals, and usually measures BVD antigen (NS2-3 or ERNS) in lysates of peripheral blood leukocytes; the new generation of antigen-capture ELISAs (ERNS capture ELISAs) is able to detect BVD antigen in blood as well as in plasma or serum samples. The best of the methods gives a sensitivity similar to virus isolation, and may be preferred in those rare cases where persistent infection is combined with seropositivity. Due to transient viraemia, the antigen ELISA appears to be less useful for virus detection in acute BVD infections. The NS2-3 ELISA may be less effective in young calves that have had colostrum due to the presence of BVDV maternal antibodies. The reverse transcription PCR (RT-PCR) is probably the most sensitive detection method for this circumstance, but the ERNS ELISA has also been shown to be a sensitive and reliable test, particularly when used with ear-notch samples (Cornish et al., 2005).

c) Immunohistochemistry

Enzyme-labelled methods are useful to detect BVDV antigen in tissue sections (Wilhelmsen et al., 1991), particularly where suitable MAbs are available. It is important that the reagents and procedures used be fully validated, and that nonspecific reactivity be eliminated. For persistently infected cattle almost any tissue can be used, but particularly good success has been found with lymph nodes, thyroid gland, skin, brain, abomasum and placenta. Skin biopsies, such as ear-notch samples, have shown to be useful for in vivo diagnosis of persistent BDV infection (Clarke et al., 1987).

d) Nucleic acid detection

The RT-PCR method can be adapted to the detection of BVD viral RNA for diagnostic purposes (Brock, 1995; Hamel et al., 1995; Kim & Dubovi, 2003; Letellier & Kerkhofs, 2003). This may have a special value where low-level virus contamination is suspected, for example in screening batches of FCS, or biological products such as vaccines (Harasawa, 1995). Caution is needed in the interpretation of results, as the detection of viral RNA does not imply per se that infective virus is present. A multiplex PCR can be used to amplify and type virus from cell culture, or direct from blood samples, by producing different sized PCR products (Gilbert et al., 1999). Newer methodologies incorporate the use of DNA fluorescently labelled probes, which confirm the identity of the PCR product, provide automated reading and can also differentiate pestiviruses in real time (McColdrick et al., 1999). Testing for virus after inoculation of cell cultures using PCR should be avoided as it may give false positive results if commercial bovine fetal serum contaminated with ruminant pestiviruses has been used in the growth medium. Primers should be selected in conserved regions of the genome, ideally the 5'-noncoding region, or the NS3 (p80 gene). Molecular tests can be prone to contamination in unskilled hands. Stringent precautions should therefore be taken to avoid DNA contamination in the test system, and rigorous controls must be mounted (see Chapter 1.1.5 Principles and methods of validation of diagnostic assays for infectious diseases).

The RT-PCR technique is also sensitive enough to enable the detection of persistently infected lactating cows in a herd of up to 100 animals or more, by testing the somatic cells within bulk milk (Drew et al., 1999; Radwan et al., 1995). A positive result indicates that at least one such animal is present in the milking herd. Follow-up virus isolation or antigen detection tests are required to identify the individual(s).

Viral nucleic acid in tissues can be detected by in situ hybridisation with enzyme-linked riboprobes (Desport et al., 1994). This is a sensitive technique that can be applied to formalin-fixed paraffin-embedded tissue, thereby allowing a retrospective analysis. Extraction of nucleic acid and RT-PCR from such samples has been described in this context, also allowing phylogenetic analysis (Bhudevi & Weinstock, 2003).

2. Serological tests

Antibody to BVDV can be detected in cattle sera by a standard virus neutralisation (VN) test or by ELISA, using one of several published methods (Edwards, 1990; Howard et al., 1985; Katz & Hanson, 1987; Paton et al., 1991). Control positive and negative standard sera must be included in every test. These should give results within predetermined limits for the test to be considered valid. ELISA for antibody in bulk milk samples can give a useful indication of the BVD status of a herd (Niskanen, 1993). A high ELISA value of 1.0 or more absorbance units indicates a high probability of the herd having been exposed to BVDV in the recent past, most likely through
one or more persistently viraemic animals being present. In contrast, a very low or negative value (≤0.2) indicates that it is unlikely that persistently viraemic animals are present. Further categorisation has been suggested for intermediate values, but this is dependent on the husbandry system in use. ELISA values have been shown to be an unreliable indicator of the presence of persistently infected animals on farms, due to differing husbandry (Zimmer et al., 2002), and also due to the presence of viral antigen in bulk milk, which may interfere with the antibody assay itself (Obritzhauser et al., 2002). Determination of the antibody status of a small number of young stock (9–18 months) has also been suggested as an indicator of recent exposure to BVDV (Houe et al., 1995), but these are likewise dependent on the degree of contact between different groups of animals in the herd. Rapid ‘spot tests’ can be used for initial screening as part of BVD control and eradication schemes (Lindberg & Alenius, 1999).

a) **Virus neutralisation test**

Because it makes the test easier to read, most laboratories use highly cytopathogenic, laboratory-adapted strains of BVDV for VN tests, although immune-labelling techniques are now available that allow simple detection of the growth or neutralisation of noncytopathogenic strains where this is considered desirable. No single strain is likely to be ideal for all circumstances, but in practice one should be selected that detects the highest proportion of serological reactions in the local cattle reactions. Two widely used cytopathogenic strains are ‘Oregon C24V’ and ‘NADL’. Low levels of antibody to BVD type 2 virus may not be detectable by a neutralisation test using type 1 strain of the virus, and vice versa (Fulton et al., 1997). It is important that BVD type 1 and BVD type 2 be used in the test and not just the one that the diagnostician thinks is present, as this can lead to under reporting.

An outline protocol for a microtitre VN test is given below (Edwards, 1990):

i) The test sera are heat-inactivated for 30 minutes at 56°C.

ii) From a starting dilution of 1/5, serial twofold dilutions of the test sera are made in a cell-culture grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For each sample, two or four wells are used at each dilution depending on the degree of precision required. Control positive and negative sera should also be tested.

iii) An equal volume (e.g. 50 µl) of a stock of cytopathogenic strain of BVDV containing 100 TCID₅₀ (50%) tissue culture infective dose is added to each well. A back titration of virus stock is also done in some spare wells to check the potency of the virus (acceptance limits 30–421 TCID₅₀).

iv) The plate is incubated for 1 hour at 37°C.

v) A flask of suitable cells (e.g. bovine turbinate, bovine testis) is trypsinised and the cell concentration is adjusted to 3 × 10⁵/ml. 50 µl of the cell suspension is added to each well of the microtitre plate.

vi) The plate is incubated at 37°C for 4–5 days, either in a 5% CO₂ atmosphere or with the plate sealed.

vii) The wells are examined microscopically for CPE. The VN titre for each serum is the dilution at which the virus is neutralised in 50% of the wells. This can be calculated by the Spearman–Kärber method. A seronegative animal will show no neutralisation at the lowest dilution (1/5), equivalent to a final dilution of 1/10.

b) **Enzyme-linked immunosorbent assay**

Both indirect and blocking types of test can be used (Howard et al., 1985; Katz & Hanson, 1987; Paton et al., 1991). A number of commercial kits are available. The chief difficulty in setting up the test lies in the preparation of a viral antigen of sufficient potency. The virus must be grown under optimal culture conditions using a highly permissive cell type. Any serum used in the medium must not inhibit growth of BVDV. The optimal time for harvest should be determined experimentally for the individual culture system. The virus can be concentrated and purified by density gradient centrifugation. Alternatively, a potent antigen can be prepared by treatment of infected cell cultures with detergents, such as Nonidet P40, N-decanoyl-N-methylglucamine (Mega 10), Triton X-100 or 1-octyl-beta-D-glucopyranoside (OGP). Some workers have used fixed, infected whole cells as antigen. In future, increasing use may be made of artificial antigens manufactured by expressing specific viral genes in bacterial or eukaryotic systems (Vanderheijden et al., 1993). Such systems should be validated by testing sera specific to a wide range of different virus strains. In the future, this technology should enable the production of serological tests complementary to subunit or marker vaccines, thus enabling differentiation between vaccinated and naturally infected cattle.

An example outline protocol for an indirect ELISA is given below (Edwards, 1990).

i) Roller cultures of secondary calf testis cells with a high multiplicity of infection (about one), are inoculated with BVDV strain Oregon C24V, overlaid with serum-free medium and incubated for 24 hours at 37°C.
ii) The cells are scraped off and pelleted. The supernatant medium is discarded. The pellet is treated with two volumes of 2% OGP in PBS for 15 minutes at 4°C, and centrifuged to remove the cell debris. The supernatant antigen is stored in small aliquots at –70°C, or freeze-dried. Non-infected cells are processed in parallel to make a control antigen.

iii) The antigen is diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6. Alternate rows of an ELISA-grade microtitre plate are coated with virus and control antigens overnight at 4°C. The plates are then washed in PBS with 0.05% Tween 20 or Tween 80 (PBST) before use in the test.

iv) Test sera are diluted 1/50 in serum diluent (0.5 M NaCl; 0.01 M phosphate buffer; 0.05% Tween 20; 0.001 M ethylene diamine tetra-acetic acid; 1% polyvinyl pyrrolidone, pH 7.2) and added to virus- and control-coated wells for 1 hour at 37°C. The plates are then washed five times in PBST.

v) Rabbit anti-bovine IgG peroxidase conjugate is added at a predetermined dilution (in serum diluent) for 1 hour at 37°C, then the plates are again washed five times in PBST.

vi) A suitable enzyme substrate is added, such as hydrogen peroxide/tetramethyl benzidine. After colour development, the reaction is stopped with sulphuric acid and the absorbance is read on an ELISA plate reader. The value obtained with control antigen is subtracted from the test reaction to give a net absorbance value for each serum.

vii) It is recommended to convert net absorbance values to sample:positive ratio (or percentage positivity) by dividing net absorbance by the net absorbance on that test of a standard positive serum that has a net absorbance of about 1.0. This normalisation procedure leads to more consistent and reproducible results.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Infection via the oropharynx and respiratory tract is probably the most important route of transmission of BVDV on farms. Protection against spread in this way would have a beneficial effect on controlling disease due to the virus, particularly in the young animal. The formulation of a vaccine that will provide protection to the fetus is also required in order to prevent the wide range of syndromes that result from in utero infection (Brownlie et al., 1995).

A standard vaccine for protection against infection has not yet been developed, but a number of commercial preparations are available in, for example, Europe and North America. Traditionally, BVD vaccines have been based on a cytopathogenic strain of the virus and fall into two classes: modified live virus or inactivated vaccines.

Although live virus vaccines are available in some countries, they should be used under careful veterinary control because a cytopathogenic strain may precipitate mucosal disease by superinfection of persistently viraemic animals, while in pregnant cattle, a noncytopathogenic component of the vaccine may cross the placenta and infect the fetus as described in Section B.b. Live virus vaccine may also be immunosuppressive and precipitate other infections. On the other hand, modified live virus vaccines may only require a single dose. Properly constituted vaccines containing killed virus are safe to use but, to obtain satisfactory levels of immunity, they usually require booster vaccinations, which may be inconvenient. A combined vaccination protocol using inactivated followed by live vaccine may reduce the risk of adverse reaction to the live strain (Frey & Eicken, 1995).

Experimental inactivated vaccines based on baculovirus-expressed BVD viral glycoprotein E2 have been described. They offer a future prospect of ‘marker vaccines’ when used in connection with a complementary serological test (Bruschke et al., 1999). However, it should be noted that such vaccines for the closely related classical swine fever virus have not proven so effective, probably because of their inability to induce a strong cell-mediated immune response.

BVDV is particularly important as a hazard in the manufacture of biological products for other diseases because of the high frequency of contamination of batches of FCS used as a culture medium supplement (Harasawa, 1995). Particular attention should be paid to sera designed for administration to animals, or used as a growth supplement in embryo transfer or in-vitro fertilisation procedures. Serum used for such purposes should be treated so as to assure sterility. It is recommended that post-treatment tests, such as are detailed in Chapter 1.1.7 Tests for sterility and freedom from contamination of biological materials, be used to ensure that serum is free of BVDV.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.
1. Seed management

a) Characteristics of the seed

An ideal vaccine should contain a strain (or strains) of virus that has been shown to give protection against the wide diversity of antigenicity that has been demonstrated by BVDV. A good appreciation of the antigenic characteristics of individual strains can be obtained by screening with panels of MAbs (Paton et al., 1995). The identity of the seed virus should be confirmed by sequencing (Ridpath et al., 1994).

The emergence of genotype 2 BVD has raised questions regarding the degree of protection conferred by type 1 vaccines against genotype 2. An in-vitro study of the neutralising ability of sera induced by one vaccine revealed broad reactivity with diverse strains from Europe and the USA, including type 2 strains (Hamers et al., 2002). Other work has shown that vaccine derived from one genotype can afford a degree of protection from the other (Cortese et al., 1998; Dean & Leyh, 1999; Makoschey et al., 2001). However, the efficacy of vaccination of whatever genotype, particularly with a killed vaccine, in preventing transplacental transmission is less predictable, as viraemia is rarely completely prevented.

Isolates of cytopathic virus are often mixed with the noncytopathic biotype. The separation and purification of the two biotypes from an initial mixed culture depends on either three cycles of a limiting dilution technique for the noncytopathogenic virus, or three cycles of plaque selection for the cytopathogenic virus. Purity of the cytopathogenic virus should be confirmed by at least one additional passage at limiting dilution. When isolates have been cloned, their identity should be confirmed by direct or indirect staining with specific antibody linked to fluorescein or enzyme.

b) Method of culture

Both biotypes will grow in a variety of cell cultures of bovine origin. Standard procedures may be used, with the expectation for harvesting noncytopathogenic virus on days 5–7 and cytopathogenic virus on days 2–4. The details for optimal yield depend on several factors, including the cell culture and isolate used and the initial seeding rate of virus (Howard et al., 1994).

c) Validation as a vaccine

All vaccines should pass standard tests for safety and efficacy. It is crucial to ensure that the cell cultures and fetal bovine serum included in culture medium be free from adventitious BVDV and antibody (described in Section B), and other microorganisms. Live vaccines must either be demonstrated to be safe in pregnant cattle (i.e. no transmission to the fetus), or should be licensed with a warning not to use them in pregnant animals. Live vaccines containing cytopathogenic strains should have an appropriate warning of the risk of inducing mucosal disease in persistently infected cattle.

Efficacy tests of BVD vaccines in non-pregnant cattle are limited by the difficulty of establishing a satisfactory challenge model. Tests should include as a minimum the demonstration of seroconversion following vaccination, a reduction in virus shedding after challenge in vaccinated cattle, and a diminution in measurable clinical parameters, such as rectal temperature response and leukopenia (Bolin, 1993; Brownlie et al., 1995; Howard et al., 1994). Vaccines intended for use in adult breeding cattle should be evaluated for their efficiency in reducing transplacental transmission, ideally achieving complete prevention. In this case, a suitable challenge system can be established by intranasal inoculation of noncytopathogenic virus into pregnant cows at under 90 days gestation (Brownlie et al., 1995). Usually this system will reliably produce persistently viraemic offspring in non-immune cows.

2. Method of manufacture

There is no standard method for the manufacture of a BVD vaccine, but conventional laboratory techniques with stationary, rolled or suspension (micro-carriers) cell cultures may be used. Inactivated vaccines can be prepared by conventional methods, such as binary ethylenimine or beta-propiolactone inactivation (Howard et al., 1994; Park & Bolin, 1987). A variety of adjuvants may be used (Howard et al., 1994; Neaton, 1986).

3. In-process control

Cultures should be inspected regularly to ensure that they are free from contamination, and to monitor the health of the cells and the development or absence of CPE, as appropriate.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.
Chapter 2.4.8. – Bovine viral diarrhoea

b) Safety

It is essential that all the infectivity be removed during preparation of an inactivated vaccine, and samples should be subjected to several passages in cell culture to ensure the absence of live BVDV. It may also be necessary to ensure the absence of various proscribed agents (prior to inactivation) before use of the vaccine is permitted.

c) Potency

Ideally, the potency of the vaccine should be determined by inoculation into seronegative and virus negative calves, followed by monitoring of the antibody response; however, this is prohibitively expensive for batch control. Antigen content can be assayed by ELISA and adjusted as required to a standard level for the particular vaccine (Bolin, 1993; Ludemann & Katz, 1994). Standardised assay protocols applicable to all vaccines do not exist. Live vaccine batches may be assayed by infectivity titration.

d) Duration of immunity

There are few published data on the duration of antibody following vaccination with a commercial product. Protocols for their use usually recommend a primary course of two inoculations and boosters at yearly intervals. Only limited data are available on the antibody levels that correlate with protection against respiratory infections (Bolin & Ridpath, 1995; Howard et al., 1989) or in utero infection (Brownlie et al., 1995).

e) Stability

There are no accepted guidelines for the stability of BVD vaccines, but it can be assumed that attenuated virus vaccine (freeze-dried) should remain potent for at least 1 year if kept at 4°C. Inactivated virus vaccine could have a longer shelf life at 4°C. Lower temperatures could prolong shelf life for either type, but adjuvants in killed vaccine may preclude this.

f) Precautions

BVDV is not considered to be a human health hazard. Standard good microbiological practice should be adequate for handling the virus in the laboratory.

5. Tests on the final product

a) Safety tests

The safety of the final product formulation of both live and inactivated vaccines should be assessed in susceptible calves for any local reactions following administration, and in pregnant cattle for their effects on the unborn calf. Tests for individual batches are described in Section C.4.b.

b) Potency tests for antigenicity

BVD vaccines must be demonstrated to produce adequate immune responses, as outlined in Section C.4.c above, when used in their final formulation according to the manufacturer’s published instructions. In-vitro assays (Section C.4.c) may be used to monitor individual batches.

REFERENCE


Chapter 2.4.8. — Bovine viral diarrhoea


Chapter 2.4.8. – Bovine viral diarrhoea


**NB:** There are OIE Reference Laboratories for Bovine viral diarrhoea (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for bovine viral diarrhoea.
CHAPTER 2.4.9.

CONTAGIOUS BOVINE PLEUROPNEUMONIA

SUMMARY

Contagious bovine pleuropneumonia (CBPP) is a disease of cattle caused by Mycoplasma mycoides subsp. mycoides SC (MmmSC; SC = small colonies). It is manifested by anorexia, fever and respiratory signs such as dyspnoea, polypnoea, cough and nasal discharges in bovines. Diagnosis depends on the isolation of the aetiological agent. The main problems for control or eradication are the frequent occurrence of subacute or subclinical infections and the persistence of chronic carriers after the clinical phase.

Identification of the agent: Samples to be taken from live animals are nasal swabs and/or broncho-alveolar washings or pleural fluid obtained by puncture. Samples to be taken at necropsy are lung lesions, lymph nodes, pleural fluid and synovial fluid from those animals with arthritis. Direct examination of the exudate or smears is possible, but requires great skill.

For cultivation of the pathogen, the tissues are ground in medium containing antibiotics and inoculated into media that contain inhibitors to prevent the growth of contaminating bacteria. The growth of MmmSC takes several days.

In broth, growth is visible within 2–4 days as a homogeneous cloudiness with whirls when shaken; on agar, small colonies develop, 1 mm in diameter, with the classical ‘fried-egg’ appearance. The biochemical characteristics of MmmSC are the following: sensitivity to digitonin, reduction of tetrazolium salts, fermentation of glucose, absence of arginine hydrolysis, and the absence of or very slight phosphatase and proteolytic activities. Special media have been described that are recommended for these tests. Diagnosis is confirmed by immunological tests, such as the growth inhibition and immunofluorescence tests (both use hyperimmune sera). The polymerase chain reaction is now used as a rapid, specific, sensitive and easy to use test.

Serological tests: For diagnosis, the modified Campbell & Turner complement fixation test remains a prescribed test for international trade. However, it has significant limitations regarding sensitivity and specificity. The competitive enzyme-linked immunosorbent assay was designated as an OIE prescribed test for international trade by the OIE International Committee in May 2004. An immunoblotting test has undergone evaluation and is highly specific and sensitive.

Requirements for vaccines: Attenuated strains now recommended for vaccine production: the T1/44 and T1sr. The minimal recommended titre is $10^7$ mycoplasmas per vaccinal dose, but higher titres of at least $10^8$ are recommended.

A. INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is a contagious disease of cattle caused by Mycoplasma mycoides subsp. mycoides SC (MmmSC; SC = small colonies). CBPP has been known to occur in Europe since the 16th century but it gained a world-wide distribution only during the second half of the 19th century because of increased international trade in live cattle. It was eradicated from many countries by the beginning of the 20th century through stamping-out policies. However the disease persists in many parts of Africa. The situation in Asia is unclear. There have been no reported outbreaks in Europe since 1999. In natural conditions, MmmSC affects only the ruminants of the Bos genus, i.e. mainly bovine and zebu cattle but also the yak (Bos grunniens). MmmSC (bovine biotype) has been isolated from buffaloes in Italy (Bubalus bubalis) (Santini et al., 1992), and from sheep and goats in Africa and more recently in Portugal and in India (Srivastava et al., 2000). Among wild animals, one single case has been reported in American buffaloes (Bison bison) and none in African buffaloes (Syncerus caffer) or other wild ruminants. Wild animals do not play a role in the epidemiology of the disease. CBPP is manifested by anorexia, fever and respiratory signs, such as dyspnoea, polypnoea, cough and nasal discharges. In the case of acute outbreaks under experimental conditions, the mortality rate may be as high as
50% in the absence of antibiotic treatment. When an outbreak first occurs in an area, the mortality will be high but is often lower in the field following the primary outbreak. Clinical signs are not always evident; subacute or asymptomatic forms occur frequently as the clinical signs in affected animals subside with partial recovery. In this case their lungs show typical encapsulated lesions called ‘sequestra’. These animals may be responsible for unnoticed persistence of the infection in a herd or a region and play an important role in the epidemiology of the disease. Transmission of the disease occurs through direct contact of an infected animal with a naive one. There is no evidence of transmission through fomites as \( MmmSC \) does not persist in the environment. In most continents, control strategies are based on the early detection of outbreaks, control of animal movements and a stamping-out policy. In Africa control of the disease is based on vaccination campaigns using attenuated \( MmmSC \) strains such as T1/44 or T1sr. Although the use of antibiotics is theoretically prohibited, they are widely applied in the field. The consequences of these antibiotic treatments in terms of clinical efficacy, emergence of resistant strains, and persistence of chronic carriers have not been evaluated yet. However, recent work has shown that antibiotic treatment of cattle may greatly reduce the transmission to healthy contacts but this requires treatment of all affected cattle in a group (Hubschle et al., 2004). The \( M. mycoides \) cluster consisted of six mycoplasma species or groups of strains, originating from bovines and goats (Cottew et al., 1987; Thiaucourt et al., 2000; Pettersson et al., 1996). This cluster can be subdivided in two groups, capricolum and mycoides, comprising very closely related species. These six mycoplasmas share serological and genetic characteristics, and this causes taxonomic and diagnostic problems (Cottew et al., 1987) with standard techniques. Following phylogenetic studies, the group has now been reduced to five species, subspecies or biotypes (Manso-Silván et al., 2009) and there are no unnamed group of strains within the cluster. Specific identification of \( MmmSC \) can now be achieved by polymerase chain reaction (PCR) or the use of specific monoclonal antibodies (MAbs). The PCR can also be used to identify specifically the T1 vaccinal strains (Lorenzon et al., 2000). Although \( MmmSC \) has been considered to be a very homogeneous biotype, recent molecular techniques, such as enzymatic digestion of whole DNA or southern blotting using an insertion element as a probe, were able to identify differences among strains. A recently described technique that provides an easier way to perform molecular epidemiology of CBPP is a multi-locus sequence analysis (or typing). This technique allows the three main lineages that correlate with the geographical origins (Europe, Southern Africa, rest of Africa) to be distinguished (Lorenzon et al., 2003). Quite interestingly, the strains of European origin can be clearly differentiated from African ones (Cheng et al., 1995; Gonçalves et al., 1998; Vilei et al., 2000). Recent European strains form a particular cluster and differ from all other strains by no duplication of a long 13 kb DNA fragment (Gaurivaud et al., 2004) and deletion of a 8.4 kb fragment. They are not able to oxidise glycerol, which may account for an apparent lower pathogenicity. The sequence of the complete genome of the reference strain PG1 has been published in 2004 (Westberg et al., 2004). There is no doubt that further technical development will allow for a finer characterisation of strains. CBPP is not a zoonotic infection.

### B. DIAGNOSTIC TECHNIQUES

1. **Identification of the agent**

The causal organism can be isolated from samples taken either from live animals or at necropsy. Samples taken from live animals are nasal swabs or nasal discharges, broncho-alveolar lavage or transtracheal washing and pleural fluid collected aseptically by puncture made in the lower part of the thoracic cavity between the seventh and eighth ribs. Blood may also be cultured but mycoplasmaemia is transient (Hudson, 1971). Samples taken at necropsy are lungs with lesions, pleural fluid (‘lymph’), lymph nodes of the broncho-pulmonary tract, and synovial fluid from those animals with arthritis. The samples should be collected from lesions at the interface between diseased and normal tissue.

The agent can be detected by culture, nucleic acid methods and immunological tests described below. Bacteriological identification of the agent is more complex and can be done by biochemical tests, nucleic acid recognition methods and immunological methods. These methods are described here in general terms; however, it is recommended that the definitive identification be done by an OIE Reference Laboratory.

The presence of pathogens varies greatly with the stage of development of the lesions, and a negative result is not conclusive, particularly after treatment with an antibiotic.

When dispatching samples to the laboratory, it is advisable to use a transport medium that will protect the mycoplasmas and prevent proliferation of other bacteria (heart-infusion broth without peptone and glucose, 10% yeast extract, 20% serum, 0.3% agar, 500 International Units [IU]/ml penicillin, thallium acetate 0.2 g/litre).

The samples must be kept cool at 4°C if stored for a few days or frozen at or below –20°C for a longer period. For laboratory-to-laboratory transfer, lung fragments or pleural fluid can also be freeze-dried.
a) Culture

*M. mm* needs appropriate media to grow (Provost *et al.*, 1987) but it is not considered a fastidious mycoplasma. A key to isolation success lies in the harvest of good quality samples. Many attempts to isolate fail because the organism is labile, is often present in small quantities, and is demanding in its growth requirements. The media should contain a basic medium (such as heart-infusion or peptone), yeast extract (preferably fresh), and horse serum (10%). Several other components can be added, such as glucose, glycerol, DNA, and fatty acids, but the effects vary with the strains. To avoid growth of other bacteria, inhibitors, such as penicillin, colistin or thallium acetate, are necessary. The media can be used as broth or solid medium with 1.0–1.2% agar. All culture media prepared should be subjected to quality control and must support growth of *Mycoplasma* spp. from small inocula. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are working correctly.

After grinding in broth containing antibiotics, the lung samples are diluted tenfold to minimise contaminating bacteria and are inoculated into five tubes of broth and on to solid medium. The pleural fluid can be inoculated directly without previous dilution. Hermetic sealing of the Petri dishes or the use of incubators with controlled humidity are recommended in order to avoid desiccation. To ensure the best conditions for mycoplasma growth, a CO₂ incubator or candle jar should be used. The tubes and Petri dishes are inspected daily for 5 days. In fluid medium, a homogeneous cloudiness usually appears within 2–4 days, frequently with a silky, fragile filament called a ‘comet’, which is characteristic of *M. mm* (or *M. capricolum* subsp. *capripneumoniae*, the cause of contagious caprine pleuropneumonia). During the following days a uniform opacity develops with whirls when shaken. On agar media, the colonies are small (1 mm in diameter) and have the classical appearance of ‘fried eggs’ with a dense centre. At this stage, the indirect fluorescent antibody (IFA) test or PCR can be performed.

b) Biochemical tests

Biochemical tests were used routinely in the past but have now been superseded by other tests. Biochemical tests alone do not allow identification of a precise mycoplasma species.

For this purpose, after two or three subcultures, antibiotics should be omitted from the medium to check if the isolate is a mycoplasma or an L-form of a bacterium that will regain its original form in the medium without inhibitors. Once this test is done and after cloning (at least three colonies should be selected), the organism can be identified using biochemical tests (Al-Aubaidi & Fabricant, 1971; Freundt *et al.*, 1979).

*M. mm* is sensitive to digitonin (like all members of the order Mycoplasmatales), does not produce ‘film and spots’, ferments glucose, reduces tetrazolium salts (aerobically or anaerobically), does not hydrolyse arginine, has no phosphatase activity, and has no or weak proteolytic properties.

For these tests, special media have been developed that include the same basic ingredients (heart-infusion broth or Bacto PPLO [pleuropneumonia-like organisms] broth, horse serum, 25% yeast extract solution, 0.2% DNA solution), to which is added 1% of a 50% glucose solution for glucose hydrolysis, 4% of a 38% arginine HCl solution for arginine hydrolysis, and 1% of a 2% triphenyl tetrazolium chloride solution for tetrazolium reduction, plus a pH indicator (e.g. phenol red). (Note: a pH indicator should not be added to a medium containing triphenyl tetrazolium chloride.) For demonstration of proteolysis, growth is carried out on casein agar and/or coagulated serum agar.

Once the biochemical characteristics have been checked, one of the following immunological tests can be performed to confirm the identification: disk growth inhibition test (DGIT), fluorescent antibody test (FAT), and the dot immunobinding on a membrane filter (MF-dot) test. The isolation and identification of the CBPP agent can be difficult and time consuming and depends on careful use of the appropriate procedures and media. When possible, classical bacteriology laboratories should set up a special section for work only with mycoplasmas.

c) Nucleic acid recognition methods

The PCR is sensitive, highly specific, rapid and relatively easy to perform. It has become the method of choice for a rapid and specific *M. mm* identification. Various PCR methods have been developed; some use nonspecific primers for amplification, in which case *Mm*-specific identification is obtained by enzymatic restriction of the amplified product or by a secondary specific amplification. Other methods describe PCR primers that allow a direct specific amplification. Primers specific for the *M. mycoides* cluster (Taylor *et al.*, 1992) and for *Mm* (Dedieu *et al.*, 1994; Miserez *et al.*, 1997; Persson *et al.*, 1999) have been reported and PCR assays have been developed (Bashiruddin *et al.*, 1994; Dedieu *et al.*, 1994; Miserez *et al.*, 1997), including a technique that permits the specific identification of the T1 vaccinal strains (Lorenzon *et al.*, 2000). Using samples such as lung exudate allows the PCR to be performed directly after differential centrifugations to remove inflammatory cells and pellet mycoplasmas. For lung fragments, the PCR is applied after DNA extraction. The PCR can also be performed on urine or blood. The main advantage of the
PCR technique is that it can be applied to poorly preserved samples (contaminated or without any viable mycoplasmas as may occur following antibiotic treatment). If direct detection of DNA from the organ under test fails, specimens should be enriched by culturing them in an appropriate medium for 24–48 hours, followed by attempted detection of DNA from the culture. The PCR has become the primary tool for identification of *Mmm*SC. If a sample is PCR positive in a CBPP-free zone, the test should be confirmed by a second and different PCR to rule out any contamination problem.

The onset of real-time PCR assays should solve this possible troubleshooting as fluorescence resulting from genomic amplification is measured directly without opening the tubes. This technique has already been applied to *Mmm*SC detection (Gorton et al., 2005) and further developments are expected in the near future.

d) Immunological tests

The aetiological agent or its antigens can be demonstrated by immunochemical tests on infected tissues, tissue fluids and/or cultures of the organism. However, as some of these tests are dependent on a minimum number of organisms being present in the sample, only positive results are taken into account. Again these techniques have been superseded by the PCR.

i) *Indirect fluorescent antibody test*

The IFA test can be performed on smears from clinical material using hyperimmune rabbit serum against *Mmm*SC and labelled anti-bovine IgG. Hyperimmune bovine serum has been used, but may have cross-reactive antibodies. The test is satisfactory when applied to pleural fluid smears, but is less satisfactory with lung smears due to considerable nonspecific fluorescence. However, good results can be obtained using lung smears counterstained with Erichrome black.

ii) *Fluorescent antibody test*

The FAT is commonly performed on broth and agar cultures. It is slightly less specific than the IFA test.

*Broth culture:* Place two drops on a microscope slide. Fix for 15 minutes with methyl alcohol, and leave in contact with the labelled hyperimmune serum for 30 minutes at 37°C in a humid chamber. Rinse three times with phosphate buffered saline (PBS, pH 7.2) and examine under an epifluorescence microscope (×80).

*Colonies grown on solid medium:* Cut a block of agar supporting a number of young colonies and place on a slide with the colonies facing upwards. Place one or two drops of the labelled hyperimmune serum on the block and leave it in a humid chamber for 30 minutes. Place the block into a tube and wash twice for 10 minutes with PBS. Place the block on a slide with the colonies facing upwards and examine as before.

*Petri dish culture:* The gel should not be too thick (no more than 3 mm) and should contain as little horse serum as possible. Rinse the gel three times with PBS, flood the surface with 1 ml of labelled serum and incubate for 30 minutes in a humid chamber. Rinse four times with PBS and examine directly under the microscope. The FAT in a Petri dish is used mainly just after isolation and before cloning, as it is very useful in the case of mixed infection with several mycoplasma species.

*Interpretation of the FAT:* With broth culture, the mycoplasmas appear bright green on a dark background. However, experience is required for the FAT carried out with colonies on agar, because the background appears dark green.

iii) *Disk growth inhibition test*

The DGIT is based on the direct inhibition of the growth of the agent on a solid medium by a specific hyperimmune serum (Freundt et al., 1979). However, cross-reactions within the mycoides cluster are common and great care should be taken to differentiate *Mmm*SC (bovine biotype) from *Mmm*LC (caprine biotype; LC: large colonies). It is a simple test to perform, but some results require experience to be interpreted: small inhibition zones (less than 2 mm wide), partial inhibition with ‘breakthrough colonies’, false-negative and false-positive reactions (very rare). The quality of the hyperimmune serum used in this test is critical for good results.

iv) *Agar gel immunodiffusion test*

The agar gel immunodiffusion (AGID) test can detect the specific antigen present at the surface of *Mmm*SC and the circulating galactan invading the haemolymph system of sick animals (Griffin, 1965). Pleural fluid, ground lung fragments or even sequestrae can be tested against a hyperimmune serum in two wells cut 5 mm apart in the gel. The gel is composed of Noble agar (12 g) and thallium acetate...
v) Dot immunobinding on membrane filtration

The MF-dot test can be used for routine identification tests in the laboratory (Poumarat et al., 1991). Specific SC biotype specific MAbs have been developed to overcome cross-reactions within the mycoides cluster (Brocchi et al., 1993).

vi) Immunohistochemistry

*Mmm*SC immunoreactive sites can be detected in lung lesions using the peroxidase–antiperoxidase method on sections of paraffin-embedded material (Ferronha et al., 1990). Because the isolation of the agent is not always achieved from chronic cases and after treatment with antimicrobial drug, this test is only supplementary to the diagnosis of CBPP (Bashiruddin et al., 1999); a negative result is not conclusive.

### 2. Serological tests

Serological tests for CBPP are valid at the herd level only. Tests on single animals can be misleading, either because the animal is in the early stage of disease, before specific antibodies are produced, or it may be in the chronic stage of the disease when very few animals are seropositive.

#### a) Complement fixation (a test suitable for determining freedom from disease and a prescribed test for international trade)

The Campbell & Turner complement fixation (CF) test remains the recommended procedure (although the current method is slightly different from the original one), and it is widely used in all countries where infection occurs (Provost et al., 1987). The CF test, as a micromethod, has been harmonised in the European Union (European Commission, 2001). For antigen titration and harmonisation purposes, an international standard positive bovine serum is available from the OIE Reference Laboratory in Teramo, Italy. However, the CF test is still difficult to perform, requiring well-trained and experienced personnel.

- **Reagents**
  
i) Veronal buffer (VB), pH 7.3. A concentrated stock solution is used diluted 1/5 in sterilised double-distilled water.
  
ii) The serum samples, free from erythrocytes, must be inactivated at 56°C for 30 minutes and diluted 1/10 in VB.
  
iii) The antigen is a suspension of *Mmm*SC, previously checkerboard titrated and used at a dose of 2 complement fixing units (CF units). It must be kept at 4°C and not frozen. It is produced, tested and delivered by Internationally recognised laboratories.
  
iv) The complement (C’) is obtained from normal guinea-pig serum. It is freeze-dried and reconstituted with double-distilled water. It must be kept at −20°C after reconstitution. It is titrated by making a close dilution series in VB containing an appropriate quantity of the antigen to be used in the test. After incubation at 37°C for 2 hours, an appropriate quantity of sensitised sheep red blood cells (SRBC) is added to each dilution. The titration is read after incubation for a further hour. The highest dilution giving complete haemolysis of the SRBC equals 1 C’ unit, from which can be calculated the dilution required for 2.5 units in 25 µl.
  
v) The haemolysin is a hyperimmune rabbit serum to SRBC. The quantity used is 6 haemolytic doses read at 50% end-point (HD50 [50% haemolyzing dose]).
  
vi) The SRBC are obtained by aseptic puncture of the jugular vein. They can be preserved in Alsever’s solution or with sodium citrate. They are used in a 6% suspension.
  
vii) The haemolytic system (HS) is prepared by diluting haemolysin in VB to give a dose of 12 HD50. An equal volume of 6% SRBC suspension is added, and the system is sensitised in a water bath at 37°C for 30 minutes with periodic shaking.
  
viii) The positive bovine standard sera has been obtained from a naturally infected animals negative to antibodies against *Brucella*, bovine viral diarrhoea virus, respiratory syncytial virus, infectious bovine rhinotracheitis virus, adenovirus, bovine herpes virus 4, foot and mouth disease viruses, bovine leukosis virus, and parainfluenza 3 virus. The infected animals are also negative for adventitious viruses.
  
ix) The negative control serum (NS) is a healthy bovine serum, negative to the above microorganisms.
• Test procedure (using microplates)

i) Dispense 25 µl of the test serum samples (already diluted 1/10). Add 25 µl of antigen at a dose of 2 CF units.

ii) Add 25 µl of C’ at a dose of 2.5 units. Shake vigorously and incubate at 37°C for 30 minutes with periodic shaking.

iii) Add 25 µl of HS. Shake vigorously and incubate at 37°C for 30 minutes with periodic shaking.

It is necessary to set up the following controls:

Complement: 0.5 units, 1 unit and 2.5 units.

Haemolytic system: 75 µl of VB + 25 µl of HS.

Antigen: 25 µl of 2 CF units of antigen + 25 µl of C’ at 2.5 units + 25 µl of HS = 25 µl of veronal buffer.

Note: the microplates must be shaken vigorously twice during the incubation period. The above-mentioned controls, the PS and the NS are always used in each microplate or in a series of microplates where the same batches of reagents are used.

iv) Reading and interpreting the results: After centrifugation of the microplates at 125 \( g \) for 2 minutes, the reading is carried out based on the percentage of complement fixation observed.

Positive result: 100% inhibition of haemolysis at 1/10;

Doubtful results: 25, 50 or 75% inhibition of haemolysis at 1/10.

It is recommended that any fixation of complement, even partial (25, 50 or 75%), at a serum dilution of 1/10 should be followed by additional investigations.

The limitations of the CF test are well known. With a sensitivity of 70% and a specificity of 98% (Bellini et al., 1998), the CF test can detect nearly all sick animals with acute lesions, but a rather smaller proportion of animals in the early stages of the disease or of animals with chronic lesions. In addition, therapeutic interventions and improperly conducted prophylactic operations (partial slaughter of the herd) may increase the number of false-negative reactions. However, for groups of animals (herd or epidemiological unit) the CF test is capable of detecting practically 100% of infected groups.

The nature of the pathogenesis of the disease is such that the incubation period, during which antibodies are undetectable by the CF test, may last for several months.

Despite the high specificity of the CF test, false-positive results can occur, of which an important cause is serological cross-reactions with other mycoplasmas, particularly other members of the \( M. \) mycoides cluster.

The validity of the results has to be confirmed by post-mortem and bacteriological examination, and serological tests on blood taken at the time of slaughter.

b) Competitive enzyme-linked immunosorbent assay (a prescribed test for international trade)

A competitive enzyme-linked immunosorbent assay (C-ELISA) developed by the OIE Collaborating Centre for the diagnosis and control of animal diseases in tropical countries (see Table given in Part 4 of this Terrestrial Manual) (Le Goff & Thiaucourt, 1998), has undergone evaluation (Amanfu et al., 1998). An indirect ELISA based on the use of a lipoprotein antigen is currently being validated by the IAEA (Abdo et al., 2000; Bruderer et al., 2002). In May 2004, the C-ELISA was designated as an OIE prescribed test for international trade by the OIE International Committee. Compared with the CF test, the C-ELISA has equal sensitivity and greater specificity. Advice on the availability of reagents can be obtained from the OIE Reference Laboratories for CBPP, or the OIE Collaborating Centre for ELISA and Molecular Techniques in Animal Disease Diagnosis (see Table given in Part 4 of this Terrestrial Manual).

Validation tests (Amanfu et al., 1998; Le Goff & Thiaucourt, 1998) that have been carried out in several African and European countries would indicate i) that the true specificity of the C-ELISA has been reported to be at least 99.9%; ii) that the sensitivity of the C-ELISA and the CF test are similar; and iii) antibodies are detected by the C-ELISA in an infected herd very soon after they can be detected by the CFT and C-ELISA antibody persists for a longer period of time (Niang et al., 2006).

This C-ELISA is now provided as a ready made kit that contains all the necessary reagents including precoated plates kept in sealed aluminium foil. This kit can be obtained commercially. The kit has been especially designed to be robust and offer a good repeatability. As a consequence, sera are analysed in single wells. The substrate has been modified and is now TMB (tetramethyl benzidine) in a liquid buffer and the reading is at 450 nm. The substrate colour turns from pale green to blue in the first place and becomes
yellow once the stopping solution has been added. MAb controls exhibit a darker colour while strong positive serum controls are very pale. The cut-off point has been set at 50% and should be valid in every country.

- **Reagents**
  i) Stock antigen is prepared by washing a concentrated suspension of mycoplasma (2 mg/ml) and lysis with sodium dodecyl sulphate at 0.1%. The stock is kept at –20°C until use.
  ii) MAbs are available from the OIE Collaborating Centre for the Diagnosis and Control of Animal Diseases in Tropical Regions (see Table given in Part 4 of this Terrestrial Manual).
  iii) The conjugate DAKO P260 is diluted in PBS according to the manufacturer’s instructions, with the addition of 0.5% horse serum and 0.05% Tween 20.
  iv) Substrate is made of 1 mM ABTS (2,2’-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) and H$_2$O$_2$ in citrate buffer.

- **Test procedure**
  i) ELISA plates are coated with a lysed antigen solution in PBS, pH 7.4 (100 µl/well) and incubated overnight at 4°C.
  ii) The plates are washed once in PBS diluted 1/5 with 0.05% Tween 20.
  iii) Sera that have not been heat inactivated (diluted 1/10) and MAb diluted in PBS with 0.5% horse serum and 0.05% Tween 20 are left in contact with the antigen for 1 hour at 37°C under moderate agitation in a humid chamber. Heat-inactivated serum will not give satisfactory results.
  iv) The plates are washed twice and conjugate is added to all the wells (100 µl); the wells are then incubated for 1 hour at 37°C.
  v) The plates are washed three times and the substrate is added to all the wells (100 µl).
  vi) Reading is performed at 405 nm when the absorbance in the control MAb has reached 0.8–1.6.

c) Immunoblotting test

An immunoenzymatic test designated the immunoblotting test (IB test) has been developed and is of diagnostic value. A field evaluation indicated a higher sensitivity and specificity than the CF test. A core profile of antigenic bands, present both in experimentally and naturally infected cattle are immunodominant. The more accurate picture of the immune status of animals given by this test is due to the possibility of a more precise analysis of the host’s immune response in relation to the electrophoretic profile of MmmSC antigens; thus the test overcomes problems related to nonspecific binding. It should be used primarily as a confirmatory test, after other tests and should be used in all cases in which the CF test has given a suspected false result.

- **Preparation of antigen strips**
  i) The antigen is prepared by harvesting and washing a suspension of mycoplasma cells obtained from a 48-hour culture.
  ii) A 4% stacking/5–15% gradient-resolving SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel) is prepared and used to perform electrophoresis of the sample with appropriate molecular weight standards.
  iii) The separated proteins are transferred to a 14 × 14 cm 0.45 µm nitrocellulose membrane at 70 V constant voltage in transfer buffer (20% methanol in 193 mM glycine, 25 mM Tris/HCl, pH 8.3).
  iv) The membrane is dried and labelled on the side on which the proteins were electrophoresed. The nitrocellulose membrane is incubated in blocking buffer (PBS containing 5% skim milk, 1 M glycine and 1% egg albumin) for 2 hours at room temperature. After washing at room temperature for three 15-minute washes in 0.1% (v/v) Tween 20 in PBS, the nitrocellulose membrane is then washed again in PBS alone. The sheet is then dried and one strip cut and tested from the edge of the membrane. Specific bands are identified at 110, 98, 95, 62/60 and 48 kDa.
  v) The nitrocellulose membrane sheet is cut into strips, 0.4 cm wide and each strip is labelled. These strips are the antigen used for blotting.

- **Test procedure**

NB: The strips must be kept with the antigen side up during the procedure.
i) Serum samples for testing are diluted 1/3 and positive and negative control sera are prepared using dilution buffer (PBS containing 0.1% skim milk and 0.1% egg albumin).

ii) An antigen strip is placed in each test sample (and controls) and incubated at 37°C for 2 hours with continuous agitation. Strips are then washed, as above.

iii) Strips are incubated for 1 hour at room temperature in an appropriate dilution of peroxidase-conjugated anti-bovine IgG (H + L chains) in dilution buffer, with continuous agitation. Wash as above.

iv) Substrate is made by adding 30 mg 4-chloro-1-naphthol dissolved in 10 ml methanol to 50 ml PBS and 30 µl H₂O₂. Substrate is added to the strips, which are then left in the dark with continuous agitation and examined periodically until the protein bands are suitably dark. The reaction is stopped with distilled water.

v) Reading the results: The strips are dried and examined for the presence of the core IgG immunoblot profile of five specific antigenic bands of 110, 98, 95, 62/60 and 48 kDa. Sera giving a similar immunological profile are considered to be positive.

NB: The IB test is rather difficult to standardise as many factors can influence the final banding pattern. Of major concern is the MmmSC culture stage and the strain that has been chosen. Recent MmmSC strains of European origin are lacking the 98 kDa band. This could lead to dubious results in animals infected by such strains. Furthermore the IB test has not been thoroughly tested on bovines infected with closely related mycoplasma species such as Mmc or M. leachii.

### d) Other tests

An indirect ELISA based on the lppQ lipoprotein had been developed (Abdo et al., 2000) and its validation initiated. However its current availability is uncertain.

A rapid field slide agglutination test (SAT) with either whole blood or serum (Turner & Etheridge, 1963) has been developed to detect specific agglutinins: the antigen is a dense suspension of stained mycoplasmas that is mixed with a drop of blood or serum. Due to a lack of sensitivity, the test detects only animals in the early stages (i.e. acute phase) of the disease. It should be used only on a herd basis. A latex agglutination test has been developed that is easier to interpret than the SAT (Ayling et al., 1999).

For CBPP, the CF test and ELISAs can be used in screening and eradication programmes, but the highly specific IB test can be used as a confirmatory test. However, the IB test is not fit for mass screening and may be difficult to standardise in countries with marginal laboratory facilities so IB testing should be performed in a reference laboratory.

### C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Since the beginning of the 20th century, many vaccines against CBPP have been described (e.g. killed vaccines, and heterologous vaccines), but none of them has proven to be really satisfactory. Today, the only vaccines commonly used are produced with attenuated MmmSC strains.

#### 1. Seed management

##### a) Characteristics of the seed

Two strains are used for preparing CBPP vaccines: strain T1/44, a naturally mild strain isolated in 1951 by Sheriff & Piercy in Tanzania, and strain T1sr (Wesonga & Thiaucourt, 2000; Yaya et al., 1999). The 44th egg-passage of strain T1, called T1/44, was sufficiently attenuated to protect cattle without post-vaccinal severe reactions, however such reactions may still occur in the field although rarely. Their frequency is unpredictable. Cattle breeds should be assessed for their sensitivity before mass vaccination. It should be noted that when given by intubation, the vaccine can produce CBPP lesions (Mbulu et al., 2004); however, as the vaccine is to be injected subcutaneously, this should not create a serious disease problem (Hubschle et al., 2002).

The identity of the strain can be verified with the insertion sequence profile or by the specific PCR assay (Lorenzon et al., 2000).

The master seed strain is kept in freeze-dried form at –20°C. It is deposited at an international laboratory from African Unity, PANVAC.
b) Method of culture

For vaccine production, a system of freeze-dried seed lots originating from master seed cultures is used. These seed lots are kept at –20°C.

The media used for seed cultures are usually the same than for batch production. However there is no specific requirement, they should ensure a correct growth of the vaccine strain.

For vaccine bulk cultures, in order to avoid the risk of inadvertent cloning of vaccine seed, the whole content of a vaccine seed vial should be inoculated directly into a tube filled with production medium. A second tube may be seeded as a dilution from the first one.

2. Method of manufacture

The media used for vaccine production may differ slightly from media for isolation purpose. In the case of a vaccine production, what matters more is the final titre that can be obtained rather than the speed of growth. Furthermore the harvested mycoplasmas should withstand the freeze drying process without excessive titre loss.

Examples of vaccine culture medium are Gourlay's medium or F66, however modifications of these media are licit and may include addition of buffers.

Vaccine bulk cultures must be obtained with a maximum of three successive passages of the seed. A passage is defined here by a 1/100 dilution of a culture in the exponential phase of growth.

For example, 0.5 ml of culture from the seed are transferred to 50 ml of fresh medium and, when turbidity is observed, these 50 ml are used to seed 5000 ml of medium, which represents the final product when the optimum titre has been reached. Each vaccine producer should then evaluate the speed of growth of the vaccine strain in the medium that is used to optimise the harvest time.

A stabiliser can be added to final cultures before freeze drying. The manufacturer should ensure an homogeneous distribution in the vials and use of a proper freeze dryer to have identical titres in all the vials when the freeze drying process is finished.

3. In-process control

Good Manufacturing Practice should be observed to avoid contaminations at each step of the production and to ensure purity of the final product.

As an example, phase contrast microscopic examination of cultures easily allows the detection of contaminations by bacteria or fungi.

4. Batch control

a) Purity and identity

Suitable media must be seeded with the final product to ensure purity of the final product and absence of contamination with classical bacteria and fungi. All media should remain sterile (Provost et al., 1987). Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

Absence of contamination by other mycoplasmas must be checked. For example a growth inhibition test with the final product and a hyperimmune serum to MmMSc (preferably raised with T1/44 antigen) can be performed. The presence of mycoplasma colonies within the inhibition zone must be followed by identification of these colonies to rule out the presence of other mycoplasmas than the vaccine strain.

The identity of the vaccine strain present in the final product must be guaranteed by the producer.

For example a specific PCR can be used to identify T1 strains. In addition streptomycin resistance can be used to differentiate T1/44 from T1sr.

b) Titration

The minimum titre is 10^7 live mycoplasmas per vaccine dose, but higher titres are recommended because of the loss of titre between production plant and actual injection to animals. Titration is performed after reconstitution of the freeze-dried vaccine in the diluent recommended for vaccination and preferably with the diluent provided by the vaccine manufacturer. Titrations should be performed on at least three vials per batch. This titre must be evaluated with a titration technique that allows a precision of ± 0.25 logs. A batch
passes the test if three vials chosen randomly have titres above this limit. The manufacturer must ensure that the minimum titre is retained until the expiry date if the product is kept at the correct temperature.

c) Safety

After reconstitution, the vaccine is inoculated subcutaneously into two mice, intraperitoneally into two mice and intraperitoneally into two male guinea-pigs. None of the animals should die within the following month, and the guinea-pigs should not show signs of orchitis. Safety tests should be carried out on (at least two) cattle or zebu cattle. These are inoculated with ten vaccinal doses each, and observed for adverse effects for at least 4 weeks.

d) Potency

Potency tests are not performed routinely with production batches as there is no laboratory animal that would allow this test to be performed at low cost. Potency tests in cattle are also not performed because of the cost. Getting statistically significant protection rates would involve using at least 50 naive animals.

Potency of the final product is ensured by using a master seed lot of well known origin for which the potency test has already been performed, by strictly following the production standard protocols (avoiding multiple passages) and by ensuring that the final titres are correct.

e) Duration of immunity

Strain T1/44 confers protection for approximately 1 year (Hudson, 1971), but the protection conferred by the T1sr strain may only be 6 months long. Serological conversion (CF test) takes place in some animals. The antibodies disappear 3 months after vaccination.

f) Stability

Periodic titration of the stored vaccine allows the shelf life to be calculated. Freeze-dried vaccine must be stored at –20°C. At this temperature its storage life is at least 1 year (Provost et al., 1987), viability may even be conserved for many years without loss of titre allowing for the constitution of emergency stocks. The titres of these stocks naturally need to be controlled regularly.

g) Preservatives

For lyophilisation, stabilisers can be added. For example, dried skimmed milk can be added: 45 g/litre of culture medium. For reconstitution of a freeze-dried vaccine normal sterile saline solution (9 g/litre) is preferably used. Alternatively, a molar solution of magnesium sulphate (248 g per litre) is used at room temperature. This molar solution protects mycoplasmas against inactivation by heat (Provost et al., 1987). The purity of the salts used is important. When using magnesium sulphate as a diluent for vaccine reconstitution, it is also important to insure that the pH of the final product does not drop below 6.5 as this may induce a loss of titre (March, 2004).

h) Precautions (hazards)

Procedures for use in the field and reconstitution of freeze-dried vaccines have been described by Provost et al. (1987).

Intense reactions may appear when infected animals are vaccinated, as occurred recently following emergency vaccination campaigns in East Africa. These reactions usually occur within 2–3 days. Local reactions may also appear at the site of injection after 2–3 weeks with strain T1/44. These reactions are known as a ‘Willems reaction’, and consist of an invading oedema that leads to death if antibiotic treatment, such as tetracyclin or tylosin, is not given. Strain T1sr is completely devoid of residual pathogenicity, which makes it an alternative choice to T1/44, although the duration of immunity is shorter. Concerns were raised about the ineffectiveness of T1sr to control outbreaks in southern Africa leading to its suspension (Tulasne et al., 1996).

The general sensitivity of a given bovine population should be first tested by vaccinating sample groups (Provost et al., 1987).

5. Tests on the final product

These tests should be performed after reconstitution of a pool of at least five vials of the freeze-dried vaccine in the recommended diluent.
a) Safety

Safety tests should be carried out on cattle or zebu cattle, according to Section C.4.c.

b) Potency

The test is carried out according to the protocol described in Section C.4.d. Because CBPP cannot be easily reproduced experimentally, and due to its cost, only one potency test need be performed on each seed lot, providing the titre is satisfactory and that production parameters have not been changed.

REFERENCES


FERRONHA M.H., NUNES PETISCA J.L., SOUSA FERREIRA H., MACHADO M., REGALLA J. & PENHA GONÇALVES A. (1990). Detection of Mycoplasma mycoides subsp. mycoides immunoreactive sites in pulmonary tissue and sequestra of
Chapter 2.4.9. — Contagious bovine pleuropneumonia


Chapter 2.4.9. — Contagious bovine pleuropneumonia


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NB: There are OIE Reference Laboratories for Contagious bovine pleuropneumonia (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for contagious bovine pleuropneumonia.
CHAPTER 2.4.10.

DERMATOPHILOSIS

SUMMARY

Dermatophilosis (also known as streptothrichosis) is an exudative, pustular dermatitis that mainly affects cattle, sheep and horses, but also goats, dogs and cats, many wild mammals, reptiles and, occasionally, humans. The severe disease in ruminants is promoted by immunomodulatory effects induced by infestation with the tick, Amblyomma variegatum.

Laboratory diagnosis of dermatophilosis depends on the demonstration of the bacterium Dermatophilus congolensis in material from the skin or other organs. Sites other than the skin are rarely affected.

Identification of the agent: Dermatophilus congolensis normally affects the epidermis, causing the formation of scabs. It may be demonstrated in smears made from scabs emulsified or softened in water or in impression smears from the base of freshly removed adherent scabs. The organism is Gram positive, but its morphology is more readily appreciated in smears stained with Giemsa. In stained smears, the organism is seen as branching filaments containing multiple rows of cocci. This characteristic appearance is diagnostic. In wet or secondarily infected scabs, only free cocci may be present, so that staining by immunofluorescence is necessary. Dermatophilus congolensis is demonstrated in histopathological sections by Giemsa staining or by immunofluorescence. Dermatophilus cheloniae may be found in crocodiles, chelonids and cobras.

Isolation of D. congolensis from freshly removed scabs is straightforward, but the organism is readily overgrown by other bacteria. When cultured from contaminated sites, special techniques involving filtration, chemotaxis, or selective media are necessary.

Demonstration and identification of D. congolensis by immunofluorescence is a reliable and very sensitive method of diagnosis, but requires that laboratories make their own diagnostic antisera as these are not available commercially. Although antigenic cross-reaction with Nocardia spp. has been reported, this is likely to give only weak fluorescence. Ideally, a monoclonal antibody specific to D. congolensis should be used. Polymerase chain reaction (PCR)-based characterisation has also been developed.

Serological tests: A variety of serological tests has been used in studies of the epidemiology and pathogenesis of dermatophilosis. Antibody can be demonstrated in all but fetal blood in healthy ruminants, but the elevated levels associated with clinical infection can be used to identify animals that have been infected with the disease.

Requirements for vaccines and diagnostic biologicals: Despite the identification of several virulence factors, no vaccines are available currently.

A. INTRODUCTION

Dermatophilosis (also known as streptothrichosis, or in sheep as ‘lumpy wool disease’) is an exudative, pustular dermatitis that affects mainly cattle, sheep and horses, but also goats, dogs and cats, many wild mammals, reptiles and, occasionally, humans. Dermatophilosis is caused by the bacterium Dermatophilus congolensis, the type species of the genus Dermatophilus, which is a member of the order Actinomycetales. Dermatophilosis is the commonest skin disease of crocodiles in Australia and has an impact on farming of this species (Buenviaje et al., 1998). It is provoked by Dermatophilus cheloniae, which has also been isolated from chelonids and cobra.

There is considerable variation in the clinical appearance of the disease and in the affected areas of the body. Typically, infection gives rise to the formation of dense scabs on the skin, but in certain areas, such as the
perineum in ruminants and the pastern in horses, moist lesions with thickened, folded skin may occur. In such lesions, relatively thin scabs are found. Where lesions are exposed to prolonged wetting, with or without secondary infection, exudative lesions may be present.

Scabs characteristically comprise alternating layers of parakeratotic keratinocytes invaded with branching bacterial filaments and infiltrates of neutrophils in serous exudate. This gives a palisaded appearance in stained sections. *D. congolensis* filaments remain confined to the epidermis and very rarely infect the dermis.

Extensive acute dermatophilosis cannot be reproduced easily in experimental conditions. *Dermatophilus congolensis* itself is not highly pathogenic, and a combination of factors is necessary for the development of clinical lesions. Malnutrition, intense rainfalls and mechanical traumas have been incriminated as favouring the disease. However, where dermatophilosis has an important economic impact in West and Central Africa as well as on some Caribbean islands, the major risk factor is the infestation by *Amblyomma variegatum* ticks. Severe disease may be promoted by immunomodulatory effects of saliva secreted during tick bite (Ambrose *et al*., 1999), but the fine underlying mechanisms are not understood. Susceptibility to dermatophilosis is also greatly influenced by the genetic background of ruminant breeds, animals from temperate regions and especially dairy cattle being extremely susceptible when introduced in regions at risk.

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**
   
a) **Microscopic observation**

Diagnosis can usually be made by demonstrating the causal organism in scabs from the lesions or in exudate beneath the scabs. The organism has a characteristic microscopic appearance – its septate, branching filaments become longitudinally, as well as transversely, divided to form ribbons of spherical or ovoid cocci, each about 0.5 µm in diameter, in multiple rows. This appearance is diagnostic, provided that cocci are found in transverse rows of four or more, and is readily seen in stained preparations. However, the distinctive formation can be disrupted during the preparation of smears for examination if the material is spread too vigorously over the slide.

Impression smears may be made from the moist, concave undersurfaces of freshly removed scabs. Otherwise, thick smears are best prepared from scabs emulsified in sterile distilled water. Alternatively, scabs can be soaked overnight in sterile water or saline to sufficiently moisten them so that the undersurface of the scab can be used to make effective impression smears by firmly pressing this surface onto a microscope slide. Smears are then air-dried, fixed by heating or immersion in methanol for 5 minutes, and stained. The organism stains well in dilute carbol fuchsin or methylene blue stain, but Gram’s stain or, preferably, a 1 in 10 dilution of Giemsa stain for 30 minutes, gives better differentiation in thick smears, the darkly stained *D. congolensis* contrasting with the paler or pink counterstained background of keratinocytes and neutrophils. Gram staining does not give as good results as Giemsa because it may overstain the background and does not clearly show the characteristic laddering of the coccoid forms.

Wet or secondarily infected scabs often contain few, if any, intact filaments, and the organism may not stain Gram positive. In such material, the cocci cannot be differentiated morphologically from other coccoid bacteria, so that staining by immunofluorescence is required. However, specific antisera for immunofluorescence are not commercially available. Thin, heat-fixed smears are used. In difficult cases and when infection of organs other than the skin is suspected, histopathological examination of biopsy or necropsy material is advisable. Giemsa stain or immunofluorescence is used.

The characteristic appearance of the lesions and of the organism in smears from typical bovine dermatophilosis makes culture unnecessary in most cases. However, in the rare cases in which a Giemsa-stained smear does not give a definitive result, confirmation of the diagnosis may be made by isolating the bacterium. Cultures are made on blood agar and incubated at 37°C. Growth is accelerated under microaerophilic conditions; rough, usually haemolytic, greyish-yellow colonies, about 1 mm in diameter, are seen pitting the medium after 24 hours. Incubation in air produces similar pinpoint colonies at 24 hours that grow to about 1 mm at 48 hours. The rough colonies are formed by the branching filaments, but continued growth in air stimulates the production of the cocci, which are commonly yellow in colour. Colonies take on a smooth, often yellowish, appearance. The cocci are normally vigorously motile when taken from young cultures. The colonies must be differentiated from *Nocardia* spp. and *Streptomyces* spp., neither of which produces filaments that break up into multiple rows of motile cocci.
Chapter 2.4.10. – Dermatophilosis

b) Culture

For isolation, material can be streaked out directly from the moist undersurfaces of freshly removed, uncontaminated scabs or from scab emulsions, but the relatively slow-growing \( D. congolensis \) is readily overgrown by other bacteria. Special isolation techniques are thus required for contaminated specimens. In most specimens, free cocci, whether motile or not, will be present in emulsions of the material. Filtration of the emulsion through a 0.45 µm membrane filter is usually sufficient to reduce or eliminate contaminants and permits isolation from the filtrate, as described above. Alternatively, Haalstra’s method (Haalstra, 1965) may be used. Small pieces of scab are placed in a bijou bottle containing 1 ml of sterile distilled water and allowed to stand at room temperature for 3–4 hours. The open bottle is then placed for 15 minutes in a candle jar. Samples of the surface liquid are removed with a bacteriological loop and cultured. The method depends on the release from the scab of the motile cocci of \( D. congolensis \) and their chemotropic attraction towards the carbon-dioxide-rich atmosphere of the candle jar. A selective medium consisting of 1000 units/ml of polymyxin B in blood agar can also be used, and is effective when the contaminants are sensitive to this antibiotic.

c) Immunological methods

Immunofluorescence staining of smears or tissues is the most reliable and sensitive immunological technique for the identification of \( D. congolensis \) antigens and for the diagnosis of dermatophilosis. Polyclonal antibody obtained from animals inoculated with \( D. congolensis \) can be easily prepared using standard methods, but there is a risk of possible cross-reaction with some strains of \( Nocardia \) spp. Monoclonal antibody to species-specific antigen (How et al., 1988) is preferable. However, monoclonal antibodies have not been widely distributed and validated by interlaboratory tests. Thin, heat-fixed smears of scab emulsions, or impression smears, are stained. Known positive and negative control specimens should always be included.

d) Nucleic acid recognition methods

In absence of extensive genome sequence information, randomly amplified polymorphic DNA methods (RAPD) as well as pulsed-field gel electrophoresis (PFGE) have been used and proved to be useful for the molecular typing of \( D. congolensis \) (Larrasa et al., 2004). An alkaline ceramidase gene was cloned from RAPD fragments, and a polymerase chain reaction (PCR) using primers designed from the nucleotide sequence from this gene gave an amplification product with \( D. congolensis \) DNA. No amplification product was observed with \( M. bovis \), \( C. propinquum \) and \( D. cheloniae \), suggesting a possible use in diagnosis or detection of \( D. congolensis \) (Garcia-Sanchez et al., 2004). Alternatively, 16S rDNA sequence obtained after amplification can be used to confirm the presence of \( D. congolensis \).

2. Serological tests

Clinical diagnosis is best performed using the methods described above rather than serological methods. Antibody can be demonstrated in all but fetal blood in healthy ruminants, but levels are raised following clinical infection. The enzyme-linked immunosorbent assay (ELISA) has proved to be a sensitive and convenient assay technique, and elevation of titres above baseline values can be used in epidemiological studies to identify animals that have had the disease (Martinez et al., 1993). The test being based on a crude antigen, cross-reactivity with other bacteria can occur as in immunofluorescence. At present, the ELISA remains as a research and investigation method. Serology, either using ELISA or older methods such as haemagglutination and counter-immunoelectrophoresis, is not used for routine diagnosis of dermatophilosis where direct detection of the bacterium is easy.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

\( Dermatophilus congolensis \) produces virulence factors such as haemolysin, phospholipases, ceramidases and proteolytic enzymes, which may be used to penetrate the epidermis barrier and interact with the inflammatory response of the host. These virulence factors are considered candidate antigens for vaccines. Research on vaccines for prevention of dermatophilosis has been conducted (How et al., 1990; Sutherland & Robertson, 1988); however, no vaccine is currently available. Research in this domain is hampered by the inability to reproduce the disease experimentally and the poor understanding of skin immunity. Much emphasis has therefore been put on tick control and identification of genetic markers of resistance or susceptibility with promising results in cattle (Maillard et al., 2003).

REFERENCES

Chapter 2.4.10. – Dermatophilosis


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CHAPTER 2.4.11.

ENZOOTIC BOVINE LEUKOSIS

SUMMARY

Description of the disease: Enzootic bovine leukosis (EBL) is a disease of cattle caused by the bovine leukaemia virus (BLV), a member of the family Retroviridae. Cattle may be infected at any age, including the embryonic stage. Most infections are subclinical, but a proportion of cattle (~30%) over 3 years old develop persistent lymphocytosis, and a smaller proportion develop lymphosarcomas (tumours) in various internal organs. Natural infection has also been recorded in water buffaloes and capybaras. Clinical signs, if present, depend on the organs affected. Cattle with lymphosarcomas almost invariably die either suddenly, or weeks or months after the onset of clinical signs.

Identification of the agent: Virus can be detected in the culture supernatant following in-vitro culture of peripheral blood mononuclear cells (PBMC) from infected animals, by BLV antigen detection, by polymerase chain reaction (PCR) or by electron microscopy. Proviral DNA can also be detected in PBMC or tumours of infected animals by PCR.

Serological tests: The antibody detection methods widely used are the agar gel immunodiffusion (AGID) assay using serum and the enzyme-linked immunosorbent assay (ELISA) using serum or milk. These tests have formed the basis for successful eradication policies in many countries. Other tests, such as radio-immunoassay, can also be used. A number of AGID and ELISA kits are commercially available.

Requirements for vaccines: No vaccine against BLV is available.

A. INTRODUCTION

There may be several causes of lymphosarcomas in cattle, but the only definitely known cause is the retrovirus, bovine leukaemia virus (BLV), which causes enzootic bovine leukosis (EBL). The term sporadic bovine leukosis (SBL) is usually reserved for young animals (calves) as well as cutaneous and thymic types of lymphoma, which is defined by the age of the animal affected and the distribution of the tumours. The cause of SBL is not known. There may also be lymphosarcomatous conditions that do not fall into either the SBL or EBL categories, i.e. adult multicentric lymphoma with sporadic occurrence of unknown aetiology. Only lymphomas caused by BLV infection should be termed leukosis or enzootic bovine leukosis (Gillet et al., 2007).

Although animals can become infected with BLV at any age, tumours (lymphosarcomas) are seen typically in animals over 3 years of age. Infections are usually subclinical; only 30–70% of infected cattle develop persistent lymphocytosis, and 0.1–10% of the infected animals develop tumours. Signs will depend on the site of the tumours and may include digestive disturbances, inappetance, weight loss, weakness or general debility and sometimes neurological manifestations. Superficial lymph nodes may be obviously enlarged and may be palpable under the skin and by rectal examination. At necropsy, lymph nodes and a wide range of tissues are found to be infiltrated by neoplastic cells. Organs most frequently involved are the abomasum, right auricle of the heart, spleen, intestine, liver, kidney, omasum, lung, and uterus. The susceptibility of cattle to persistent lymphocytosis, and perhaps also to tumour development, is genetically determined.

There is conflicting evidence of the role of the virus in immunological dysfunctions or in increased culling rates. Two large-scale investigations estimated the mean decline in milk production per cow among test-positive BLV herds compared with test-negative herds as very similar at 2.5% and 2.7%, respectively (Emanuelsson et al., 1992; Ott et al., 2003). In addition, a 7% lower conception rate in BLV test-positive cows compared with test-negative cows has been reported. Increased culling rates and a greater susceptibility to other diseases with infectious aetiology, e.g. mastitis, diarrhoea and pneumonia were also demonstrated among test-positive BLV...
herds (Emanuelsson et al., 1992). Therefore despite no obvious clinical signs during the long subclinical infection period, economic losses caused by persistent BLV infections seem to be relevant.

Virus can be detected by in-vitro cultivation of peripheral blood mononuclear cells (PBMC). The virus is present in PBMC and in tumour cells as provirus integrates into the DNA of infected cells. Virus is also found in the cellular fraction of various body fluids (nasal and bronchial fluids, saliva, milk). Natural transmission depends on the transfer of infected cells, for example during parturition. Artificial transmission occurs, especially by blood-contaminated needles, surgical equipment, gloves used for rectal examinations etc. Lateral transmission in the absence of these contributory factors is usually slow (Monti et al., 2005). In regions where blood-sucking insects occur in large numbers, especially tabanids, these may transmit the virus mechanically. Viral antigens and proviral DNA can be identified in semen, milk and colostrum of infected animals (Dus Santos et al., 2007; Romero et al., 1993). Natural transmission through these secretions, however, has not clearly been demonstrated.

Although several species can be infected by inoculation of the virus, natural infection occurs only in cattle (Bos taurus and Bos indicus), water buffaloes, and capybaras. Sheep are very susceptible to experimental inoculation and develop tumours more often and at a younger age than cattle. A persistent antibody response can also be detected after experimental infection in deer, rabbits, rats, guinea-pigs, cats, dogs, sheep, rhesus monkeys, chimpanzees, antelopes, pigs, goats and buffaloes.

BLV was probably present in Europe during the 19th century, from where it spread to the American continent in the first half of the 20th century. It may then have spread back into Europe and introduced into other countries for the first time by the import of cattle from North America (Johnson & Kaneene, 1992). A number of countries are recognised as officially free from BLV infection.

Several studies have been carried out in an attempt to determine whether BLV causes disease in humans, especially through the consumption of milk from infected cows (Burmeister et al., 2007; Perzova et al., 2000). There is, however, no conclusive evidence of transmission, and it is now generally thought that BLV is not a hazard to humans.

### B. DIAGNOSTIC TECHNIQUES

#### 1. Identification of the agent

BLV is an exogenous retrovirus and belongs to the genus *Deltaretrovirus* within the subfamily *Orthoretrovirinae* and the family *Retroviridae*. It is structurally and functionally related to the primate T-lymphotropic viruses 1, 2 and 3 (STLV-1, -2, -3) and human T-lymphotropic virus(es) 1 and 2 (HTLV-1 and -2). The major target cells of BLV are B lymphocytes (Beyer et al., 2002; Gillet et al., 2007). The virus particle consists of single-stranded RNA, nucleoprotein p12, capsid (core) protein p24, transmembrane glycoprotein gp30, envelope glycoprotein gp51, and several enzymes, including the reverse transcriptase. Proviral DNA, which is generated after reverse transcription of the viral genome, integrates randomly into the DNA of the host cell where it persists without constant production of viral progeny. When infected cells are cultured in-vitro, usually by co-cultivation of PBMC with indicator cells, infectious virus is produced, most readily through stimulation with mitogens.

**a) Virus isolation**

PBMC from 1.5 ml of peripheral blood in ethylene diamine tetra-acetic acid (EDTA) are separated on a ficoll/sodium metrizoate density gradient, cultured with 2×10^6 fetal bovine lung (FBL) cells, and subsequently grown for 3–4 days in 40 ml of minimal essential medium (MEM) containing 20% fetal calf serum. Virus causes syncytia to develop in the cell monolayer of the FBL cells. Short-term cultures can be prepared by culturing PBMC in the absence of FBL cells in 24-well plates for 3 days (Miller et al., 1985). The p24 and gp51 antigens can subsequently be detected in the supernatant of the cultures by radio-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoblot or agar gel immunodiffusion (AGID), and the presence of the BLV particles or BLV-provirus can be demonstrated by electron microscopy and by PCR, respectively.

**b) Nucleic acid detection by PCR (an alternative test for international trade)**

The use of the polymerase chain reaction (PCR) to detect BLV provirus has been described by various workers (Beier et al., 1998; Blankenstein et al., 1998; Belak & Ballagi-Pordany, 1993; Rola & Kuzmak, 2002; Teifke & Vahlenkamp, 2008; Venables et al., 1997). Primers constructed to match the gag, pol and env regions of the genome have all been used with variable success. So far, nested PCR followed by gel electrophoresis is the most rapid and sensitive method. The methods described are based on primer sequences from the env gene, coding for gp51. This gene is highly conserved, and the gene and the antigen are generally present in all infected animals throughout the course of infection. The technique is restricted to
those laboratories that have the facilities for molecular virology, and the usual precautions and control procedures must be in place to ensure validity of the test results (see Chapter 1.1.5 Principles and methods of validation of diagnostic assays for infectious diseases).

PCR is mainly used as an adjunct to serology for confirmatory testing. The detection of BLV infection in individual animals by PCR can be useful in the following circumstances:

- Young calves with colostral antibodies;
- Tumour cases, for differentiation between sporadic and infectious lymphoma;
- Tumour tissue from suspected cases collected at slaughterhouses;
- New infections, before development of antibodies to BLV;
- Cases of weak positive or uncertain results in ELISA;
- The systematic screening of cattle in progeny-testing stations (before introduction into artificial insemination centres);
- Cattle used for production of vaccines, ensuring that they are BLV free.

**Sensitivity and reliability of the method**

i) **Analytical sensitivity:** Although the nested PCR assay has a theoretical sensitivities of one target molecule, in practice the analytical sensitivity is around five to ten target molecules of proviral DNA.

ii) **False-positive results:** The high sensitivity of the nested PCR may cause problems of false-positive results due to contamination between samples (Belak & Ballagi-Pordany, 1993). To minimise this, several special procedures are adopted throughout the protocol, such as the use of laminar air-flow hoods, separate rooms for different steps of the procedure, new gloves or the use of special tube openers for each individual assay and negative controls (e.g. water blanks).

iii) **False-negative results:** It should be noted that only a small proportion of the PBMC can be infected, thus limiting the sensitivity of the assay. The presence of inhibitory substances in some samples may cause false-negative results. To detect this, at least one positive control is used on every test run. In addition, assays should use internal controls (mimics) that are added to each sample. The mimic is a modified target molecule that is amplified with the same primers as the real target, but that generates a PCR product with different size, which can be visualised by agarose gel electrophoresis. The mimic is added at a low concentration that favours the amplification of the real target (Ballagi-Pordany & Belak, 1996). However, it is possible for the mimic to compete with the true target. It may therefore be necessary to analyse each sample with or without the mimic.

**Sample preparation**

PBMC are separated from EDTA blood samples by using the Ficoll-Paque separation method. Alternatively buffy coat may be used, or even whole blood, e.g. where samples have been frozen.

Tumours or other tissues should be homogenised to a 10% suspension.

**DNA extraction**

Purification of total DNA is a prerequisite for achieving optimal sensitivity. Various purification methods are commercially available and suitable for the assay.

Special precautions should be taken during all steps to minimise the risk of contamination (Belak & Ballagi-Pordany, 1993).

**Nested PCR procedure**

Several PCR protocols for the detection of BLV provirus sequences have been published (Beier et al., 1998; Rola & Kuzmak, 2001; Teifke & Vahlenkamp, 2008; Venables et al., 1997). As an example, two PCR assays based on the one developed by Belak & Ballagi-Pordany et al. (1993) and the one developed by Fechner et al., 1996 are described in detail. The BLV region used as target is the gp51 (env) gene. The sequence used for designing the primers is available from GenBank, accession No. K02120.

Method developed by Belak & Ballagi-Pordany (1993)
Chapter 2.4.11. — Enzootic bovine leukosis

i) Primer design and sequences

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence</th>
<th>Position in K02120</th>
</tr>
</thead>
<tbody>
<tr>
<td>OBLV1A</td>
<td>(5’-CTT-TGT-GTG-CCA-AGT-CTC-CCA-GAT-ACA-3’)</td>
<td>5029</td>
</tr>
<tr>
<td>OBLV6A</td>
<td>(5’-CCA-ACA-TAT-AGC-ACA-GTC-TGG-GAA-GGC-3’)</td>
<td>5442</td>
</tr>
<tr>
<td>OBLV3</td>
<td>(5’-CTG-TAA-ATG-GCT-ATC-CTA-AGA-TCT-ACT-GGC-3’)</td>
<td>5065</td>
</tr>
<tr>
<td>OBLV5</td>
<td>(5’-GAC-AGA-GGG-AAC-CCA-GTC-ACT-GTT-CAA-CTG-3’)</td>
<td>5376</td>
</tr>
</tbody>
</table>

PCR\textsuperscript{I}-product size: 440 bp; PCR\textsuperscript{II}-product size: 341 bp; Mimic-product size: 761 bp.

ii) Reaction mixtures

Reaction mixtures are blended (except DNA sample and mimic) before adding to the separate reaction tubes. One negative control (double distilled H\textsubscript{2}O) per five samples, and one positive control should be added. Total volumes of mixtures are calculated by multiplying the indicated volumes by the total number of samples, including controls, plus one. Taq polymerase is used in a premade 1/10 dilution.

DNA samples and mimic\textsuperscript{1} (Ballagi-Pordany & Belak, 1996) should be added in separate rooms in the laboratory: laboratory room 1 for DNA preparations and mimics, and laboratory room 2 for PCR\textsuperscript{II}-products, to minimise contamination.

a) Reagents added in clean laboratory room

This mixture may be prepared in advance and stored at 4°C for up to 1 month.

<table>
<thead>
<tr>
<th>Reagents per reaction (conc.)</th>
<th>PCR\textsuperscript{I}</th>
<th>PCR\textsuperscript{II}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double-distilled H\textsubscript{2}O (standardised)</td>
<td>21 µl</td>
<td>21 µl</td>
</tr>
<tr>
<td>10 × PCR buffer (Perkin Elmer)</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>dNTP (10 mM)</td>
<td>4 × 1 µl</td>
<td>4 × 1 µl</td>
</tr>
<tr>
<td>Bovine serum albumin (1 mg/ml)</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

**Primers (10 pmol/µl):**

- OBLV1A: 1.5 µl –
- OBLV6A: 1.5 µl –
- OBLV3: – 1.5 µl
- OBLV5: – 1.5 µl

In total: 38 µl 38 µl

The following should be added just before starting the PCR

<table>
<thead>
<tr>
<th>Reagents per reaction (conc.)</th>
<th>PCR\textsuperscript{I}</th>
<th>PCR\textsuperscript{II}</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl\textsubscript{2} (25 mM)</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>Taq polymerase (1 unit/reaction)</td>
<td>2 µl</td>
<td>2 µl</td>
</tr>
<tr>
<td>Mineral oil</td>
<td>2 drops</td>
<td>2 drops</td>
</tr>
</tbody>
</table>

In total: 45 µl 45 µl

\textsuperscript{1} Available from Dr S. Belák, Department of Virology, National Veterinary Institute, Box 585, Biomedical Centre, S-751 23, Uppsala, Sweden.
Chapter 2.4.11. – Enzootic bovine leukosis

b) **Reagents added in laboratory room 1 (DNA) or 2 (PCRII)**

<table>
<thead>
<tr>
<th>Reagents per reaction (conc.)</th>
<th>PCR I reaction</th>
<th>PCR II reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA sample* (or water*)</td>
<td>5 µl</td>
<td>–</td>
</tr>
<tr>
<td>PCR I product</td>
<td>–</td>
<td>5 µl</td>
</tr>
<tr>
<td>In total:</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

iii) **PCR thermoprofiles**

**PCR I-thermoprofile**
- 5 × 94°C/45 seconds, 60°C/60 seconds, 72°C/90 seconds
- 30 × 94°C/45 seconds, 55°C/60 seconds, 72°C/90 seconds
- 1 × 72°C/420 seconds ≥20°C

**PCR II-thermoprofile**
- 5 × 94°C/45 seconds, 58°C/60 seconds, 72°C/90 seconds
- 30 × 94°C/45 seconds, 53°C/60 seconds, 72°C/90 seconds
- 1 × 72°C/420 seconds ≥20°C

iii) **Laboratory procedure**

Mix PCR I-reagents as described in step ii use separate gloves or tube openers for each individual tube when adding the DNA samples. Put the samples on ice. Heat the thermoblock to 80°C. Place samples in the thermoblock and start the PCR-programme (step iii).

Mix PCR II-reagents as described in step ii. Use separate gloves or tube openers for each individual tube when adding the PCR I-product. Put the samples on ice. Heat the thermoblock to 80°C. Put samples in the thermoblock and start the PCR II-programme (step iii).

- **Agarose gel electrophoresis**
  
  Take the PCR II-products to the electrophoresis laboratory. Load approximately 10–15 µl of the samples and 23 µl loading buffer on a 2% agarose gel containing 0.01% ethidium bromide (alternative, safer products are available to visualise PCR products, e.g. RedSafe™ or GelRed™). Using 0.5 × Tris/borate/EDTA (TBE) buffer, electrophoresis is performed with 90 mA for 2 hours. To control the size of the amplification products, a 100 bp ladder is recommended. Analysis of PCR products is done by UV illumination.

- **Interpretation of the results**
  
  i) Positive samples should have PCR products of the expected size (341bp), similar to the positive control.
  
  ii) Negative samples should have no PCR products of the expected size (341bp) but mimic product (144 bp) should be present.
  
  iii) Unclear results: the assay must be repeated if the positive controls (mimic or external positive control are negative, or if the negative water controls are positive.

- **Confirmatory testing**
  
  For confirmatory identification, the PCR products can be sequenced, hybridised to a probe, or analysed by restriction fragment length polymorphism (RFLP) analysis (Fechner et al., 1997).
Method developed by Fechner et al. (1996)

i) **Primer design and sequences**

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Env-Sequence (5’–3’)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLV-env-1</td>
<td>TCT-GTG-CCA-AGT-CTC-CCA-GAT-A</td>
<td>5032–5053</td>
</tr>
<tr>
<td>BLV-env-2</td>
<td>AAC-AAC-AAC-CTC-TGG-GAA-GGG</td>
<td>5629–5608</td>
</tr>
<tr>
<td>BLV-env-3</td>
<td>CCC-ACA-AGG-GCG-GCG-CCG-GTT-T</td>
<td>5099–5121</td>
</tr>
<tr>
<td>BLV-env-4</td>
<td>GCG-AGG-CCG-GGT-CCA-GAG-CTG-G</td>
<td>5542–5552</td>
</tr>
</tbody>
</table>


ii) **Reaction mixtures**

Reaction solutions are mixed (except DNA sample) before adding to the separate reaction tubes. One negative control (double distilled H₂O) per five samples, and one positive control should be added. Total volumes of mixtures are calculated by multiplying the indicated volumes by the total number of samples, including controls, plus one.

The first PCR can be performed using a 50 µl reaction volume. For one reaction, the assay is optimised to 5 µl (10×) PCR buffer, 20 µl DNA (~1 µg of DNA), 1.25 µl each of the env-specific primers BLV-env-1 and BLV-env-2 (20 pmol/µl), 0.15 dNTP (each 25 mM), 3 µl MgCl₂ (25 mM), 0.25 µl Taq polymerase (1.25U), and 19.1 µl of distilled H₂O. The reaction follows the temperature profile: 2 minutes denaturation at 94°C; 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C and 60 seconds at 72°C; followed by 4 minutes at 72°C.

The nested PCR can be performed using a 50 µl reaction volume. For one reaction, the assay is optimised to 3 µl PCR product of the first PCR, 5 µl (10×) PCR buffer, 1.25 µl each of the env-specific primers BLV-env-3 and BLV-env-4 (20 pmol/µl), 0.15 dNTP (each 25 mM), 0.25 µl Taq polymerase (1.25U), and 36.1 µl of distilled H₂O. The reaction follows the temperature profile: 2 minutes denaturation at 94°C; 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C and 60 seconds at 72°C; followed by 4 minutes at 72°C.

iii) **Laboratory procedure**

Mix PCR-reagents for the first or nested PCR and use separate gloves or tube openers for each individual tube when adding the DNA samples. Put the samples on ice. Heat the thermoblock to 94°C. Place samples in the thermoblock and start the PCR-programmes accordingly.

- **Agarose gel electrophoresis**

Load approximately 10 µl of the nested PCR products with 20 µl loading buffer on a 2% agarose gel containing 0.01% ethidium bromide. Using 0.5 × Tris/borate/EDTA (TBE) buffer, electrophoresis is performed with 90 mA for 2 hours. To control the size of the amplification products, a 100 bp ladder is recommended. Analysis of PCR products is done by UV illumination.

- **Interpretation of the results**

i) **results**: Positive samples should have PCR products of the expected size (444 bp), similar to the positive control.

ii) **results**: Negative samples should have no PCR products of the expected size (444 bp).

iii) **results**: The assay must be repeated if the positive control remained negative, or if the negative water controls are positive.

- **Confirmatory testing**

For confirmatory identification, the PCR products can be sequenced, hybridised to a probe, or analysed by restriction fragment length polymorphism (RFLP) analysis (Fechner et al., 1997).

### 2. Serological tests

Infection with the virus in cattle is lifelong and gives rise to a persistent antibody response. Antibodies can first be detected 3–16 weeks after infection. Maternally derived antibodies may take up to 6 or 7 months to disappear.
There is no way of distinguishing passively transferred antibodies from those resulting from active infection. Active infection, however, can be confirmed by the detection of BLV provirus by the PCR. Passive antibody tends to protect calves against infection. During the periparturient period, cows may have serum antibody that is undetectable by AGID because of an antibody shift from the dam’s circulation to her colostrum. Therefore, when using the AGID test, a negative test result on serum taken at this time (2–6 weeks pre- and 1–2 weeks post-partum) is not conclusive and the test should be repeated. However, the AGID can be performed at this stage with first-phase colostrum.

The antibodies most readily detected are those directed towards the gp51 and p24 of the virus. Most AGID tests and ELISAs in routine use detect antibodies to the glycoprotein gp51, as these appear earlier. Methods of performing these tests have been published (Dimmock et al., 1987; European Commission, 2009). ELISAs are usually more sensitive than the AGID tests.

Weak positive and negative OIE reference sera for use in ELISA are available in freeze-dried, irradiated form from the OIE Reference Laboratory in Germany (see Table given in Part 4 of this Terrestrial Manual). The calibration of these sera is based on the accredited OIE reference serum, named E05, which has been validated against the former reference serum E4 by different AGID and ELISAs.

a) Enzyme-linked immunosorbent assay (a prescribed test for international trade)

Either an indirect or blocking ELISA may be used. Assays based on both of these are available commercially; different kits may be required for serum or milk samples. Some ELISAs are sufficiently sensitive to be used with pooled samples. ELISAs are carried out in solid-phase microplates. BLV antigen is used to coat the plates either directly or by the use of a capture polyclonal or monoclonal antibody (MAB). The antigen is prepared from the cell culture supernatant of persistently BLV-infected cell lines. Fetal lamb kidney (FLK) cells are most commonly used for commercial tests (Van der Maaten & Miller, 1976). Since 2004, a new BLV-producing cell line, PO714, which is free from other viral infections and contains a provirus of the Belgian subgroup, has been made available (Beier et al., 2004). The antigen is used at a predetermined dilution (e.g. 1/10) in phosphate buffered saline (PBS). In kit form, the plates are sometimes purchased precoated. Some preservatives may be added to milk samples to prevent souring. Preserved samples will not usually deteriorate significantly if stored for up to 6 weeks at 4°C.

• Blocking enzyme-linked immunosorbent assay – serum ELISA

The following method is suitable for antibody detection in single or pooled serum samples.

  • Test procedure

i) Coating the plate

All wells are coated with BLV antibody, prediluted in coating buffer (100 µl/well), the plate is sealed and incubated for 18 hours at 4°C. A wash cycle (standard wash) is performed, which is three washes filling wells to the top, with a 3-minute soak in between each wash, and then the plate is blotted. BLV antigen is added, prediluted in wash buffer (100 µl/well), the plate is sealed and incubated for 2 hours at 37°C. A standard wash cycle is performed.

ii) Preparation and addition of samples and controls

The positive and negative control sera are prediluted (1/2) in wash buffer and the solution is added to four wells per control (100 µl/well). For testing pooled samples, 80 sera may be bulked then diluted (1/2) using wash buffer and the solution is added to two wells (100 µl/well) per sample. Single samples should be diluted 1/100 using wash buffer and the solution added to two wells (100 µl/well) per sample. After plating out the samples, the plate is sealed and incubated for 18 hours at 4°C. A brief wash is performed by filling the wells and immediately emptying them.

iii) Preparation and addition of conjugates and substrate

Prediluted biotinylated antibody is added (100 µl/well) to all wells – predilute using wash buffer + 10% fetal calf serum – the plate is sealed and incubated on a rocking table for 1 hour at 37°C. A standard wash is performed as described earlier. The peroxidase-conjugated avidin is prediluted in wash buffer and the solution is added to all wells (100 µl/well). The plate is sealed and incubated on a rocking table for 30 minutes at 37°C. A standard wash is performed. 100 µl orthophenylene diamine substrate is added to all wells, the plate is covered and left in the dark for 9 minutes. The reaction is stopped with 100 µl of 0.5 M sulphuric acid per well.
• Reading and interpretation of results

The plate reader is blanked on air and the absorbance is read at 490 nm. For dual wave-length readers a reference filter between 620 nm and 650 nm is used. Results are read within 60 minutes after the addition of stop solution.

The absorbance of the negative control should be about 1.1 ± 0.4; if the absorbance is below 0.7, the colour development time in step iii above (preparation and addition of conjugates and substrate) should be increased. Conversely, the time should be shortened if the absorbance is above 1.5. The absorbance of the positive control should be less than the absorbance of the negative control × 0.25.

A sample is positive when the absorbance of each of the two test wells is identical with or less than the mean absorbance of the four negative wells × 0.5.

A sample is negative when the absorbance of each of the two test wells is identical with or higher than the mean absorbance of the four negative control wells × 0.65.

For samples giving values between the absorbance of the negative control × 0.5 and × 0.65 it is recommended to retest the animal, using a sample taken 1 month later.

• Sensitivity of the enzyme-linked immunosorbent assay

The sensitivity of pooled milk ELISAs can be evaluated using the OIE weak positive and reference sera. Assays should give a positive result on OIE reference sera E05 diluted in negative milk 250 times more than the number of individual milks in the pool (EU Directive 88/406). For example, for pools of 60 milks, E05 should be diluted 1/250 × 60 = 1/15000. For individual milk samples the positive OIE reference sera E05 diluted 1/250 in negative milk must be positive.

Where pooled serum samples are tested, the OIE reference serum E05 must test positive at a dilution 10 times higher than the number of individual animals in the pool. For example, for a pool of 50 individual samples, the OIE reference serum diluted 1/500 in negative serum should give a positive result. In assays where serum samples are tested individually, OIE reference serum E05 diluted 1/10 must be positive.

• Indirect enzyme-linked immunosorbent assay — Milk ELISA

The following method is suitable for antibody detection in pooled milk samples.

• Controls

Strong positive, weak positive, negative milk and diluent controls should be included in each assay. A strong positive control should be prepared by diluting the OIE reference serum E05 1/25 in negative milk. A weak positive control should be prepared by diluting, in negative milk, the OIE reference serum E05 25 times the number of individual milk samples in the pool under test. The milk used for diluting the OIE reference serum controls should be unpasteurised, cream free and preserved.

• Example test procedure

i) Milk samples must be stored, undisturbed in a refrigerator until a definite cream layer has formed (24–48 hours), or alternatively, centrifuged at 2000 rpm for 10 minutes, the cream layer should be removed prior to testing.

ii) A BLV antigen and a control negative antigen are precoated in alternate columns in the plate. 100 µl of test sample is added to 100 µl wash buffer in the plate to make a 1/2 dilution, adding to two control antigen wells and two BLV antigen wells.

iii) The plate is sealed and mixed on a shaker.

iv) The plate is incubated between 14 and 18 hours at 2–8°C.

v) 300 µl per well of wash diluent is added and discarded, and then 200 µl per well wash diluent is added, shaken for 10 seconds and discarded. Finally, 300 µl of wash diluent is added and soaked for 3 minutes and discarded.

vi) 200 µl per well of anti-bovine IgG-horseradish peroxidase affinity-purified conjugate diluted in wash diluent is added and the plate is incubated for 90 minutes at room temperature.

vii) The plate is washed by adding 300 µl of wash diluent per well; this is then discarded and a further 300 µl of wash diluent is added. This is left to soak for 3 minutes and discarded. Steps vi and vii are repeated.
viii) 200 µl of ABTS (2,2’-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) substrate (prewarmed to 25°C) is added and the plate is incubated for 20 minutes at room temperature in the dark. The reaction may be stopped by adding 50 µl of stopping solution.

- Reading and interpreting the results

The plate reader is blanked on air and the absorbance is read at 405 nm. All microplate wells must be read within 2 hours of addition of stopper. The absorbance readings of the wells containing negative antigen are subtracted from the readings of wells containing the positive antigen. The two net absorbance values for each test sample should be averaged. The same applies for the replicate weak positive controls. Replicates should be within 0.1 absorbance units of each other.

For the test to be considered valid, the averaged net absorbance of the weak positive (WP) controls should be 0.2–0.6 absorbance units. The net absorbance of the strong positive control should be >1.0 absorbance units. The net absorbance of the negative and diluent controls should be less than the lower limit of the inconclusive range.

Assuming that the above criteria are met:

i) Test samples are positive if their net absorbance value is greater than or equal to that of the WP control.

ii) Test samples are inconclusive if their net absorbance value is 75% or less of the net absorbance value of the WP control.

i.e. if the WP control net absorbance = 0.40
then the lower limit of the inconclusive range = 0.40 × 0.750 = 0.30
and samples of ≥0.40 are considered positive.

iii) Test samples are negative if their net absorbance value is less than the lower limit of the ‘inconclusive’ range (<0.30 in the example).

b) Agar gel immunodiffusion (a prescribed test for international trade)

The AGID test is a specific, but not very sensitive, test for detecting antibody in serum samples from individual animals. It is, however, unsuitable for milk samples (except first colostrums) because of lack of specificity and sensitivity. The AGID is simple and easy to perform and has proven to be highly useful and efficient as a basis for eradication schemes. Reference sera are included with commercial AGID test kits.

i) **Agar gel:** A 0.8–1.2% solution of agar or agarose is prepared in 0.2 M Tris buffer, pH 7.2, with 8.5% NaCl. One method of preparing the agar is to dissolve 24.23 g of Tris methylamine in 1 litre of distilled water and adjust to pH 7.2 with 2.5 M HCl. Sodium chloride (85 g) is dissolved in 250 ml Tris/HCl and made up to 1 litre. Agarose (8 g) is added and the mixture is heated in a pressure cooker or autoclave at 4.55 kg/sq. cm for 10 minutes. The mixture is dispensed in 15 ml aliquots, which can be stored at 4°C for up to approximately 6 weeks.

ii) **Antigen:** The antigen must contain specific glycoprotein gp51 of BLV. Antigen is prepared in a suitable cell culture system, such as permanently infected FLK cell monolayers. The cells used to produce the BLV antigen should be free from noncytopathic bovine viral diarrhoea virus and of bovine retroviruses, bovine immunodeficiency-like virus (lentivirus), and bovine syncytial virus (spumavirus). After 3–4 days' culture at 37°C, the growth medium is replaced with maintenance medium. The cells are harvested after 7 days using standard trypsin/versene solution. The cell suspension is centrifuged at 500 g for 10 minutes. Cells are resuspended in growth medium; 30% of the cells are returned to the culture vessel and the remainder is discarded. All culture supernatants are collected. The supernatants are concentrated 50–100-fold by available methods. This can be done by concentration in Visking tubing immersed in polyethylene glycol, or by precipitation with ammonium sulphate followed by ultrafiltration, or by precipitation in polyethylene glycol followed by desalting and size separation on a polyacrylamide bead column. The antigen contains gp51 predominantly, but may also contain p24.

The antigen may be standardised for glycoprotein gp51 by titration against the OIE reference serum E05 as follows: a twofold dilution of the antigen preparation is made. The highest dilution that, when tested against undiluted OIE reference serum E05, gives a precipitation line equidistant between the antigen and the serum will contain one unit. Two units of antigen are used in the test.

iii) **Known positive control serum:** The positive control serum comes from a naturally or experimentally infected animal (cattle or sheep). The precipitation line formed should be a sharp distinct line midway between the antigen and the control serum wells. A dilution of the control positive serum that gives a weak positive result should be included in the test as an indicator of the test’s sensitivity.
iv) *Known negative control serum:* Serum from uninfected animals (cattle, sheep) is used.

v) *Test sera:* Sera from any species of animal are suitable.

### Test procedure

i) The agar is melted by heating in a water bath and poured into Petri dishes (15 ml per Petri dish of diameter 8.5 cm). The poured plates are allowed to cool at 4°C for about 1 hour before holes are cut in the agar. A punch is used that cuts a hexagonal arrangement of six wells round a central well. Various dimensions of wells can be used; one satisfactory pattern has been produced using wells of 6.5 mm in diameter with 3 mm between wells. For best results, agar plates are used the same day that they are poured and cut.

ii) Antigen is placed in the central wells of the hexagonally arranged patterns. Test sera are placed alternately with positive control serum in the outer wells. There should be one control pattern per plate with positive control serum, weak positive control serum and negative control serum in the place of test sera.

iii) The test plates are kept at room temperature (20–27°C) in a closed humid chamber, and read at 24, 48 and 72 hours.

iv) *Interpretation of the results:* A test serum is positive if it forms a specific precipitation line with the antigen and forms a line of identity with the control serum. A test serum is negative if it does not form a specific line with the antigen and if it does not bend the line of the control serum. Nonspecific lines may occur; these do not merge with or deflect the lines formed by the positive control. A test serum is a weak positive if it bends the line of the control serum towards the antigen well without forming a visible precipitation line with the antigen; the reaction is inconclusive if it cannot be read either as negative or positive. A test is invalid if the controls do not give the expected results. Sera giving inconclusive or weak positive results can be concentrated and retested.

### C. REQUIREMENTS FOR VACCINES

Despite advances in research on experimental vaccines, there is as yet no vaccine available commercially for the control of EBL.

### REFERENCES


Chapter 2.4.11. – Enzootic bovine leukosis


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NB: There are OIE Reference Laboratories for Enzootic bovine leukosis (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the OIE Reference Laboratory for any further information on diagnostic tests and reagents for enzootic bovine leukosis.
CHAPTER 2.4.12.

HAEMORRHAGIC SEPTICAEMIA

SUMMARY

Haemorrhagic septicaemia (HS) is a major disease of cattle and buffaloes characterised by an acute, highly fatal septicaemia with high morbidity and mortality. It is caused by certain serotypes of Pasteurella multocida that are geographically restricted to some areas of Asia, Africa, the Middle East and southern Europe.

The diagnosis of HS depends on the isolation of the causative organism, P. multocida, generally from the blood or bone marrow of a dead animal, by cultural and biological methods, and the identification of the organism by biochemical, serological and molecular methods.

Isolation and identification of the agent: Pure cultures of P. multocida can be obtained by streaking materials on to artificial media and the subsequent identification on the basis of the morphological, cultural, and biochemical characteristics of P. multocida.

Conventionally, the identification of the specific serotype is carried out using one or more serological methods. These include rapid slide agglutination, indirect haemagglutination for ‘capsular’ typing using sheep red blood cells coated with bacterial extracts, ‘somatic’ typing by agar gel immunodiffusion tests using heat-treated cell extracts, or agglutination using acid-treated cells. Confirmation of the isolates can be made using molecular techniques.

Serology: Serological tests for detecting specific antibodies are not normally used for diagnostic purposes.

Requirements for vaccines and diagnostic biologicals: Effective vaccines against haemorrhagic septicaemia are formalin-killed bacterins, or dense bacterins with adjuvants. The latter enhance the level and prolong the duration of immunity.

Seed cultures for the production of vaccines should contain capsulated organisms. Vaccines are standardised as to their bacterial density on the basis of turbidity tests and dry bacterial weight. Potency tests are carried out in mice and/or rabbits.

A. INTRODUCTION

Haemorrhagic septicaemia (HS) is a major disease of cattle and buffaloes occurring as catastrophic epizootics in many Asian and African countries, resulting in high mortality and morbidity (Bain et al., 1982; Carter & De Alwis, 1989; De Alwis, 1992; Mustafa et al., 1978; Singh et al., 1996). The disease has been recorded in wild mammals in several Asian and European countries (Carigan et al., 1991; Rosen, 1981). In many Asian countries disease outbreaks mostly occur during the climatic conditions typical of monsoon (high humidity and high temperatures). The disease is caused by Pasteurella multocida, a Gram-negative coccobacillus residing mostly as a commensal in the nasopharynx of animals. The Asian serotype B:2 and the African serotype E:2 (Carter and Heddleston system), corresponding to 6:B and 6:E (Namioka-carter system), are mainly responsible for the disease. In wild ruminants, serotype B:2,5 is predominantly present while serotype B:3,4 also has been reported from fallow deer (Aalbæk et al., 2009). The association of other serotypes, namely A:1, A:3 with a HS-like condition in cattle and buffaloes in India has been recorded (Kumar et al., 1996). The geographical distribution of HS includes some areas of Asia, Africa, the Middle East and southern Europe. It has never been confirmed in Mexico, Central or South America.

Clinically, HS caused by B:2 or E:2 strains is typified by fever, respiratory distress with nasal discharge, and frothing from the mouth, leading eventually to recumbency and death. Infection with serotypes A:1 and A:3 predominantly involves pneumonia and death. Septicaemia is the main characteristic feature in all forms of the disease. The incubation period varies from 3 to 5 days. In peracute cases, sudden death without clinical signs
may be observed (Carter & De Alwis, 1989; De Alwis, 1992). Water buffaloes are generally more susceptible to HS than cattle and show more severe forms of disease with profound clinical signs. Severe subcutaneous oedema of the mandible, neck and brisket is a distinctive feature of the disease. In endemic areas mortality is largely confined to older calves and young adults.

At post mortem most animals succumbing to HS typically show marked swelling of the neck caused by severe blood-tinged oedema. There are also abundant petechial haemorrhages in many tissues and organs, particularly in serosal membranes. The thoracic, pericardial and abdominal cavities may contain serosanguinolent fluid. The lungs are notably congested and oedematous, and foam is generally present in the nasal cavity, trachea and bronchi. Microscopically, there is interstitial pneumonia and pulmonary oedema as well as focal infiltrates of neutrophils and macrophages in many tissues. All these lesions are similar to those observed in severe sepsis and septic shock.

Massive epizootics may occur in endemic as well as non-endemic areas (Carter & De Alwis, 1989; De Alwis, 1992). HS has been identified as a secondary complication in cattle and water buffaloes following outbreaks of foot and mouth disease (FMD). Case fatality approaches 100% if treatment is not followed at the initial stage of infection (Carter & De Alwis, 1989; De Alwis, 1992).

The diagnosis of the disease is based on the clinical signs, gross lesions, morbidity and mortality patterns. Confirmation requires the isolation and characterisation of the pathogen using conventional and molecular techniques. There are no confirmed reports of human infections with *P. multocida* B2 and E2; however, other serotypes do cause human infections and precautions should be taken to avoid exposure. The organism should be handled in biosafety level 2 laboratories.

**B. DIAGNOSTIC TECHNIQUES**

1. Isolation and identification of the agent

   **• Cultural and biochemical methods**

   True septicaemia in HS occurs at the terminal stage of the disease, therefore blood samples should be taken from sick animals immediately before death. Animals in the early stages of the disease may not contain *P. multocida* in blood. The bacteria are also not consistently present in the nasal secretions or body fluids of sick animals.

   A blood sample or swab collected from the heart is satisfactory only if taken immediately after death. If the animal has been dead for a long time, bone marrow from a long bone can be used for bacterial isolation. If there is no facility for post-mortem examination, blood can be collected from the jugular vein by incision or aspiration. Blood samples in any standard transport medium should be dispatched on ice and well packed to avoid any leakage. If the blood samples are not transported to the laboratory within a few hours, they can be frozen. However repeated freezing and thawing may destroy the organism and is not advisable.

   Blood smears from affected animals are stained with Gram, Leishman’s or methylene blue stains. The organisms appear as Gram-negative, bipolar-staining short bacilli. No conclusive diagnosis can be made on the basis of direct microscopic examinations alone.

   Blood samples, or swabs eluted into 2–3 ml sterile physiological saline, are cultured. Alternatively, the surface of a long bone is disinfected with alcohol and split open. The marrow is extracted aseptically and cultured. Direct culture is usually satisfactory only if the material is fresh and free from contaminants or post-mortem invaders that would otherwise overgrow any *Pasteurella* present.

   For biological examinations, a small volume (0.2 ml) of eluted blood swabs or a portion of bone marrow in saline is inoculated subcutaneously or intramuscularly into mice. The mouse usually serves as a biological ‘screen’ for extraneous organisms. If viable *P. multocida* is present, the mice die 24–36 hours following inoculation, and a pure growth of *P. multocida* can be seen in blood smears. Pure cultures of *P. multocida* can usually be grown from blood of the mice, even when the original samples come from relatively old carcasses. The organism can be identified by its morphological and cultural characteristics, biochemical reactions and serological tests.

   A suitable bacteriological medium for *Pasteurella* is casein/sucrose/yeast (CSY) agar containing 5% blood. The composition of this medium is casein hydrolysate (3 g), sucrose (3 g), yeast extract (5 g), sodium chloride (5 g), anhydrous dipotassium hydrogen orthophosphate (3 g), and distilled water to 1 litre. The pH is adjusted to 7.3–7.4, after which 1.5% agar is added. The medium is autoclaved at 1 bar for 15 minutes. After cooling to 45–50°C, 5% calf blood (antibody-free *P. multocida*) is added (Wijewardana *et al.*, 1986). Conventional blood agar may also be used.
Freshly isolated *P. multocida* forms smooth, greyish glistening translucent colonies, approximately 1 mm in diameter, on blood agar after 24 hours’ incubation at 37°C. Colonies grown on CSY agar are larger. Old cultures, particularly those grown on media devoid of blood, may produce smaller colonies. *Pasteurella multocida* does not grow on MacConkey agar. Gram-stained blood or tissue smears show Gram-negative, short, ovoid, bipolar-staining coccobacilli. A degree of pleomorphism will be noted, particularly in old cultures, with longer rods of varying length. The bipolar staining will be more evident with methylene blue or Leishman’s stain.

HS organisms produce oxidase, catalase and indole, and will reduce nitrates. They do not produce hydrogen sulphide or urease, and fail to use citrate or liquefy gelatin. Glucose and sucrose are always fermented with the production of acid only. Most strains also ferment sorbitol. Some strains ferment arabinose, xylose and maltose, whereas salicin and lactose are almost invariably not fermented.

One property of HS-causing strains of *P. multocida* is the ability to produce the enzyme hyaluronidase (Carter & Chengappa, 1980). Having identified the genus and species by cultural characteristics and biochemical tests, hyaluronidase production may then be used as a specific test for HS-causing pasteurellae. It should be noted that B serotypes other than B:2 (or 6:B), and type E, are hyaluronidase negative.

A hyaluronic-acid-producing culture is streaked across the centre of a dextrose starch agar plate. The **Pasteurella** culture to be tested for hyaluronidase production is streaked at right angles. The plates are incubated at 37°C for 18 hours. Originally, hyaluronic-acid-producing *Streptococcus equi* was used, but a convenient culture for this purpose is a capsulated mucoid *P. multocida* type A culture. At the point of intersection, the mucoid growth of the hyaluronic acid producer will diminish into a thin line of growth, indicating the production of hyaluronidase by the test culture. Use of freshly prepared plates and a humidified incubator will facilitate hyaluronic acid production and, thereby, the interpretation of the test.

**Serotyping methods**

Several serotyping tests are used for the identification of the HS-causing serotypes of *P. multocida*. These consist of a rapid slide agglutination test (Namioka & Murata, 1961a), an indirect haemaggulitination (IHA) test for capsular typing (Carter, 1955), an agglutination test using hydrochloric-acid-treated cells for somatic typing (Namioka & Murata, 1961b), the agar gel immunodiffusion (AGID) test (Anon, 1981; Heddeston et al., 1972; Wijewardena et al., 1982), and the counter immuno-electrophioreis test (CIEP) (Carter & Chengappa, 1981).

Hyperimmune antisera for most of these tests are prepared against specific reference strains in rabbits. Cultures in CSY broth (6–8-hours old) are seeded on to CSY blood agar medium. After overnight incubation (18–20 hours) the growth is washed into physiological saline containing 0.3% formalin. The turbidity of the cell suspension is adjusted to that of MacFarland’s tube No. 4. Rabbits are inoculated intravenously at 3–4-day intervals with 0.2, 0.5, 1.0, 1.5 and finally, 2.0 ml of this suspension. The rabbits are inoculated subcutaneously or intramuscularly 1 week after the last injection with 0.5 ml of a similar, but live, suspension. The animals are bled 10 days later. The serum is stored at –20°C, but small quantities for regular use are stored at 4°C with the addition of 1/10,000 merthiolate.

**a) Rapid slide agglutination test (capsular typing)**

A single colony is mixed with a drop of saline on a slide, a drop of antiserum is added, and the slide is warmed gently. A coarse, floccular agglutination appears within 30 seconds. Old cultures may give a fine, granular agglutination that takes longer to appear.

**b) Indirect haemagglutination test (capsular typing)**

This was originally performed using antigen-sensitised human type ‘O’ red blood cells (RBCs) (Carter, 1955), but more recently sheep RBCs have been used (Sawada et al., 1985; Wijewardana et al., 1986). The antigen is prepared as follows:

A 6–8-hour broth culture of a reference strain is seeded on to CSY blood agar plates and incubated overnight at 37°C. The growth is harvested in 3 ml physiological saline containing 0.3% formalin. This suspension is then heated at 56°C for 30 minutes, centrifuged at 3000 g for 15 minutes at 4°C, and the clear supernatant fluid is stored at –20°C. If a refrigerated centrifuge is not available, centrifugation at 1500 g for 30 minutes gives a supernatant fluid. This is used as the antigen extract. A similar procedure is followed for preparing an antigen extract from an unknown strain that is to be typed.

Sheep blood is collected aseptically into an anticoagulant and centrifuged at 500 g for 10 minutes. The packed RBCs are washed three times in sterile physiological saline. The antigen extract from an unknown strain prepared by the method described above is used to sensitise the RBCs or absorbed on to the RBCs. This is done by adding 15 volumes of the antigen extract to the RBCs and incubating the mixture for 1 hour at 37°C with frequent shaking. The sensitised RBCs are recovered by centrifugation, washed three times in
sterile physiological saline, and made up to a final 1% suspension in physiological saline. The type-specific hyperimmune antiserum (three volumes) is absorbed by the addition of packed RBCs (one volume) for 30 minutes at room temperature, and then centrifuged at 500 g for 10 minutes to pellet the RBCs. The absorbed antiserum is then inactivated by heating at 56°C for 30 minutes.

The test itself can be carried out in tubes or plates, and is performed in two rows. The test described below is for standard microtitre plates.

i) The capsular extract of the unknown strain is prepared as described above and used to sensitishe the sheep RBCs. The known type-specific hyperimmune sera raised in rabbits against types A, B, D and E are diluted as follows:

ii) Using four separate rows of wells, the first wells are filled with 0.72 ml saline followed by 0.4 ml in the next six wells or more.

iii) The type-specific hyperimmune sera are each separately diluted in each row by adding 0.08 ml of the serum to the first well and mixing with a pipette. From this well 0.4 ml is transferred to the next well, mixed, and the process carried on until well seven. This constitutes 1/10 dilution in the first well and a doubling dilution thereafter.

iv) All the wells are each filled with 0.4 ml of antigen-adsorbed/sensitised RBCs, shaken slightly and left at room temperature. By the addition of the sensitised blood, the serum dilutions in the wells are doubled, i.e. 1/20 in well one, 1/40 in the second, and so on. A positive, negative and saline control are included in each test run.

v) The first reading is taken after 2 hours and a final reading after 18 hours. A course agglutination of the RBCs along the sides of the concave wells is taken as a positive reading, and the formation of a button at the centre of the wells as negative. An arbitrary score of 1–4 is given depending on the size of the agglutination. An unknown strain is identified with the hyperimmune serum that has agglutination. In the absence of agglutination with all sera, the strain is considered to be untypeable.

While IHA can be used for typing unknown strains, the test itself is more efficient when dealing with serotypes B and E and is more reliable as a quantitative test against these strains.

c) Agar gel immunodiffusion tests

AGID tests are used for what is described as ‘capsular’ as well as ‘somatic’ typing, depending on the antigens and antisera used. The double-diffusion technique is employed. Wells are punched in the solid agar in a circular pattern with one centre well surrounded by six peripheral wells.

i) Capsular typing: The gel medium is 1.0% Noble agar, or equivalent product, in 0.2 M phosphate buffer containing merthiolate at a final concentration of 1/10,000 (Anon, 1981; Wijewardena et al., 1982). Antigens and antisera are the same as for capsular typing by the IHA method (Carter, 1955). The standard antiserum is placed in the centre well, and the test antigens are placed in the peripheral wells alternately with standard homologous antigen.

ii) Somatic typing: The gel medium consists of special Noble agar, or equivalent product, at a concentration of 0.9% in 0.85% sodium chloride solution.

iii) For antigen preparation, the growth from each plate is harvested in 1 ml of 8.5% sodium chloride containing 0.3% formalin. The suspension is heated at 100°C for 1 hour, the cells are sedimented by centrifugation, and the supernatant fluid is used as antigen.

iv) Antisera against 16 somatic types (Heddleston et al., 1972) are prepared in chickens. Oil-emulsified bacterin1 (1 ml) is injected subcutaneously into the mid-portion of the neck of 12–16-week-old male birds. A further injection is made 3 weeks later of 1 ml intramuscularly into the breast, 0.5 ml on each side of the sternum. The birds are bled 1 week later, and the serum is separated and preserved with 0.01% thiomersal and 0.06% phenol. Sera are tested against all somatic types and sera that cross-react are discarded. Some antisera preparations against B:2 may cross react with the somatic type 5.

v) The test antigen is placed in the centre well and antisera against the different serotypes are placed in the peripheral wells. All haemorrhagic septicaemia serotypes (Asian and African) will react with type 2 antiserum. Cross-reactions may occur with type 5.

1 The bacterial antigens in broth are covered by a light mineral oil (adjuvant) and then emulsified (stabilised) with an emulsifying agent, in this case lanolin or lanoline (wool fat). This has to be done as the watery phase with the bacteria (broth) will not mix with the oily phase (adjuvant). The proportion of oil to emulsifying agent will vary with different batches of lanolin and will have to be adjusted accordingly. The higher the percentage of lanoline, the higher the stability of the emulsion.
d) **Counter immunoelectrophoresis**

CIEP offers a rapid method for the identification of capsular types B and E cultures.

i) *Preparation of capsular substance:* Capsular substance is prepared in the same manner as described for the IHA test.

ii) *Preparation of hyperimmune antiserum:* Antisera are prepared in rabbits as for the IHA test.

iii) *Medium for CIEP:* The medium for the CIEP consists of agarose (2.0 g), barbitone sodium (2.06 g), diethyl barbituric acid (0.37 g), distilled water (180 ml), and 1/1000 merthiolate (20 ml).

iv) *Veronal acetate buffer (barbitone buffer):* The barbitone buffer consists of barbitone sodium (29.24 g), anhydrous sodium acetate (11.70 g), 0.1 N hydrochloric acid (180 ml), and distilled water to 3 litres. The pH should be 8.8.

v) *Preparation of slides:* The electrophoresis plates are prepared by precoating glass slides (57 mm × 70 mm) with 12 ml volumes of the medium. Seven wells, 4 mm in diameter and 7 mm apart, are cut in a row. A parallel set of wells is cut 6 mm (centre to centre) away from the other set of wells.

vi) *Test procedure:* The well on the side of the cathode is loaded with a 20 µl volume of capsular antigen, while an equal volume of type-specific antiserum is loaded on to the well on the side of the anode. Controls included in the test are 0.85% sodium chloride solution against positive antiserum, and capsular extract against negative rabbit serum as well as positive and negative control samples. The electrophoresis tank is filled with barbitone buffer, pH 8.8. The antigen and antiserum are electrophoresed for 30 minutes at 150 V (25 V/cm). The plates are then examined for precipitation lines.

vii) *Interpretation of the results:* The presence of a distinct line between the antigen and antiserum wells is considered to be a positive result.

e) **Agglutination tests (somatic antigen)**

The somatic ‘O’ antigen is prepared by a method similar to that described previously for the IHA test (Namioka, 1978; Namioka & Murata, 1961b). A 6–8-hour test culture is seeded on to CSY blood agar and incubated overnight. The growth is harvested in 2–3 ml of physiological saline containing 0.3% formalin per plate, and centrifuged at 3000 g for 15 minutes at 4°C (or 1200–1500 g for 30–45 minutes at room temperature). The deposited bacteria are resuspended in 25 ml normal HCl saline (0.85% saline in a normal HCl solution) to give an opacity approximately equivalent to Brown’s opacity tube No. 6, and incubated overnight. The suspension is again centrifuged, the supernatant fluid is discarded, and the cell residue is washed three times successively in phosphate buffered saline (PBS) at pH 5.0, 6.0 and 7.0, respectively.

Finally, a suspension of the residual cells, equivalent to Brown’s opacity tube No. 6, is prepared in PBS at pH 7.0. Any suspensions that autoagglutinate should be discarded.

Antisera are prepared against whole bacterial cell suspensions of the reference strains B:2 (Asian HS), E:2 (African HS) and 11:B (Australian 989, non-HS). Agglutination tests are carried out on a slide and the test antigen is used against the three types of sera. A fine granular agglutination indicates a specific somatic agglutination. Tests carried out against the standard antigens will facilitate reading and interpretation. When nonspecific partial agglutination occurs, the tests carried out with tenfold dilutions of the serum against the test and standard antigens will help to identify somatic antigen.

f) **Serotype designation**

Broadly, two typing systems are adopted. One is ‘capsular’ typing by Carter’s IHA test (Carter, 1955) or by AGID tests (Anon, 1981; Wijewardena et al., 1982). The other is ‘somatic’ typing by the method of Namioka & Murata (Namioka, 1978; Namioka & Murata, 1961b; 1961c), and by the method of Heddleston et al. (1972). It is generally agreed that designation of serotypes should be based on a somatic–capsular combination. Two systems commonly in use are the Namioka–Carter and the Carter–Heddleston systems. In the former system, Asian and African HS serotypes are designated 6:B and 6:E, respectively, while in the latter system they are designated B:2 and E:2, respectively.

g) **Antimicrobial susceptibility testing**

Antimicrobial susceptibility testing (AST) is particularly necessary for *P. multocida* for which resistance to commonly used antimicrobial agents has been reviewed by Kehrenberg et al. (2001). AST methods are described in Guideline 3.1 *Laboratory methodologies for bacterial antimicrobial susceptibility testing*. The agar disk diffusion method has been used to test common fast-growing bacterial pathogens and is recognised to work well with *P. multocida* (Bauer et al., 1966). Reliable results can be obtained with disk
diffusion tests that use standardised methodology and zone diameter measurement correlated with minimum inhibitory concentration (MIC) and the behaviour of strains among clinically susceptible and resistant categorisations. Selection of the most appropriate antimicrobial agents to test is a decision best made by each laboratory in accordance with the needs of veterinary practitioners and the drugs available for veterinary use in the country. The following agents have proven their clinical efficacy: penicillin, amoxicillin (or ampicillin), cephalothin, cefetilov, cefquinome, streptomycin, gentamicin, spectinomycin, florfenicol, tetracycline, sulfonamides, trimethoprim/sulfamethoxazole, erythromycin, tilmicosin, enrofloxacin (or other fluoroquinolones) and norfloxacin.

- **Nucleic acid recognition methods**

  a) *Pasteurella multocida*-specific PCR assay

  PCR technology can be applied for rapid, sensitive and specific and/or detection of *P. multocida* (Miflin & Blackall, 2001; Townsend et al., 1998a). The rapidity and high specificity of two of the *P. multocida*-specific assays (Miflin & Blackall, 2001; Townsend et al., 1998a) provide optimal efficiency without the need for additional hybridisation. The *P. multocida*-specific PCRs (Miflin & Blackall, 2001; Townsend et al., 1998a) identify all subspecies of *P. multocida*. The Miflin & Blackall PCR (Miflin & Blackall, 2001) was described as giving a false positive with both *P. avium* biovar 2 and *P. canis* biovar 2, while the Townsend et al. PCR (1998a) gave a false positive with *P. canis* biovar 2 (it has not been tested against *P. avium* biovar 2). Recently, both *P. avium* biovar 2 and *P. canis* biovar 2 have been re-named as *P. multocida* (Christensen et al., 2004) – meaning that both the Townsend et al. (1998a) and the Miflin & Blackall (Miflin & Blackall, 2001) PCR assays are now regarded as being specific for *P. multocida*. Some difficulties remain as it is now known that sucrose-negative *P. multocida*-like organisms from large cat bite wounds form two groups. While both are positive in the Miflin & Blackall *P. multocida*-specific PCR (2001) only one group has been confirmed as true *P. multocida* by other genotypic methods (Christensen et al., 2005). The Townsend et al. (1998a) PCR is described in the following paragraph).

  A fraction of an isolated colony of the suspect organism is transferred directly into the PCR mixture. Alternatively, template DNA can be obtained from 2 µl of either a mixed or pure broth culture. All currently used methods for the preparation of template DNA produce reproducible results with the KMT1 primers (Townsend et al., 1998a), and allow detection of ≤10 organisms per reaction. The sensitivity and specificity of the *P. multocida*-specific PCR offer the most compelling argument for the use of PCR technology in laboratory investigation of suspected HS cases. *Pasteurella multocida* can be detected regardless of the purity of the specimen, an advantage if the specimen is from an old carcass or from tonsil or nasal swabs. In such cases, the swab should be inoculated in 2 ml CSY broth and incubated on a roller for 2–4 hours; 2 µl of the culture is then added directly to the PCR mixture prior to amplification.

  Primer sequences (Townsend et al., 1998a):

  **P.-multocida**-specific PCR: 
  KMT1T7 5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3'
  KMT1SP6 5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3'

  PCR conditions:

  Template DNA is added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTP), 2 mM MgCl₂, 3.2 pmol of each primer and 0.5 u Taq DNA polymerase. Thermocycler parameters are as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute; with a final extension at 72°C for 7 minutes. 5 µl of each sample is electrophoresed on a 2% agarose gel in 1 × Tris-acetate running buffer (TAE) at 4 V/cm for 1 hour. The gel is stained with 1% ethidium bromide and DNA fragments are viewed by UV transillumination.

  b) *Pasteurella multocida* multiplex capsular PCR typing system

  Identification of the genes involved in the biosynthesis of the *P. multocida* A:1 (Chung et al., 1998) and B:2 (Boyce et al., 2000) polysaccharide capsules provided the required information to determine the biosynthetic region of the remaining three serogroups (D, E, and F) (Boyce et al., 2000). Moreover, with the use of serogroup specific multiplex PCR, conflicting results as regards to typing of some strains could be confirmed (Townsend et al., 2000). With this knowledge, serogroup-specific sequences were identified for use as primers in a multiplex capsular PCR-typing system (Townsend et al., 2001). The *P.-multocida*-specific primers are included as an internal control for species identification. In the multiplex capsular PCR typing system, the amplicon band giving the typing result may be unclear. In such cases, removal of the *P. multocida*-specific primers (KMT1T7, KMT1SP6) from the mixture can improve the result.
Chapter 2.4.12. – Haemorrhagic septicaemia

Primer sequences (Townsend et al., 2001):

**Multiplex capsular PCR:**
- CAPA-FWD: 5'-TGC-CAA-AAT-CGC-AGT-CAG-3'
- CAPA-REV: 5'-TTG-CCA-TCA-TTG-TCA-GTG-3'
- CAPB-FWD: 5'-CAT-TTA-TCG-AAAG-CTC-CAC-C-3'
- CAPB-REV: 5'-GCC-CGA-GAG-TTT-CAA-TCA-3'
- CAPD-FWD: 5'-TTA-CAA-AAG-AAA-GAC-TAG-GAG-CGC-3'
- CAPD-REV: 5'-CAT-CTA-CCC-CTC-CAA-CTA-CAG-3'
- CAPE-FWD: 5'-TCC-GCA-GAA-AAT-TGA-TAT-GTC-3'
- CAPE-REV: 5'-GCT-TGC-TGC-TTGTT-ATT-TTG-TC-3'
- CAPF-FWD: 5'-AAT-CGG-AGA-ACG-CAG-AAA-TCA-G-3'
- CAPF-REV: 5'-TTC-CGC-CGT-CAA-TTA-CTC-TG-3'
- KMT1T7: 5'-ATT-CGC-TAT-TTA-CCC-AGT-GG-3'
- KMT1SP6: 5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3'

**Size of resulting fragments:**
- Serogroup A: CAPA-FWD/CAPA-REV 1044 bp
- Serogroup B: CAPB-FWD/CAPB-REV 760 bp
- Serogroup D: CAPD-FWD/CAPD-REV 657 bp
- Serogroup E: CAPE-FWD/CAPE-REV 511 bp
- Serogroup F: CAPF-FWD/CAPF-REV 851 bp

**PCR conditions:**
Template DNA is added to the PCR mixture (total volume of 25 µl) containing 1 x PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTP), 2 mM MgCl₂, 3.2 pmol of each primer and 1 u Taq DNA polymerase.

In the original publication (Townsend et al., 2001) it is suggested to use a standard cycling programme as per *P.-multocida*-specific PCR assay. However, the cycling programme should be optimised to and validated for the model of thermocycler in use. Agarose gel electrophoresis is as described above.

c) **HS-causing type-B-specific PCR assay**

Presumptive identification of HS-causing type-B-specific *P. multocida* is also possible by PCR amplification (Townsend et al., 1998a). Type B cultures with the predominant somatic antigen being either type 2 or 5 are identified by the amplification of a ~620 bp fragment with the KTSP61 and KTT72 primers.

**Primer sequences (Townsend et al., 1998a):**
- HS-causing type-B-specific PCR (KTT72, KTSP61):
  - KTT72: 5'-AGG-CTC-GTT-TGG-ATT-ATG-AAG-3'
  - KTSP61: 5'-ATC-CGC-TAA-CAF-CTC-3'

Conditions for HS-causing type-B-specific PCR are as described for *P. multocida*-specific PCR. The usefulness of these primers has been reported for identification of serogroup B strains.

HS-causing type-B-specific PCR primers can also be used in a multiplex PCR with the *P.-multocida*-specific primers, dramatically decreasing the time required for *P. multocida* detection and presumptive identification of the HS-serotype. Multiplex PCR conditions are as described above except that 3.2 pmol of each of the four primers and 1 u Taq DNA polymerase are used. The use of the multiplex *P.-multocida*-specific/HS-causing type-B-specific PCR on suspect organisms can confirm the identity and provide a presumptive serotype within 3–4 hours, in comparison with biochemical analysis and conventional serotyping, which can take up to 2 weeks.

d) **Pasteurella multocida** type A specific PCR

Primers useful for typing of serogroup A strains with several somatic types have been reported to be useful in specific identification of isolates (Gautam et al., 2004).

**Primers:**
- RGPMA5: 5'-AAT-GT-TTG-CGA-TAG-TCC-GTT-AGA-3'
- RGPMA6: 5'-ATT-TGG-CGC-CAT-ATC-ACA-GTC-3'
PCR conditions:

Template DNA (50 ng) is added to the PCR mixture (total volume of 25 µl) containing 1 x PCR buffer, 200 µM each dNTPs, 1.5 mM MgCl₂, 20 pmol of each primer and 1 unit Taq DNA polymerase. Standard amplification conditions are as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 45 seconds, 56°C for 45 seconds, 72°C for 6 minutes. Amplified products are separated by agarose gel electrophoresis (1.5% agarose gel) in 0.5 x TBE buffer at 5 v/cm for 2 hours.

The PCR amplification yields a product of 564 bp.

The test can be applied on direct culture, boiled cell lysate and infected tissues.

e) Genotypic differentiation of isolates

Once presumptive (or definitive) identification has been made, further differentiation of isolates can be achieved by genotypic fingerprinting methods. Restriction endonuclease analysis with the enzyme Hhal has proved useful for characterisation of type B HS-serotypes. Among 71 P. multocida capsule serogroup B isolates, 20 DNA fingerprint profiles were observed. With HS-causing serogroup strains, 13 unique profile among 54 isolates resembling the profile of the somatic serotype 2 reference strain have been reported (Wilson et al., 1992). In contrast, while a single Hhal profile was observed among 13 serogroup E isolates, differentiation of these strains was possible following HpaII digestion. HpaII appear to generate finer subdivisions than those achieved with the use of Hhal (Wilson et al., 1995). Ribotyping and large DNA separation by pulsed-field gel electrophoresis also provide useful discrimination of serogroup B and E P. multocida isolates (Townsend et al., 1997a). Genetic diversity of HS-causing Pasteurella multocida strains of animal or bird origin could be obtained by sequence analysis of the 16S rRNA gene. A study in the United Kingdom using 79 field isolates recovered from various species revealed nineteen 16S rRNA types that clustered into two distinct phylogenetic lineages (Davies, 2004). On the other hand, sequence analyses of Indian isolates of P. multocida serogroup B from different animal species did not reveal considerable variation (Dey et al., 2007). Multilocus sequence typing (MLST), a sequence-based typing system based on seven housekeeping genes has been used to identify strain diversity of bovine isolates of P. multocida (Davies et al., 2004). However, these techniques are largely used for research purposes and require specialised equipment. Moreover these profiles are not unique to country of origin or host species.

PCR fingerprinting is feasible for any laboratory with PCR capability, with several methods previously used for P. multocida differentiation. Random amplified polymorphic DNA (RAPD) analysis and arbitrarily primed PCR (AP-PCR), respectively, have been shown to be useful for epidemiological studies of P. multocida isolated from rabbits (Chaslus-Dancla et al., 1996). Repetitive sequence PCR analysis of P. multocida has provided useful for discrimination of avian and swine isolates, although all HS-causing strains analysed demonstrated similar profiles (Townsend et al., 1997b; 1998b). However, molecular variability among HS-causing strains of P. multocida belonging to serogroup B has been found recently. Using repetitive extragenic palindromic (REP)-PCR, enterobacterial repetitive intragenic consensus (ERIC)-PCR and single primer PCR, genotypic differentiation among five P. multocida serogroup B isolates have been reported (Biswas et al., 2004). RAPD and AP-PCR analysis of HS-causing P. multocida isolates have not been previously described.

2. Serological tests

Serological tests for detecting antibodies are not normally used for diagnosis. The IHA test can be used for this purpose, following a method broadly similar to that described for capsular typing above. High titres detected by the IHA test are indicative of recent exposure to HS. As HS is a disease that occurs mainly in animals reared under unsophisticated husbandry conditions, where disease-reporting systems are also poor, there is often considerable delay in notification of outbreaks. Deaths occur very suddenly and no carcasses are available for examination when notification is made. In such situations, high IHA titres from 1/160 up to 1/1280 or higher among in-contact animals surviving in affected herds, are indicative of recent exposure to HS for the purpose of diagnosis.

C. REQUIREMENTS FOR VACCINES

1. Background

a) Rationale and intended use of the product

The three types of vaccines used against HS are bacterins, alum-precipitated vaccine (APV) and oil-adjuvanted vaccine (OAV). To provide sufficient immunity with bacterins, repeated vaccination is required.
Administration of dense bacterins can give rise to shock reactions, which are less frequent with the APV and almost nonexistent with the OAV.

A live HS vaccine prepared using an avirulent \textit{P. multocida} strain B:3,4 (Fallow deer strain) has been used for control of the disease in cattle and water buffaloes over 6 months of age in Myanmar since 1989. It is administered by intranasal aerosol application (Carter \textit{et al.}, 1991; Myint \textit{et al.}, 2005). The vaccine has been recommended by the Food and Agriculture Organization of the United Nations (FAO) as a safe and potent vaccine for use in Asian countries. However, there is no report of its use in other countries and killed vaccines are the only preparations in use by the countries affected with HS.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed

i) Biological characteristics

A local isolate of \textit{P. multocida} representing the prevalent serotype is used. A well-capsulated, stable culture that produces large colonies of approximately 2 mm in diameter on CSY blood agar must be maintained. Seed cultures should be stored as semisolid nutrient agar stab cultures at room temperature, or as lyophilised cultures.

A calf is infected with the culture, and, within 2–3 hours of its death, blood is collected aseptically from the heart and stored at –20°C in 1 ml aliquots. A fresh aliquot is used for each new batch of vaccine. It is permissible to subculture this aliquot once or twice, provided the colony size does not diminish. A blood aliquot is thawed, plated on to CSY blood agar, and the growth is tested for agglutinability by the appropriate antiserum on a slide. A good culture will give a coarse floccular agglutination in under 30 seconds. A poor culture will yield only a fine granular agglutination.

ii) Quality criteria (sterility, purity, freedom from extraneous agents)

Seed lots must be shown to be:

- \textit{Pure}: Free from adventitious agents.
- \textit{Safe}: Produce no adverse reaction in the target species when given as recommended.
- \textit{Efficacious}: Stimulate effective immunity as indicated by potency tests.

The necessary tests are described in Section C.2.b.iv, below.

b) Method of manufacture

i) Procedure

For vaccine production, dense suspensions of bacteria are necessary. They should have a minimum bacterial content of 1.5 g dry weight per litre of suspension. There are two methods of producing dense suspensions. The first is to culture on solid medium in Roux flasks and harvest in formalinised physiological saline, by which means suspensions of any density can be achieved. This is laborious as each flask must be harvested separately and tested for purity. The second and recommended method is the use of a large vessel with aerated cultures in a medium that specifically supports \textit{P. multocida}.

There are two types of aeration process – by vortexing and sparging. Sterile air is provided by a compressor. In vortex aeration, the culture is stirred by an impeller shaft operating in the air stream, whereas in sparging aeration, the air is dispersed through a sparger. Intermittent aeration seems to produce denser growth (Thomas, 1968). The more finely dispersed the air, the better is the bacterial growth. Vessels of 20–40 litres are usually employed, and incubation is at 37°C. In continuous culture systems, once a maximum density has been reached, usually within 15 hours, about 25% of the working volume is harvested and replaced hourly. The harvests of continuous cultures are collected in relatively small volumes into separate vessels, but, after several days, the density diminishes, presumably through loss of capsular antigen. For this reason, batch cultures are preferred. If batch culture vessels are inoculated at a rate of 50 ml/litre of medium, maximum turbidity is obtained within 15–18 hours, when the growth can be terminated by the addition of formalin to a final concentration of 0.5%. This procedure, where a large inoculum is employed and the growth is terminated within a short period, helps to minimise the chances of contamination. The turbidity is standardised against a reference containing the equivalent dry weight/volume of 1.5 g/litre.
Dense cultures are also obtained using fermenters, where heat sterilisation of the tanks and culture can be carried out in situ, with automatic temperature, pH and aeration control devices. Liquid sterilisation systems by filtration, for heat-labile components, can also be built into the fermenter. A 100 litre batch fermenter will yield a minimum of 66,000 doses (each of 3 ml) of OAV, and even more doses if the density is high enough for dilution to a reference standard equivalent to 1.5 g/litre, dry weight/volume.

OAV is made by the emulsification of equal volumes of a light mineral oil and the bacterial suspension, with 5% pure anhydrous lanolin as emulsifying agent. The mineral oil and lanolin are first sterilised and, on cooling to 40°C, 0.5% formalin is added to the mixture. The bacterial suspension is added slowly and emulsification is continued for a further 10 minutes. Following overnight storage, the mixture is re-emulsified, bottled and stored at 4°C for 2 weeks prior to use.

APV is prepared by first adjusting the turbidity of the suspension to the reference standard as above, and diluting it with an equal volume of 0.5% formalinised physiological saline. The pH is adjusted to 6.5, and a hot 20% solution of potash alum is added to give a final concentration of 1% alum. After overnight storage with continuous agitation, the vaccine is bottled for use.

ii) Requirements for substrates and media

A suitable sterilised medium for the aerated culture method is casein hydrolysate (2 g), sucrose (6 g), yeast extract (6 g), sodium chloride (5 g), anhydrous dipotassium hydrogen orthophosphate (8.6 g), anhydrous potassium dihydrogen orthophosphate (1.36 g), and distilled water to 1 litre. A denser growth is obtained if the casein, sucrose and yeast are prepared as a concentrate, filter-sterilised or autoclaved for 10 minutes at 107°C, and transferred aseptically into the tank that has previously been heat-sterilised with the rest of the ingredients.

iii) In-process controls

Proper concentration of bacterial growth, the capsulation of the bacteria, purity of culture and efficient inactivation all need to be checked.

iv) Final product batch tests

Sterility and purity

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

Safety

Two seronegative cattle are vaccinated with twice the recommended dose and observed for 10–14 days for adverse effects. Five mice are inoculated intramuscularly with 0.2 ml each of the vaccine, and observed for 5 days. The blood of any mouse that dies is cultured for P. multocida.

Batch potency

Potency tests can be carried out by any of the following methods:

i) Vaccination of cattle followed by direct challenge or passive mouse protection tests using the bovine sera. This procedure is not very feasible as cattle take a long time to develop adequate immunity after OAV;

ii) Vaccination of rabbits followed by direct challenge or passive mouse protection test using the rabbit sera; or

iii) Potency tests in mice, the most feasible method of the three.

Each of 50 mice is vaccinated intramuscularly with 0.2 ml of vaccine, and again 14 days later. On day 21, the mice are divided into ten groups of five, each group being challenged with respective dilutions of a 6–8-hour broth culture of a field strain in the range $10^{-1} - 10^{-10}$. 50 unvaccinated controls are similarly challenged, and all mice are observed for 5 days. The median lethal dose ($LD_{50}$) can then be calculated in order to obtain an indication of the dose that is sufficient to protect cattle: vaccines prepared in the manner described give at least $10^4$ units protection in the vaccinated mice.
c) Requirements for authorisation

i) Safety requirements

Target and non-target animal safety
See chapter 1.1.6.

Reversion-to-virulence for attenuated/live vaccines
See chapter 1.1.6.

Environmental consideration
See chapter 1.1.6.

ii) Efficacy requirements

For animal production

A single dose of vaccine administered to young calves 4–6 months of age will protect susceptible animals for 3–4 months when APV is used, and for 6–9 months when OAV is used.

The vaccine should be administered by deep intramuscular injection. The use of nylon 5 ml volume syringes for a 3 ml dose and a gauge 14–15 needle is advised, and the recommended age for primary vaccination is 4–6 months. For routine, prophylactic vaccination, a single dose of OAV at 4–6 months, a booster 3–6 months later, and annual revaccination thereafter, is recommended. Where husbandry practices are such that reaching individual animals at appropriate times is impracticable, annual vaccination of all animals over 4 months of age, preferably before the breeding season, and vaccination of all calves under 1 year of age, 6 months later, is recommended. In the face of an outbreak in vaccinated animals, one dose of APV, followed by one dose of OAV, is recommended.

Leakage of OAV into subcutaneous tissue can occasionally give rise to fibrous lumps at sites of injection. Rarely, abscesses may develop if sterility conditions are not observed, though most animals are resistant to such infections. APV may occasionally cause shock reactions.

For control and eradication

Not applicable.

iii) Stability

The OAV emulsion should be pure white, and should stick to glass like paint. If the emulsion shows signs of cracking, it should be discarded. Separation of a thin layer of oil on the surface is permissible. It can be stored at 4–8°C for 6 months without any significant loss of potency. It must not be frozen. Increase in the content of lanolin improves stability, but also increases the viscosity – a distinct disadvantage. Use of other emulsifying agents such as ‘Arlacel’ helps to produce thinner, stable emulsions.

3. Vaccines based on biotechnology

Not applicable at present

REFERENCES


Chapter 2.4.12. — Haemorrhagic septicaemia


Chapter 2.4.12. — Haemorrhagic septicaemia


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744

OIE Terrestrial Manual 2012
CHAPTER 2.4.13.

INFECTIOUS BOVINE RHINOTRACHEITIS/INFECTIOUS PUSTULAR VULVOVAGINITIS

SUMMARY

Definition of the disease: Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV), caused by bovine herpesvirus 1 (BoHV-1), is a disease of domestic and wild cattle. The virus is distributed worldwide, but has been eradicated from Austria, Denmark, Finland, Sweden, Italy (Province of Bolzano), Switzerland, Norway and parts of Germany (the ‘Oberfranken’ and ‘Oberpfalz’ districts of Bavaria). Control programmes are running in several other countries, for example in Germany and Italy.

Description of disease: The disease is characterised by clinical signs of the upper respiratory tract, such as a (muco)purulent nasal discharge, hyperaemia of the muzzle (red nose disease) and by conjunctivitis. Signs of general illness are fever, depression, inappetence, abortions and reduced milk yield. The virus can also infect the genital tract and cause pustular vulvovaginitis and balanoposthitis. Post-mortem examinations reveal rhinitis, laryngitis and tracheitis. Mortality is low, and most infections run a subclinical course. Secondary bacterial infections can lead to more severe respiratory disease, and BoHV-1 could play a role in multifactor diseases such as ‘shipping fever’.

Identification of the agent: The virus can be isolated from nasal or genital swabs from animals with respiratory signs, vulvovaginitis or balanoposthitis, taken during the acute phase of the infection, and, in severe cases, from various organs collected at post-mortem. Following infection, BoHV-1 may persist in infected animals in a latent state in sensory neurons, e.g. in the trigeminal or sacral ganglia. The virus can be reactivated and this results in virus shedding (re-excretion) without exhibition of clinical disease. Therefore, antibody-positive animals have to be classified as infected with BoHV-1 (with two exceptions: serological responses induced by vaccination with an inactivated vaccine or by colostral antibodies).

For virus isolation, various cell cultures of bovine origin are used, for example, secondary lung or kidney cells or the Madin–Darby bovine kidney cell line (MDBK). The virus produces a cytopathic effect in 2–4 days. It is identified by neutralisation or antigen detection methods using monospecific antisera or monoclonal antibodies. BoHV-1 isolates can be further subtyped by DNA restriction enzyme analysis (RFLP) into subtypes 1.1 and 1.2. BoHV-1.2 isolates can be further differentiated into 2a and 2b. Development of rhinotracheitis or vulvovaginitis/balanoposthitis depends more on the route of infection than on the subtype of the virus. The virus previously referred to as BoHV-1.3, a neuropathogenic agent, is now classified as BoHV-5.

Viral DNA detection methods have been developed, and the polymerase chain reaction technique is increasingly used in routine diagnosis including real-time polymerase chain reaction (PCR).

Serological tests: The virus neutralisation test and various enzyme-linked immunosorbent assays (ELISA; indirect or gB-blocking) are most widely used for antibody detection. With the ELISAs, antibodies can be detected in serum or plasma, and with lower sensitivity in milk or bulk milk samples.

Requirements for vaccines and diagnostic biologicals: Inactivated and attenuated live vaccines are available. The vaccines protect cattle clinically in case of infection and markedly reduce the subsequent shedding of field virus. Although vaccination may not prevent field virus infection of individual animals, spreading of wild-type virus in infected herds is efficiently reduced. The vaccines must not induce disease, abortion, or any local or systemic reaction, and must be genetically stable.
BoHV-1 glycoprotein E deleted mutant marker vaccines are now generally available (live or inactivated). The use of a gE-antibody-ELISA makes it possible to distinguish field virus infected cattle from cattle vaccinated with such a marker vaccine (DIVA strategy).

A. INTRODUCTION

Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV), caused by bovine herpesvirus 1 (BoHV-1), is a disease of domestic and wild cattle. BoHV-1 is a member of the genus Varicellovirus in the subfamily Alphaherpesvirinae, which belongs to the Herpesviridae family, order Herpesvirales. The viral genome consists of double-stranded DNA that encodes for about 70 proteins, of which 33 structural and more than 15 nonstructural proteins have been identified. The viral glycoproteins, which are located in the envelope on the surface of the virion, play an important role in pathogenesis and immunity. BoHV-1 can be differentiated into subtypes 1.1, 1.2a and 1.2b (Metzler et al., 1985). The BoHV-1.2 subtypes may be less virulent than subtype 1.1 (Edwards et al., 1990). The former BoHV-1.3, which may act as a neuropathogenic agent in calves, has been reclassified as BoHV-5 (Magyar et al., 1993). BoHV-1 shares antigenic and genetic close relationships with other ruminant alphaherpesviruses: BoHV-5, caprine herpesvirus 1, cervid herpesvirus 1 (red deer), cervid herpesvirus 2 (reindeer), bubaline herpesvirus 1 and elk herpesvirus 1 (Thiry et al., 2006).

After an incubation period of 2–4 days, serous nasal discharge, salivation, fever, inappetence, and depression become evident. Within a few days the nasal and ocular discharges change to mucopurulent. Where natural mating is practised, genital infection can lead to pustular vulvovaginitis or balanoposthitis. However, most infections run a very mild or subclinical course (Van Oirschot et al., 1993). Uncomplicated cases of respiratory or genital disease caused by BoHV-1 last about 5–10 days. Secondary bacterial or viral agents may contribute to a multifactor disease complex resulting in severe respiratory disease of young animals (‘shipping’ or ‘crowding fever’).

After infection via the airborne route, BoHV-1 replicates to high titres in mucous membranes of the upper respiratory tract and in the tonsils. Subsequently, the virus disseminates to conjunctivae and reaches the trigeminal ganglia by neuronal axonal transport. After genital infection, BoHV-1 replicates in the mucous membranes of the vagina or prepuce, and becomes latent in the sacral ganglia. The viral DNA remains in the neurons of the ganglia, probably for the entire life of the host (status of latency). Stress, such as transport and parturition, but also the application of corticosteroids can induce reactivation of the latent infection. Consequently, the virus may switch between latent and lytic infection and may be shed intermittently into the environment and spread to contact animals.

BoHV-1 infection elicits an antibody response and a cell-mediated immune response within 7–14 days. The immune response is presumed to persist life-long, although it may fall below the detection limit of some tests after a number of years. Maternal antibodies are transferred via colostrum to the young calf, which is consequently protected against BoHV-1-induced clinical disease (Mechor et al., 1987). Maternal antibodies have a biological half-life of about 3 weeks, but may be detected occasionally in animals up to 9 months old, and rarely in animals over this age.

The virus is distributed world-wide, with the exception of the BoHV-1-free countries, paralleling the distribution of domestic cattle. Other Artiodactyla (e.g. goats, sheep, water buffaloes, camels) may be infected with BoHV-1. After infection, nasal viral shedding is detected for 5–14 days, with peak titres of 10^8–10^{10} TCID_{50} (50% tissue culture infective doses) per ml of nasal secretion. The semen of an infected bull may contain BoHV-1, and the virus can thus be transmitted by natural mating and artificial insemination (Parsonson & Snowdon, 1975).

Prevention and control of BoHV-1 infections are based on thorough farm management including hygienic measures, vaccination schedules and removal of infected animals. Ideally, a 4-week quarantine period is imposed for newly introduced cattle, if the cattle are not from certified BoHV-1-free farms. Only cattle that are BoHV-1-seronegative are then admitted to a free herd. Natural mating should be avoided and only semen from BoHV-1-negative bulls should be used.

Vaccines usually prevent the development of clinical signs and markedly reduce the shedding of virus after infection, but do not completely prevent infection. Several eradication campaigns have been carried out or are currently running in different countries including test-and-removal programmes and/or vaccination campaigns (see Section C).

BoHV-1 infection may be suspected on the basis of clinical, pathological and epidemiological findings. However, to make a definite diagnosis, laboratory examinations (serology and/or virus detection) are required. A complete diagnostic procedure in the laboratory is aimed at detecting the causative virus (or viral components) and the
specific antibodies they induce. Nevertheless, because of latent infection induced by BoHV-1, detection of antibodies could be sufficient for the determination of the BoHV-1 status of individual animals.

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

   a) **Collection and processing of samples**

   Nasal swabs are collected from several (from five to ten) affected cattle in the early phase of the infection. These cattle still have serous rather than mucopurulent nasal discharge. In cases of vulvovaginitis or balanoposthitis, swabs are taken from the genitals. The swabs should be vigorously rubbed against the mucosal surfaces. The prepuce can also be washed with saline; the washing fluid is then collected. The specimens are suspended in transport medium (cell culture medium containing antibiotics and 2–10% BoHV-1-free fetal bovine serum to protect the virus from inactivation), cooled at 4°C, and rapidly submitted to the laboratory.

   During necropsy, mucous membranes of the respiratory tract, and samples of the tonsil, lung and bronchial lymph nodes are collected for virus detection. In cases of abortion, the fetal liver, lung, spleen, kidney and placental cotyledons are examined. Samples should be kept on ice and sent to the laboratory as quickly as possible.

   After arrival at the laboratory, swabs are agitated at room temperature for 30 minutes in the transport medium to elute virus. Following removal of the swabs, the transport medium is clarified by centrifugation at 1500 g for 10 minutes. Tissues are homogenised to a 10–20% (w/v) suspension in cell culture medium before centrifugation at 1500 g for 10 minutes. The supernatants of these specimens are filtered through 0.45 µm filters and used for virus isolation.

   The isolation of virus from semen needs some special adaptations, because the seminal fluid contains enzymes and other factors that are toxic to the cells and inhibit viral replication (see below).

   b) **Virus isolation**

   For virus isolation, bovine cells of various origins can be used. Primary or secondary bovine kidney, lung or testis cells, cell strains derived from bovine fetal lung, turbinate or trachea, and established cell lines, such as the Madin–Darby bovine kidney cell line (MDBK), are suitable for BoHV-1 propagation. Cell cultures can be grown in glass or plastic tubes, plates or dishes. When 24-well plastic plates are used, a 100–200 µl volume of the supernatants described above is inoculated into these cell cultures. After a 1-hour adsorption period, the cultures are rinsed and maintenance medium is added. The serum used as a medium supplement in the maintenance medium should be free of antibodies against BoHV-1. The cell cultures are observed daily for CPE, which usually appears within 3 days after inoculation. It is characterised by grape-like clusters of rounded cells gathered around a hole in the monolayer; sometimes giant cells with several nuclei may be observed. Experience is needed to recognise this characteristic appearance. When, after 7 days, no CPE has appeared, a blind passage must be made. The cell culture is freeze–thawed and clarified by centrifugation, and the supernatant is used for inoculation of fresh monolayers (Brunner et al., 1988; Edwards et al., 1983).

   To identify the recovered virus as BoHV-1, the supernatant of the culture should be neutralised with a monospecific BoHV-1 antiserum or neutralising monoclonal antibody (MAb). For this purpose, serial tenfold dilutions of the test supernatant are made, and to each dilution monospecific BoHV-1 antiserum or negative control serum is added. Following incubation at 37°C for 1 hour, the mixtures are inoculated into cell cultures; 3–5 days later, the neutralisation index is calculated. The neutralisation index is the virus titre (in log_{10}) in the presence of negative control serum minus the virus titre in the presence of specific antiserum. If the neutralisation index is greater than 1.5, the isolate may be considered to be BoHV-1. To shorten the virus isolation procedure, two specimens may be inoculated into cell culture: one that has been preincubated with monospecific antiserum and another that has been preincubated with negative control serum. If the CPE is inhibited by the monospecific antiserum, the isolate can be considered to be BoHV-1, although definitive confirmation would require molecular characterisation to distinguish it from related ruminant alphaherpesviruses.

   An alternative method of virus identification is the direct verification of BoHV-1 antigen in cells around the CPE by an immunofluorescence or immunoperoxidase test (Kaashoek et al., 1994) with conjugated monospecific antiserum or MAb. Furthermore, the supernatant can be used as template for restriction endonuclease fragment length polymorphism (RFLP) (see Section B.1.e) and polymerase chain reaction (PCR) (see Section B.1.c) analyses.
• **Virus isolation from semen (a prescribed test for international trade)**

0.05 to 0.1 ml of raw semen should be tested with two passages in cell culture. Raw semen is generally cytotoxic and should be prediluted (e.g. 1/10) before being added to cell cultures. A similar problem may sometimes arise with extended semen. For extended semen, an approximation should be made to ensure that the equivalent of a minimum of 0.1 ml raw semen is examined (e.g. a minimum of 0.5 ml extended serum). Multiple diluted samples may need to be tested with this procedure to reach a volume equivalent to 0.1 ml raw semen (e.g. 5 × 1 ml of a 1/10 diluted sample of extended semen). A suitable test procedure is given below. See also Brunner *et al.* (1988).

- **Test procedure**
  1. Dilute 200 µl fresh semen in 2 ml fetal bovine serum (free from antibodies against BoHV-1) with antibiotics.
  2. Mix vigorously and leave for 30 minutes at room temperature.
  3. Inoculate 1 ml of the semen/serum mixture into a monolayer of susceptible cells (see virus isolation above) in a six-well tissue culture plate.
  4. Incubate the plates for 1 hour at 37°C.
  5. Remove the mixture, wash the monolayer twice with 5 ml maintenance medium, and add 5 ml maintenance medium to each well.
  6. Include BoHV-1 negative and positive controls in the test. Special caution must be taken to avoid accidental contamination of test wells by the positive control, for example always handling the control last, and using separate plates.
  7. Observe plates under a microscope daily for the appearance of a CPE. If a CPE appears, confirmatory tests for BoHV-1 are made by specific neutralisation or immunolabelling methods (see above).
  8. If there is no CPE after 7 days, the cultures are frozen and thawed, clarified by centrifugation, and the supernatant is used to inoculate fresh monolayers.
  9. The sample is considered to be negative, if there is no evidence of a CPE after 7 days’ incubation of the passaged cultures.

c) **Nucleic acid detection**

During the past decade, various methods for the detection of BoHV-1 DNA in clinical samples have been described, including DNA–DNA hybridisation and the PCR. The PCR is also increasingly used in routine diagnostic submissions (Moore *et al.*, 2000). Compared with virus isolation, the PCR has the primary advantages of being more sensitive and more rapid: it can be performed in 1–2 days. It is also possible to detect episomal DNA of non-replicating virus in sensory ganglia (Van Engelenburg *et al.*, 1993), such as the trigeminal ganglion, in the latent phase of infection. The disadvantage is that PCR analyses are prone to contamination and therefore precautions have to be taken to prevent false-positive results. Risk of contamination is markedly reduced by new PCR techniques, such as real-time or quantitative PCR (see below) (Abril *et al.*, 2004; Lovato *et al.*, 2003).

So far PCR has been used mainly to detect BoHV-1 DNA in artificially (Kramps *et al.*, 1993) or naturally (Van Engelenburg *et al.*, 1993) infected semen samples. It is important to thoroughly optimise the PCR conditions, including the preparation of the samples, the concentration of Mg²⁺, primers and polymerase, and the cycle programmes. The target region for amplification must be present in all BoHV-1 strains, and its nucleotide sequence must be conserved. The TK, gB, gC, gD and gE genes have been used as targets for PCR amplification. In addition, PCRs based on detection of gE sequences can be used to differentiate between wild-type virus and gE-deleted vaccine strains (Fuchs *et al.*, 1999; Schyns *et al.*, 1999). Discrimination between infection with virulent IBR strains and infection with live attenuated strains is not possible with the PCR technique, and RFLP is used for this purpose. Specific PCRs have been developed that are able to discriminate between BoHV-1, BoHV-5 and other related alphaherpesviruses (Ashbaugh *et al.*, 1997; Ros *et al.*, 1999).

Experimentally, PCR was found to be more sensitive than virus isolation: in egg yolk-extended semen samples obtained from experimentally infected bulls, PCR detected five times as many positives as did virus isolation (Van Engelenburg *et al.*, 1995). The detection limit of validated PCR assays amounts to only a few genome copies per PCR reaction. Nevertheless, false-negative results cannot be excluded. To identify possible false-negative results, it is recommended to spike an internal control template into the reaction tube of the semen sample to be amplified by the same primers. Such a control template may be constructed by inserting, for example, a 100 base-pair fragment into the target region. This control template also makes it possible to semi-quantify the amount of DNA that is detected (Ros *et al.*, 1999; Van Engelenburg *et al.*, 1993). When using an internal control, extensive testing is necessary to ensure that PCR amplification of the
added internal control does not compete with the diagnostic PCR and thus lower the analytical sensitivity (see also Chapter 1.1.5 Principles and methods of validation of diagnostic assays for infectious diseases). DNA extraction and quality of the DNA preparations can also be controlled by amplification of cellular sequences (housekeeping genes) or by addition of ‘artificial’ DNA sequences prior to extraction procedures (e.g. green fluorescent protein, non-BoHV-related viruses) as internal controls. To enhance the sensitivity and specificity of the BoHV-1 PCR, real-time PCR systems are the methods of choice.

- **Real-time polymerase chain reaction (a prescribed test for international trade)**

The following real-time PCR test method has been developed to detect BoHV-1 in extended bovine semen destined for trade. The method has been validated according to chapter 1.1.5, and includes a comprehensive international inter-laboratory comparison involving six collaborating laboratories with specialist status in IBR testing (Wang et al., 2008).

A number of studies have shown that PCR assays are more sensitive than virus isolation (Smits et al., 2000; Van Engelenburg et al., 1995; Vlieck et al., 1994; Wang et al., 2008; Wiedmann et al., 1993). Real-time PCR has been used for the detection of BoHV-1 and BoHV-5 in experimentally infected cattle and mice (Abril et al., 2004; Lovato et al., 2003) and a series of conventional PCR assays have been used for the detection of BoHV-1 DNA in artificially or naturally infected bovine semen samples (Deka et al., 2005; Grom et al., 2006; Masri et al., 1996; Van Engelenburg et al., 1993; Weiblen et al., 1992; Wiedmann et al., 1993; Xia et al., 1995). Conventional detection of amplified PCR products relies on gel electrophoresis analysis (Rola et al., 2003). Sequence-specific primers have been selected to amplify different parts of conserved glycoprotein genes of the BoHV-1 genome, including glycoprotein B (gB) gene (Grom et al., 2006; Santurde et al., 1996), gC gene (Smits et al., 2000; Van Engelenburg et al., 1995), gD gene (Smits et al., 2000; Wiedmann et al., 1993), gE gene (Grom et al., 2006), and the thymidine kinase (tk) gene (Moore et al., 2000; Yason et al., 1995).

Real-time PCR differs from standard PCR in that the amplified PCR products are detected directly during the amplification cycle using a hybridisation probe, which enhances assay specificity. Real-time PCR assays have several advantages over the conventional PCR methods. Real-time PCR assays are able to provide sensitivity close or equal to nested PCR methods with a much lower risk of contamination. The amplification and detection of target is conducted simultaneously, and tubes have not to be opened for product analysis on agarose gels. There is no post-amplification PCR product handling, which significantly reduces the risk of contamination, and it is possible to perform quantitative analysis.

The real-time PCR described here uses a pair of sequence-specific primers for amplification of target DNA and a 5'-nuclease oligoprobe (TaqMan) for detection of amplified products. The oligoprobe is a single, sequence-specific oligonucleotide, labelled with two different fluorophores, the reporter/donor, 5-carboxyfluorescein (FAM) at the 5’ end, and the acceptor/quencher 6-carboxytetramethylrhodamine (TAMRA) at the 3’ end. This real-time PCR assay is designed to detect viral DNA of all BoHV-1 strains, including subtype 1 and 2, from extended bovine semen. The assay selectively amplifies a 97 base-pair sequence of the glycoprotein B (gB) gene. Details of the primers and probes are given in the protocol outlined below.

- **Sample preparation, equipment and reagents**

  i) The samples used for the test are, typically, extended bovine semen stored in liquid nitrogen. The semen samples can be transported to the laboratory in liquid nitrogen, or shipped at 4°C, and stored in liquid nitrogen or at −70°C (for long-term storage) or 4°C (for short-term storage). Storing semen at 4°C for a short period (up to 7 days) does not affect PCR test result.

  ii) Three straws from each batch of semen should be processed. Duplicate PCR amplifications should be carried out for each DNA preparation (six amplifications in total) to ensure the detection of DNA in samples containing low levels of virus.

  iii) A real-time PCR detection system, and the associated data analysis software, is required to perform the assay. A number of real-time PCR detection systems are available from various manufacturers. In the procedure described below, a RotorGene 3000, Corbett Research Ltd, Australia, was used. Other real-time PCR detection systems can also be applied. Other equipment required for the test includes a micro-centrifuge, a heating block, a boiling water bath, a micro-vortex, magnetic stirrer and micropipettes. Real-time PCR assays are able to detect very small amounts of target nucleic acid molecules therefore appropriate measures are required to avoid contamination\(^1\). Furthermore, a minimum of one negative sample should be processed in parallel to estimate the risk of low level contamination.

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\(^1\) Sources of contamination may include product carry-over from positive samples or, more commonly, from cross-contamination by PCR products from earlier experiments. Samples and reagents should be handled in separate areas, with separate equipment for reagent and sample preparation and amplification/detection.
iv) The real-time PCR assay described here involves two separate procedures. Firstly, BoHV-1 DNA is extracted from semen using Chelex-100 chelating resin, along with proteinase K and DL-Dithiothreitol (DTT). The second procedure is the PCR analysis of the extracted DNA template in a real-time PCR reaction mixture: Platinum Quantitative PCR SuperMix-UDG, Invitrogen Technologies (note that there are a number of other commercial real-time PCR amplification kits available from various sources and the particular kits selected need to be compatible with the real-time PCR platform selected). The required primers and probes can be synthesised by various commercial companies. In this protocol, all the primers and probes used were supplied by Sigma-Genosys.

• Extraction of DNA
  
  i) In a screw top 1.5 ml tube, add:
  
  Chelex 100 sodium (Sigma) (10% w/v in distilled deionised water) 100 µl.
  Proteinase K (10 mg/ml, Sigma) 11.5 µl
  DL-Dithiothreitol (1 M, Sigma) 7.5 µl
  Nuclease-free water 90 µl
  Semen sample 10 µl
  
  Mix gently by pipetting 2.
  
  ii) The samples are incubated at 56°C for 30 minutes and then vortexed at high speed for 10 seconds.
  
  iii) Subsequently, the tubes are incubated in a boiling water bath for 8 minutes and then vortexed at high speed for 10 seconds.
  
  iv) The tubes are centrifuged at 10,000 g for 3 minutes.
  
  v) The supernatant 3 is transferred into a new microtube and can be used directly for PCR, or stored at –20°C.

• Preparation of reagents
  
  The real-time PCR reaction mixture (Platinum Quantitative PCR SuperMix-UDG, or other reaction mixture) is normally provided as a 2 × concentration ready for use. The manufacturer’s instructions should be followed for application and storage.
  
  Working stock solutions for primers and probe are made with nuclease-free water at the concentration of 4.5 µM and 3 µM, respectively. The stock solutions are stored at –20°C and the probe solution should be kept in the dark. Single-use aliquots can be prepared to limit freeze–thawing of primers and probes and extend their shelf life.

• Real-time PCR test procedure
  
  i) Primers and probe sequences
  
  Selection of the primers and probe are outlined in Abril et al. (2004) and described below.
  
  Primer gB-F: 5’-TGT-GGA-CCT-AAA-CCT-CAC-GGT-3’ (position 57499–57519 GenBank®, accession AJ004801)
  
  Primer gB-R: 5’-GTA-GTC-GAG-CAG-ACC-CGT-GTC-3’ (position 57595–57575 GenBank®, accession AJ004801)
  
  TaqMan Probe: 5’-FAM-AGG-ACC-GCG-AGT-TCT-TGC-CGC-TAMRA-3’ (position 57525–57545 GenBank®, accession AJ004801)
  
  ii) Preparation of reaction mixtures
  
  The PCR reaction mixtures are prepared in a separate laboratory room. For each PCR test, appropriate controls should be included. As a minimum, a no template control (NTC, reagents only), appropriate negative controls, i.e. 1 per 10 test samples, and two positive controls (moderate and weak positive) should be included. Each test sample and control is tested in duplicate. The PCR amplifications are carried out in a volume of 25 µl.
  
  a) PCR reagent mixtures are added in a clean room (no viral cultures, DNA extracts or post-amplification products should be handled here)

    | Component                          | Concentration |
    |------------------------------------|---------------|
    | 2 × Platinum Quantitative PCR SuperMix-UDG | 12.5 µl       |
    | ROX reference dye (optional)       | 0.5 µl        |
    | Forward primer (gB-F, 4.5 µM)      | 1 µl          |
    | Reverse primer (gB-R, 4.5 µM)      | 1 µl          |
    | Probe (3 µM)                        | 1 µl          |
    | Nuclease free water                | 4 µl          |

2  It is important that the Chelex 100 solution is homogeneous while pipetting, as Chelex 100 sodium is not soluble. This can be achieved by putting the vessel containing Chelex-100 solution on a magnetic stirrer while pipetting.

3  Some DNA samples can become cloudy and a thin white membrane may form occasionally after freezing and thawing. This appears to have no influence on the PCR performance. No heating or re-centrifuging of the samples is necessary.
b) 5 µl of the DNA template are added to the PCR reagent mixture to a final volume of 25 µl. DNA samples are prepared and added to the PCR mix in a separate room.

iii) Real-time (TaqMan) polymerase chain reaction

The PCR tubes are placed in the real-time PCR detection system in a separate, designated PCR room. The PCR detection system is programmed for the test as follows:

**PCR Reaction Parameters**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>One cycle</td>
<td>Hold 50°C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>One cycle</td>
<td>Hold 95°C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>45 cycles</td>
<td>Hold 95°C</td>
<td>15 seconds</td>
</tr>
<tr>
<td></td>
<td>Hold 60°C</td>
<td>45 seconds</td>
</tr>
</tbody>
</table>

iv) Analysis of real-time PCR data

The threshold level is usually set according to the manufacturers’ instructions for the selected analysis software used. Alternatively, virus isolation negative semen samples, from seronegative animals, can be run exhaustively (e.g. up to 60 amplification cycles) to determine the background reaction associated with the detection system used.

• Interpretation of results

• Test controls

Positive and negative controls, as well as reagent controls, should be included in each PCR test. Negative semen, from seronegative bulls, can be used as a negative control. Positive semen from naturally infected bulls is preferable as a positive control. However, this might be difficult to obtain. Alternatively, positive controls can be derived from negative semen spiked with defined quantities of BoHV-1 virus.

• Test results

**Positive result:** Any sample that has a cycle threshold (Ct) value equal or less than 45 is regarded as positive. The positive control should have a Ct value within an acceptable range (± 3 Ct values) as previously determined by repeatability testing. To minimise the risk of contamination by the positive control, a dilution resulting in a Ct value of about 30 to 33 should be used.

**Negative result:** Any sample that shows no Ct value is regarded as negative. Negative control and no template control should have no Ct values.

d) Viral antigen detection

Nasal, ocular or genital swabs can be directly smeared onto glass cover-slips, or, following centrifugation, the cell deposit (see Section B.1.a) may be spotted onto cover-slips. These cover-slips are subjected to a standard direct or indirect fluorescent antibody test. In a direct immunofluorescence test, the monospecific antiserum is conjugated to a fluorescent dye e.g. fluorescein isothiocyanate (FITC), whereas in the indirect procedure, the anti-species immunoglobulin secondary antibody is conjugated to a fluorochrome. To obtain reliable results, it is necessary to sample several animals in a herd that have fever and a slight, serous nasal discharge. Smears should be air-dried and fixed in acetone. Smears from nasal swabs from cattle with a purulent or haemorrhagic nasal discharge are often negative (Terpstra, 1979). The advantage of this antigen-detection technique is that it can lead to a same-day diagnosis. However, the sensitivity of this procedure is lower than that of virus isolation (Edwards et al., 1983) or PCR. Positive and negative controls must be included in each test.

Tissues collected at post-mortem can be examined for the presence of BoHV-1 antigen by immunofluorescence analyses of frozen sections. Immunohistochemistry may also be applied for BoHV-1 detection and determination of the antigen location in the tissues. MAbs are increasingly being used for detecting BoHV-1 antigen, leading to enhanced specificity of the test. However, such MAbs must be carefully selected, because they must be directed against conserved epitopes that are present on all isolates of BoHV-1.

Another possibility for direct rapid detection of viral antigen is the use of an enzyme-linked immunosorbent assay (ELISA). Antigen can be captured by MAbs or polyclonal antibodies coated on a solid phase, usually

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4 These PCR parameters are adapted to the RotorGene 3000, Corbett Research Ltd, Australia, and may vary with different PCR platforms.

5 PCR Taq polymerase systems from different commercial sources may require a prolonged initial denaturation (95°C) time up to 10 minutes. Please follow the manufacturer’s instructions.
on microplates. Amounts of antigen equivalent to $10^4$–$10^5$ TCID$_{50}$ of BoHV-1 are required in order to obtain reliable positive results (Collins et al., 1988). This may not be unrealistically high, because titres of $10^8$–$10^9$ TCID$_{50}$/ml of nasal fluid can be excreted by cattle 3–5 days after infection with BoHV-1. Sensitivity can be increased by amplification systems (see Edwards & Gitao, 1987).

In contrast to virus isolation, no cell culture facilities are required for direct antigen detection techniques and a laboratory diagnosis can be made within 1 day. The disadvantages are the lower sensitivity of direct antigen detection and the extra requirement to perform additional virus isolation, if the isolate is required for further studies.

e) Differentiation of bovine herpesvirus 1 subtypes and of ruminant alphaherpesviruses related to bovine herpesvirus 1

By using appropriate MAbs for immunofluorescence, radioimmunoprecipitation, immunoperoxidase or immunoblot assays, BoHV-1 subtype 1 and subtype 2b can be differentiated (Rijswijk et al., 1999; Wyler et al., 1989). Restriction endonuclease digestion of viral DNA enables differentiation between BoHV-1 subtypes. RFLP analysis includes extraction of the DNA from virions or from infected cells, digestion of the isolated DNA by restriction endonucleases, and separation of the resulting fragments by agarose gel electrophoresis. Differentiation of the BoHV-1 subtypes 1, 2a and 2b by HindIII endonuclease digestion is based on the molecular weight of three relevant DNA fragments (I, K and L) (Metzler et al., 1985). RFLP techniques are of limited diagnostic value, but may be useful in epidemiological studies. Furthermore, RFLP pattern of virus isolates can be compared with that of live vaccine strains.

When differentiation is required between antigenically and genetically related alphaherpesviruses (BoHV-1, BoHV-5 [caprine herpesvirus 1, cervid herpesvirus 1 and 2, elk herpesvirus 1, bubaline herpesvirus 1]), improved methods are available using monoclonal antibodies (Keuser et al., 2004) or PCR amplification and sequencing (Ros et al., 1999).

f) Interpretation of results

The isolation of BoHV-1 from a diseased animal does not unequivocally mean that this virus is the cause of the illness. It may, for instance, be a latent virus that has been reactivated due to stressful conditions. A confirmatory laboratory diagnosis must be made from a group of animals and must be accompanied by seroconversion from negative to positive, or a four-fold or higher increase in antibody titres. Paired serum samples collected 3–4 weeks apart are examined in a serological test for the presence of specific antibodies (see Section B.2).

2. Serological tests

Serological tests can be used for several purposes:

i) To diagnose an acute infection: paired serum samples from the acute and convalescent stages of infection of the same animals are examined in one test. A seroconversion from negative to positive or a four-fold or higher increase in antibody titres is considered to prove an acute infection.

ii) To demonstrate absence of infection, for instance, for international trade purposes.

iii) To determine the prevalence of infection in seroepidemiological studies.

iv) To support eradication programmes and subsequent surveillance.

v) For research purposes, for instance, the evaluation of the antibody response after vaccination and challenge infection.

Virus neutralisation (VN) tests (Bitsch, 1978) and various ELISAs (Kramps et al., 1993) are usually used for detecting antibodies against BoHV-1 in serum. Because virus latency is a normal sequel to BoHV-1 infection, the identification of serologically positive animals provides a useful and reliable indicator of infection status. Any animal with antibodies to the virus is considered to be a carrier and potential intermittent excretor of the virus. The only exceptions are calves that have acquired passive colostral antibodies from their dam, and noninfected cattle vaccinated with inactivated vaccines. There is also a risk that calves infected under cover of maternal immunity may become serologically negative while carrying a reactivatable latent infection (Lemaire et al., 2000).

In general, BoHV-1 serological tests can be divided into conventional and marker tests. Up to now, the only serological marker tests available are the BoHV-1 gE-antibody blocking ELISAs (Van Oirschot et al., 1997). Animals vaccinated with gE-deleted marker vaccines can be discriminated from field-virus infected animals by a negative serological reaction for gE. For conventional serology, VNT, BoHV1-antibody blocking ELISAs indirect ELISAs may be used.
ELISAs, including the gE-ELISA, are increasingly used for the detection of antibodies in (bulk) milk samples (Wellenberg et al., 1998a), but have some limitations. By testing bulk milk, a positive gB-specific test indicates that the infection has already spread in the herd (Frankena et al., 1997). With the gE-blocking ELISA, bulk milk gives a positive reaction when more than 10–15% of the herd is infected ((Wellenberg et al., 1998b). Consequently, it is not possible to declare a herd to be free from BoHV-1 infection with these tests on the basis of bulk or pooled milk samples, and a negative gE- or gB-ELISA bulk milk test should be followed up with individual blood samples from all cattle in the herd. However, indirect ELISAs optimised for use with bulk milk samples of up to 50 individual cows can indicate reliably the BoHV-1 status of these animals. For general surveillance purposes, bulk milk tank tests can give an estimate of BoHV-1 prevalence in a herd, an area or country (Nylin et al., 2000). These should be supplemented by serum testing (individual or pooled) from non-milking herds. For monitoring a BoHV-1 status in dairy herds, bulk milk samples of up to 50 animals should be tested 3–4 times per year with a suitable indirect ELISA. In herds of more than 50 cows, several bulks of milk from up to 50 animals should be tested. Positive bulk milk results have to be confirmed by testing individual blood samples from all animals included in a positive bulk milk sample.

In an extensive study, tests for the detection of antibodies as routinely used by national reference laboratories in Europe were evaluated (Kramps et al., 2004). Twelve reference laboratories from 12 European countries participated in this study. Fifty three serum samples and 13 milk samples, originating from several countries, were sent in duplicate under code to the participating laboratories. The serum samples included the three European reference sera EU1 (antibody positive), EU2 (antibody weak positive and defined as borderline sample) and EU3 (antibody negative) (Perrin et al., 1994). It was concluded that VNT and gB-specific ELISAs are the most sensitive tests for the detection of antibodies in serum. Owing to the very high sensitivity of the gB-blocking ELISAs, gB-antibody weak positive results can often not be confirmed by alternative test systems (indirect ELISA, VNT). Recently, new indirect BoHV-1 ELISAs have been developed that are highly sensitive and specific. The results of these ELISAs are comparable with those obtained using gB-blocking ELISAs (Beer et al., 2003).

gE-ELISAs are less sensitive and specific than the conventional test systems. In addition, seroconversion against gE can be delayed, especially in vaccinated animals, and is often not detectable before day 21 to 35 post-infection. Furthermore, second generation indirect ELISAs were found to be the most sensitive tests for the detection of BoHV-1-specific antibodies in milk. Moreover, it has observed that commercial ELISAs perform better than home-made ELISAs.

a) Virus neutralisation (a prescribed test for international trade)

VN tests are performed with various modifications. Tests vary with regard to the virus strain used in the test protocol, the starting dilution of the serum, the virus/serum incubation period (1–24 hours), the type of cells used, the day of final reading and the reading of the end-point (50% versus 100%) (Perrin et al., 1993). Among these variables, the virus/serum incubation period has the most profound effect on the sensitivity of the VNT. A 24-hour incubation period may score up to 16-fold higher antibody titres than a 1-hour incubation period (Bitsch, 1978), and is recommended where maximum sensitivity is required (e.g. for international trade purposes). Various bovine cells or cell lines are suitable for use in the VN test, including secondary bovine kidney or testis cells, cell strains of bovine lung or tracheal cells, or the established Madin–Darby bovine kidney (MDBK) cell line.

A suitable protocol for a VN test is shown below.

i) Inactivate sera, including control standard sera, for 30 minutes in a water bath at 56°C.

ii) Make doubling dilutions of test sera in cell culture medium. Start with undiluted serum and continue to 1/1024 horizontally in a 96-well flat-bottomed cell-culture grade microtitre plate, at least three wells per dilution and 50 µl volumes per well. Dilutions of a positive control serum, and of weak positive and negative internal control sera, are also included in the test. An extra well with undiluted test serum is used for toxicity control of sera.

iii) Add 50 µl per well of BoHV-1 stock at a dilution in culture medium calculated to provide 100–200 TCID_{50} per well. In the toxicity control wells, add 50 µl of culture medium in place of virus. Add 100 µl of culture medium to ten empty wells for cell controls.

iv) Make at least four tenfold dilutions of the residual virus stock (back titration) in culture medium, using 50 µl per well and at least four wells per dilution.

v) Incubate the plates for 24 hours at 37°C.

vi) Add 100 µl per well of the cell suspension at 3 × 10^4 cells per well.

vii) Incubate the plates for 3–5 days at 37°C.

viii) Read the plates microscopically for CPEs. Validate the test by checking the back titration of virus (which should give a value of 100 TCID_{50} with a permissible range of 30–300 TCID_{50}), the control sera
and the cell control wells. The positive control serum should give a titre of ± 1 twofold dilution (±0.3 log₁₀ units) from its target value. The weak positive serum should be positive. The negative serum should give no neutralisation (equivalent to a final dilution of 1/2 at the neutralisation stage). In the cell control wells, the monolayers should be intact.

ix) The test serum results are expressed as the reciprocal of the dilution of serum that neutralised the virus in 50% of the wells. If 50% of the wells with undiluted serum neutralised the virus, the (initial dilution) titre is read as 1 (1/2 using the final dilution convention). If all the undiluted and 50% of the wells with 1/2 diluted serum neutralised the virus, the (initial dilution) titre is 2 (final dilution 1/4). For qualitative results, any neutralisation at a titre of 1 or above (initial dilution convention) is considered to be positive. If cytotoxicity is observed in the control wells, the sample is reported to be toxic (no result) unless neutralisation of the virus without cytotoxicity is observed at higher dilutions and a titre can be read without ambiguity. Where cytotoxicity of a serum interferes with the interpretation of the neutralising activity of the sample, changing the medium in the wells of the lowest two or three dilutions 16–24 hours after the addition of cells may remove the cytotoxic effects.

b) Enzyme-linked immunosorbert assay (a prescribed test for international trade)

ELISAs for the detection of antibody against BoHV-1 appear to be gradually replacing VN tests. A standard procedure for ELISA has not been established. Several types of ELISA are commercially available, including indirect and blocking ELISAs, some of which are also suitable for detecting antibodies in milk (Kramps et al., 2004). For reasons of standardisation in a country or state, it may be desirable to compare the quality of the kits and to perform batch release tests by previously defined criteria in the national reference laboratory, before it is used by other laboratories in the country.

There are a number of variations in the ELISA procedures. The most common are: antigen preparation and coating, the dilution of the test sample, the incubation period of antigen and test sample, and the substrate/chromogen solution. Before being used routinely, an ELISA should be validated with respect to sensitivity, specificity and reproducibility (see chapter 1.1.5). For this purpose, a comprehensive panel of well defined (e.g. by VN test) strong positive, weak positive and negative sera has to be tested. However, it is recommended to use commercially available ELISAs that have been shown to perform better than homemade assays (Kramps et al., 2004).

- **Indirect enzyme-linked immunosorbert assay**

The principle of an indirect ELISA is based on the binding of BoHV-1-specific antibodies present in the test sample to immobilised BoHV-1 antigen. The bound antibodies are detected using enzyme-labelled ant bovine immunoglobulin antiserum. The presence of antibodies in the test sample will result in colour development after addition of the substrate/chromogen solution.

- **Blocking enzyme-linked immunosorbert assay**

The principle of a blocking or competitive ELISA is based on blocking the binding of an enzyme-labelled BoHV-1 antiserum or anti-BoHV-1 MAb to immobilised antigen by antibodies in the test sample. The presence of antibodies in the test sample results in reduced colour development after addition of the substrate/chromogen solution. An example of a gB blocking ELISA procedure is given below:

i) Prepare the antigen by growing BoHV-1 in cell cultures. When extensive CPE is observed, cells and medium are frozen at −20°C. After thawing, the resulting cellular lysate is centrifuged for 4 hours at 8500 g. The virus-containing pellet is suspended in a small volume of phosphate buffered saline (PBS), cooled on ice and disrupted using an ultrasonic disintegrator. The antigen preparation is then centrifuged for 10 minutes at 800 g, and inactivated by adding detergent (final concentration of 0.5% Nonidet P 40). The antigen preparation is used at an appropriate dilution to coat the microtitre plates. Many alternative methods of antigen production are described in the published literature.

ii) Coat the microtitre plates with antigen by adding 100 µl of diluted antigen (in 0.05 M carbonate buffer, pH 9.6) to each well. Seal the plates with tape, incubate at 37°C overnight, and store at −20°C.

iii) Before the test is performed, wash the plates with 0.05% Tween 80. Add 100 µl negative serum (fetal calf serum, FCS), 100 µl of each of the serum test samples and 100 µl of positive, weak positive and negative control sera. Usually, serum samples are tested undiluted. Shake, seal the plates and incubate overnight at 37°C. With some ELISAs, it is necessary to heat sera for 30 minutes at 56°C before testing in order to avoid nonspecific responses.

iv) Wash the plates thoroughly and add 100 µl of an anti-BoHV-1-gB-monoconal antibody/horseradish peroxidase conjugate at a predetermined dilution, and incubate again for 1 hour at 37°C. The monoclonal antibody must be selected carefully for its specificity to gB of BoVH-1.
v) Wash the plates and add freshly prepared substrate/chromogen solution (e.g. 0.05 M citric acid buffer, pH 4.5, containing 2,2’-azino-bis-[3-ethylbenzothiazoline]-6-sulphonic acid [ABTS; 0.55 mg/ml] and a 3% solution of freshly added H₂O₂ [5 µl/ml]), and incubate for the appropriate time (1–2 hours at room temperature).

vi) Measure the absorbance of the plates on a microplate photometer at 405 nm.

vii) Calculate for each test sample the blocking percentage \[
\frac{(\text{OD FCS} - \text{OD test sample})}{\text{OD FCS}} \times 100\%
\]

vii) A test sample is considered to be positive if it has a blocking percentage of e.g. 50% of higher. The test is valid if the positive and weak positive control sera are positive and the negative control serum reacts negatively. The acceptable limits for control and cut-off values must be determined for the individual assay.

c) Standardisation

In each serological test, appropriate controls of strong positive, weak positive and negative serum should be included. A scientific group in Europe, initiated by the group of artificial insemination veterinarians of the European Union (EU), has agreed on the use of a strong positive (EU1), a weak positive (EU2) and negative serum (EU3) for standardisation of BoHV-1 tests in laboratories that routinely examine samples from artificial insemination centres (Perrin et al., 1994). These sera have been adopted as OIE international standards for BoHV-1 tests and are available in limited quantities at the OIE Reference Laboratories for IBR/IPV. Prescribed tests for international trade purposes (VN or ELISA) must be capable of scoring both the strong and weak positive standards (or secondary national standards of equivalent potency) as positive. Because of the limited availability of the international standard sera, there is a need to prepare a new extended panel of reference lyophilised serum (and milk) samples taken from infected as well as from vaccinated animals. This panel should be used to validate newly developed tests and to harmonise tests between laboratories. Additional reference sera are available in limited quantities from the OIE Reference Laboratories (e.g. R1, R2 and R3 as positive, weak positive and very weak positive standard sera from the OIE Reference Laboratory in Germany).

d) Nonspecific reactivity in BoHV-1-serology and ‘pseudo-vaccines’

Nonspecific reactivity of sera in the BoHV-1-ELISAs should be taken into consideration, and is more often seen for the marker test than for the conventional serology. There are several reasons for nonspecific reactions:

• Batch variation of the ELISA used;
• Samples were tested very early after collection (freshness phenomenon);
• Samples were collected within 4 weeks after vaccination (vaccination phenomenon);
• Bad sample quality (e.g. haemolysed samples).

Therefore the following measures should be considered:

• Validation of each test batch, and batch release tests have to be implemented;
• Samples should be stored at 4°C and should not be tested before 24–48 hours after sample collection;
• Samples should be subjected to a freeze–thaw cycle (~20°C) and to subsequent heat inactivation (30 minutes/56°C);
• Cattle should not be serologically tested for BoHV-1 prior to 4 weeks after any vaccination;
• gE-ELISAs should not be used for classification of unvaccinated animals.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Background

a) Rationale and intended use of the product

Several attenuated and inactivated BoHV-1 vaccines are currently available. The vaccine strains have usually undergone multiple passages in cell culture. Some of the vaccine virus strains have a temperature-
sensitive phenotype, i.e. they do not replicate at temperatures of 39°C or higher. Attenuated vaccines are administered intranasally or intramuscularly. Inactivated vaccines contain high levels of inactivated virus or portions of the virus particle (glycoproteins) supplemented with an adjuvant to stimulate an adequate immune response. Inactivated vaccines are given intramuscularly or subcutaneously. Vaccination against BoHV-1 is used to protect animals from the clinical outcome of infection, and as an aid in control and eradication programmes.

Marker or DIVA (differentiation of infected from vaccinated animals) vaccines are now available in various countries. These attenuated or inactivated marker vaccines are based on deletion mutants (deletion of gE) or on a subunit of the virion, for instance glycoprotein D. The use of such marker vaccines in conjunction with companion diagnostic tests allows the distinction between infected and vaccinated cattle (DIVA principle), and provides the basis for BoHV-1 eradication programmes in countries or regions with a high prevalence of field-virus infected animals. Intensive vaccination programmes can reduce the prevalence of infected animals (Bosch et al., 1998; Mars et al., 2001), which could be monitored by using an appropriate diagnostic test. In situations where it is economically justifiable, the residual infected animals could be slaughtered, resulting in a region free from BoHV-1. Control and eradication of BoHV-1 was started in some countries in the early 1980s. Different policies have been used due to differences in herd prevalence, breeding practices and disease eradication strategies. To date, in the European Union, only gE-deleted DIVA vaccines (live as well as killed) have been marketed and used for control or eradication programmes. As there is no proven advantage of conventional non-marker BoHV-1 vaccines, gE-deleted marker vaccines (live or inactivated) should be the vaccines of choice.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are of general nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed

i) Biological characteristics

The vaccine is prepared using a seed-lot system. Origin, passage history and storage conditions of the master seed virus (MSV) must be recorded. A virus identity test must be performed on the MSV. The seed lot contains BoHV-1 strains have to be attenuated to yield a live vaccine strain. The strains can be attenuated by multiple passages in cell cultures, by adapting virus to grow at low temperatures (temperature-sensitive mutants), or by genetic engineering, for example, by deleting one or more viral genes (e.g. the BoHV-1 glycoprotein E) that are nonessential for replication. There should be some means of distinguishing the live vaccine virus from field viruses (for example temperature-specific growth patterns or restriction fragment length polymorphisms). Strains used for the preparation of inactivated vaccines need not be attenuated. The seed lot must be free from contaminants.

ii) Quality criteria (sterility, purity, freedom from extraneous agents)

The seed lot is tested for absence of extraneous viruses and absence from contamination with bacteria, fungi or mycoplasma. The following extraneous viruses should be specifically excluded in BoHV-1 vaccines: adenovirus, Akabane virus, bovine coronavirus, bovine herpesviruses 2, 4 and 5, bovine parvovirus, bovine respiratory syncytial virus, bovine viral diarrhoea virus and atypical pestiviruses, bovine rotavirus, vaccinia virus, and the viruses of Aujeszky's disease, bluetongue, bovine ephemeral fever, bovine leukaemia, bovine papilloma, bovine papular stomatitis, cowpox, foot and mouth disease, lumpy skin disease, malignant catarhhal fever, parainfluenza 3, rabies, rinderpest, and vesicular stomatitis. As bovine viral diarrhoea virus (either CPE and/or non-CPE) has regularly been found to be a contaminant of vaccines, special attention should be paid to the absence of BVDV. In addition, new atypical pestiviruses (HoBi or HoBi-like) have to be taken into consideration as possible contaminants.

b) Method of manufacture

i) Procedure

The cells used for vaccine production are prepared using a seed-lot system. The virus should be cultured on established cell lines that have been shown to be suitable for vaccine production, for example the Madin–Darby bovine kidney (MDBK) cell line. The history of the cell line must be known. The cell line must be free from extraneous agents and may be tested for tumorigenicity.

ii) Requirements for substrates and media

All substances used for the manufacture of vaccines must be free from contaminants. Cells should be used that are not further than 20 passages from the master cell seed. The seed virus should not be
more than five passages from the MSV. Genetically engineered vaccine virus strains are treated in the same way as conventionally attenuated vaccine virus strains. When sufficient cells are grown, infection of the cell line with the vaccine virus takes place. The addition of antibiotics is normally restricted to cell culture fluids. The supernatant fluid is harvested at times when the virus (antigen) production peaks. For live vaccines, the supernatant is clarified, mixed with a stabiliser, freeze-dried and bottled. For the production of classical inactivated vaccines, the supernatant is homogenised before the inactivating agent is added in order to ensure proper inactivation. After the inactivation procedure, a test for ensuring complete inactivation of the virus is carried out. The test should include at least two passages in cells. The inactivated virus suspension is then mixed with an adjuvant and bottled. The manufacture of vaccines must comply with guidelines for Good Manufacturing Practice (GMP).

iii) In-process controls

Working cell seed and working virus seed must have been shown to be free from contaminants. The cells must show inconspicuous morphology before being inoculated with virus. The CPE is checked during cultivation. Uninoculated control cells must have retained their morphology until the time of harvesting. A virus titration is performed on the harvested supernatant. During the production of inactivated vaccines, tests are performed to ensure inactivation. The final bulk must be tested for freedom from contaminants.

iii) Final product batch tests

The following tests must normally be performed on each batch. Example guidelines for performing batch control can be found in EU directives, the European Pharmacopoeia and the United States Department of Agriculture’s Code of Federal Regulations.

Sterility/purity

Bacteria, fungi, mycoplasma and extraneous viruses must not be present. Tests for sterility and freedom from contamination of biological material may be found in chapter 1.1.7.

Safety

For inactivated vaccines, a twofold dose of vaccine, and for live vaccines, a tenfold dose of vaccine, must not produce adverse effects in young BoHV-1 seronegative calves.

Batch potency

It is sufficient to test one representative batch for efficacy, as described in Section C.1.c.ii. In the case of live vaccines, the virus titre of each batch must be determined and must be not higher than 1/10 of the dose at which the vaccine has been shown to be safe, and no lower than the minimum release titre. In the case of inactivated vaccines, the potency is tested using another validated method, for instance, efficacy assessment in calves.

c) Requirements for authorisation

i) Safety requirements

Target and non-target animal safety

A quantity of virus equivalent to ten doses of vaccine should (a) not induce significant local or systemic reactions in young calves; (b) not cause fetal infection or abortion, and (c) not revert to virulence during five serial passages in calves. For inactivated vaccine, a double dose is usually administered. The reversion to virulence test is not applicable to inactivated vaccines.

Reversion-to-virulence for attenuated/live vaccines

The selected final vaccine strain should not revert to virulence during a minimum of five serial passages in calves.

Environmental consideration

Attenuated vaccine strains should not be able to perpetuate autonomously in a cattle population (R0 <1).

ii) Efficacy requirements

For animal production

This must be shown in vaccination challenge experiments under laboratory conditions. Example guidelines are given in a monograph of the European Pharmacopoeia (Third Edition (1997). Briefly, the vaccine is administered to ten 2–3-month-old BoHV-1 seronegative calves. Two calves are kept as
controls. All the calves are challenged intranasally 3 weeks later with a virulent strain of BoHV-1 that gives rise to typical clinical signs of a BoHV-1 infection. The vaccinated calves should show no or only very mild signs. The maximum (peak) virus titre in the nasal mucus of vaccinated calves should be at least 100 times lower than that in control calves. The virus excretion period should be at least 3 days shorter in vaccinated than in control calves.

An efficacious BoHV-1 vaccine should induce protective immunity for at least 1 year, although many existing vaccines have not been tested to this standard.

For control and eradication

In addition to the above-mentioned criteria, BoHV-1-vaccines for control and eradication should be marker vaccines (e.g. gE-deleted vaccines) allowing the differentiation of infected from vaccinated animals (DIVA-strategy). Several gE-deleted vaccines (inactivated preparations as well as modified live vaccines) are commercially available.

iii) Stability

For live vaccines, virus titrations should be carried out 3 months beyond the indicated shelf life. In addition, tests for determining moisture content, concentrations of preservatives, and pH are performed. For inactivated vaccines, the viscosity and stability of the emulsion are also tested.

The efficacy of preservatives should be demonstrated. The concentration of the preservative and its persistence throughout shelf life should be checked. The concentration must be in conformity with the limits set for the preservative.

3. Vaccines based on biotechnology

a) Vaccines available and their advantages

There is a glycoprotein E (gE)-deleted inactivated vaccine available which is based on a recombinant strain. The vaccine is comparable to other gE-deleted vaccines and is licensed by European Medicines Agency (EMA) for use in the European Union.

Additional recombinant vaccines like gD-subunits or genetically engineered deletion mutants of BoHV-1 (e.g. with deletions of gE and/or gG) are described and available as prototypes.

Advantages of BoHV-1 vaccines based on biotechnology could be the possibility of additional marker features for the differentiation of infected from vaccinated animals (DIVA; e.g. gB-antibody-ELISAs for gD-subunit vaccines or gG-antibody-ELISAs for the respective deletion mutants).

b) Special requirements for biotechnological vaccines, if any

Recombinant vaccines, which are destined for use in the European Union have to be licensed by EMA.

REFERENCES


Chapter 2.4.13. — Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis


* * *

NB: There are OIE Reference Laboratories for Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for infectious bovine rhinotracheitis/infectious pustular vulvovaginitis.
CHAPTER 2.4.14.

LUMPY SKIN DISEASE

SUMMARY

Lumpy skin disease (LSD, knopvelsiekte) is a pox disease of cattle characterised by fever, nodules on the skin, mucous membranes and internal organs, emaciation, enlarged lymph nodes, oedema of the skin, and sometimes death. The disease is of economic importance as it can cause a temporary reduction in milk production, temporary or permanent sterility in bulls, damage to hides and death due to secondary bacterial infections. Various strains of capripoxvirus are responsible for the disease. These are antigenically indistinguishable from strains causing sheep pox and goat pox yet distinct at the genetic level. LSD has a partially different geographical distribution from sheep and goat pox, suggesting that cattle strains of capripoxvirus do not infect and transmit between sheep and goats. Transmission of LSD virus is thought to be predominantly by insects, natural contact transmission in the absence of insect vectors being inefficient. Lumpy skin disease occurs in most African countries. The most recent outbreaks outside Africa occurred in the Middle East in 2006 and 2007 and in Mauritius in 2008.

Identification of the agent: Laboratory confirmation of LSD is most rapid using a polymerase chain reaction (PCR) method specific for capripoxviruses or by the demonstration of typical capripox virions in biopsy material or desiccated crusts using the transmission electron microscope in combination with a clinical history of a generalised nodular skin disease and enlarged superficial lymph glands in cattle. Capripoxvirus is distinct from parapoxvirus, which causes bovine papular stomatitis and pseudocowpox, but cannot be distinguished morphologically from cowpox and vaccinia virus, both orthopoxvirus infections of cattle. Neither of these, however, causes generalised infection and both are uncommon in cattle. LSD virus will grow in tissue culture of bovine, ovine or caprine origin, although maximum yield is obtained using lamb testis or bovine dermis cells. Capripoxvirus causes a characteristic cytopathic effect and intracytoplasmic inclusion bodies, and is distinct from the virus of pseudo-LSD (Allerton – herpes mammilitis), which is a herpesvirus producing syncytia and intranuclear inclusion bodies. The antigen of capripoxvirus can be demonstrated in tissue culture using immunoperoxidase or immunofluorescent staining and the virus can be neutralised using specific antisera.

An antigen-detection enzyme-linked immunosorbent assay (ELISA) using a polyclonal detection serum raised against a recombinant immunodominant antigen of capripoxvirus and an antibody-detecting ELISA based on the purified whole virus have been described. Genome detection using capripoxvirus-specific primers for the fusion protein gene and attachment protein gene has been reported and several conventional and real-time PCR methods have been published for use on blood, tissue and semen samples.

Serological tests: The virus neutralisation test is the most specific serological test, but because immunity to LSD infection is predominantly cell mediated, the test is not sufficiently sensitive to identify animals that have had contact with LSD virus and developed only low levels of neutralising antibody. The agar gel immunodiffusion test and indirect immunofluorescent antibody test are less specific due to cross-reactions with antibody to other poxviruses. Western blotting using the reaction between the P32 antigen of LSD virus with test sera is both sensitive and specific, but is difficult and expensive to carry out. The use of this or another appropriate antigen, expressed by a suitable vector, in an ELISA offers the prospect of an acceptable and standardised serological test.

Requirements for vaccines: All strains of capripoxvirus examined so far, whether derived from cattle, sheep or goats, share immunising antigens. Attenuated cattle strains, and strains derived from sheep and goats have been used as live vaccines.
A. INTRODUCTION

Lumpy skin disease (LSD) was first seen in Zambia in 1929, spreading into Botswana by 1943 (Haig, 1957), and then into South Africa, where it affected over eight million cattle causing major economic loss. In 1957 it entered Kenya, associated with an outbreak of sheep pox (Weiss, 1968). In 1970 LSD spread north into the Sudan, by 1974 it had spread west as far as Nigeria, and in 1977 was reported from Mauritania, Mali, Ghana and Liberia. Another epizootic of LSD between 1981 and 1986 affected Tanzania, Kenya, Zimbabwe, Somalia and the Cameroon, with reported mortality rates in affected cattle of 20%. However, the true extent of this epizootic was not clear, and it probably affected a considerable area of central Africa. Lumpy skin disease outbreaks tend to be sporadic, depending upon animal movements, immune status, and wind and rainfall patterns affecting vector populations. For example the recurrence of outbreaks in Egypt and Israel after an absence of 17 years could be attributed to a combination of these factors (Brenner et al., 2006). LSD must be considered to have the potential to become established outside Africa. The principle method of transmission is mechanical by arthropod vector (Carn & Kitching, 1995a; Chihota et al., 2006). The principle method of transmission is mechanical by arthropod vector (Carn & Kitching, 1995a; Chihota et al., 2006).

The severity of clinical signs of LSD (Neethling virus infection or “knopvelsiekte”), depends on the strain of capripoxvirus and the host cattle breed. Bos taurus is more susceptible to clinical disease than Bos indicus; the Asian buffalo has also been reported to be susceptible. Within Bos taurus, the fine-skinned Channel Island breeds develop more severe disease, with lactating cows appearing to be the most at risk. However, even among groups of cattle of the same breed kept together under the same conditions, there is a large variation in the clinical signs presented, ranging from subclinical infection to death (Carn & Kitching, 1995b). There may be failure of the virus to infect the whole group, probably depending on the virulence of the virus isolate, immunological status of the host and vector prevalence.

The incubation period under field conditions has not been reported, but following inoculation is 6–9 days until the onset of fever. In the acutely infected animal, there is an initial pyrexia, which may exceed 41°C and persist for 1 week. All the superficial lymph nodes become enlarged. In lactating cattle there is a marked reduction in milk yield. In severe cases, nodules of 2–5 cm in diameter develop over the body, particularly on the head, neck, udder and perineum between 7 and 19 days after virus inoculation (Coetzer, 2004). These nodules involve the dermis and epidermis and may initially exude serum, but over the following 2 weeks necrotic plugs may appear penetrating the full thickness of the hide. At the appearance of clinical signs, the discharge from the eyes and nose becomes mucopurulent, and keratitis may develop. Nodules may also develop in the mucous membranes of the mouth and alimentary tract, particularly the abomasum and in the trachea and the lungs, resulting in primary and secondary pneumonia. The nodules on the mucous membranes of the eyes, nose, mouth, rectum, udder and genitalia quickly ulcerate, and by then all secretions, ocular and nasal discharge and saliva contain LSD virus. The limbs may be oedematous and the animal is reluctant to move. Pregnant cattle may abort, and there are reports of aborted fetuses being covered in nodules. Bulls may become permanently or temporarily infertile and the virus can be excreted in the semen for prolonged periods (Irons et al., 2005). Recovery from severe infection is slow; the animal is emaciated, may have pneumonia and mastitis, and the necrotic plugs of skin, which may have been subject to fly strike, are shed leaving deep holes in the hide (Prozesky & Barnard, 1982).

LSD virus is not transmissible to humans.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

• Sample collection, submission and preparation

Material for virus isolation and antigen detection should be collected by biopsy or at post-mortem from skin nodules, lung lesions or lymph nodes. Samples for virus isolation and antigen-detection enzyme-linked immunosorbent assay (ELISA) should be collected within the first week of the occurrence of clinical signs, before the development of neutralising antibodies (Davies, 1991; Davies et al., 1971). Samples for genome detection by polymerase chain reaction (PCR) may be collected when neutralising antibody is present. Following the first appearance of the skin lesions, the virus can be isolated for up to 35 days and viral nucleic acid can be demonstrated by PCR for up to 3 months (Tuppurainen et al., 2005; Weiss, 1968). Buffy coat from blood collected into heparin or EDTA (ethylene diamine tetra-acetic acid) during the viraemic stage of LSD (before generalisation of lesions or within 4 days of generalisation), can also be used for virus isolation. Samples for histology should include tissue from the surrounding area and should be placed immediately following collection into ten times the sample volume of 10% formalin.

Tissues in formalin have no special transportation requirements. Blood samples with anticoagulant for virus isolation from the buffy coat should be placed immediately on ice and processed as soon as possible. In practice, the samples may be kept at 4°C for up to 2 days prior to processing, but should not be frozen or kept at ambient conditions.
temperatures. Tissues for virus isolation and antigen detection should be kept at 4°C, on ice or at –20°C. If it is necessary to transport samples over long distances without refrigeration, the medium should contain 10% glycerol; the samples should be of sufficient size that the transport medium does not penetrate the central part of the biopsy, which should be used for virus isolation.

Material for histology should be prepared by standard techniques and stained with haematoxylin and eosin (H&E) (Burdin, 1959). Lesion material for virus isolation and antigen detection is minced using sterile scalpel blade and forceps and then ground with a pestle in a sterile mortar with sterile sand and an equal volume of sterile phosphate buffered saline (PBS) containing sodium penicillin (1000 international units [IU]/ml), streptomycin sulphate (1 mg/ml), mycostatin (100 IU/ml) or fungizone (amphotericin, 2.5 µg/ml) and neomycin (200 IU/ml). The suspension is freeze–thawed three times and then partially clarified by centrifugation using a bench centrifuge at 600 g for 10 minutes. In cases where bacterial contamination of the sample is expected (such as when virus is isolated from skin samples), the supernatant can be filtered through a 0.45 µm pore size filter after the centrifugation step. Buffy coats may be prepared from unclotted blood by centrifugation at 600 g for 15 minutes, and the buffy coat carefully removed into 5 ml of cold double-distilled water using a sterile Pasteur pipette. After 30 seconds, 5 ml of cold double-strength growth medium is added and mixed. The mixture is centrifuged at 600 g for 15 minutes, the supernatant is discarded and the cell pellet is suspended in 5 ml of fresh GMEM. Alternatively, the buffy coat may be separated from a heparinised sample by using a Ficoll gradient.

a) Virus isolation on cell culture

LSD virus will grow in tissue culture of bovine, ovine or caprine origin, although primary or secondary culture of bovine dermis cells or lamb testis (LT) cells are considered to be the most susceptible, particularly those derived from a breed of wool sheep. One ml of clarified supernatant or buffy coat, is inoculated on to a 25 cm² culture flask at 37°C and allowed to absorb for 1 hour. The culture is then washed with warm PBS and covered with 10 ml of a suitable medium, such as GMEM, containing antibiotics and 2% fetal calf serum. If available, tissue culture tubes containing LT cells and a flying cover-slip, or tissue culture microscope slides, are also infected.

The flasks are examined daily for 7–14 days for evidence of cytopathic effect (CPE). Contaminated flasks should be discarded. Infected cells develop a characteristic CPE consisting of retraction of the cell membrane from surrounding cells, and eventually rounding of cells and margination of the nuclear chromatin. At first only small areas of CPE can be seen, sometimes as soon as 2 days after infection; over the following 4–6 days these expand to involve the whole cell sheet. If no CPE is apparent by day 14, the culture should be freeze–thawed three times, and clarified supernatant inoculated on to fresh LT culture. At the first sign of CPE in the flasks, or earlier if a number of infected cover-slips are being used, a cover-slip should be removed, fixed in acetone and stained using H&E. Eosinophilic intracytoplasmic inclusion bodies, which are variable in size but up to half the size of the nucleus and surrounded by a clear halo, are diagnostic for poxvirus infection. The CPE can be prevented or delayed by inclusion in the medium of specific anti-LSD serum. The herpesvirus of pseudo-LSD produces a Cowdry type A intranuclear inclusion body. Formation of syncytia is not usually a feature of capripoxvirus infection (although they may be seen in Madin-Darby bovine kidney [MDBK] cells), unlike the herpesvirus causing pseudo-LSD.

Strains of capripoxvirus that cause LSD have been adapted to grow on the chorioallantoic membrane of embryonated chicken eggs and African green monkey kidney (Vero) cells. This is not recommended for primary isolation. Ovine testis secondary cell line (OA3.Ts) has been evaluated for the propagation of capripoxvirus isolates (Babiuk et al., 2007).

b) Electron microscopy

Before centrifugation, material from the original biopsy suspension is prepared for examination under the transmission electron microscope by floating a 400-mesh hexagon electron microscope grid, with pieoform-carbon substrate activated by glow discharge in pentyamine vapour, on to a drop of the suspension placed on paraffin or a wax plate. After 1 minute, the grid is transferred to a drop of Tris/EDTA buffer, pH 7.8, for 20 seconds and then to a drop of 1% phosphotungstic acid, pH 7.2, for 10 seconds. The grid is drained using filter paper, air-dried and placed in the electron microscope. The capripox virion is brick shaped, covered in short tubular elements and measures approximately 290 × 270 nm. A host-cell-derived membrane may surround some of the virions, and as many as possible should be examined to confirm their appearance (Kitching & Smale, 1986).

The virions of capripoxvirus are indistinguishable from those of orthopoxvirus, but, apart from vaccinia virus and cowpox virus, which are both uncommon in cattle and do not cause generalised infection, no other orthopoxvirus causes lesions in cattle. However, vaccinia virus may cause generalised infection in young immunocompromised calves. In contrast, orthopoxviruses are a common cause of skin disease in domestic buffalo causing buffalo pox, a disease that usually manifests as pock lesions on the teats, but may cause...
skin lesions at other sites, such as the perineum, the medial aspects of the thighs and the head. Orthopoxviruses that cause buffalo pox cannot be readily distinguished from capripoxvirus by electron microscopy. The virions of parapoxvirus that cause bovine papular stomatitis and pseudocowpox are smaller, oval in shape and each is covered in a single continuous tubular element that appears as striations over the virion. The capripoxvirus is also distinct from the herpesvirus that causes pseudo-LSD (Allerton – herpes mammillitis).

**c) Fluorescent antibody tests**

Capripoxvirus antigen can also be identified on the infected cover-slips or tissue culture slides using fluorescent antibody tests. Cover-slips or slides should be washed and air-dried and fixed in cold acetone for 10 minutes. The indirect test using immune cattle sera is subject to high background colour and nonspecific reactions. However, a direct conjugate can be prepared from sera from convalescent cattle (or from sheep or goats convalescing from capripox) or from rabbits hyperimmunised with purified capripoxvirus. Uninfected tissue culture should be included as a negative control as cross-reactions can cause problems due to antibodies to cellular components.

**d) Agar gel immunodiffusion**

An agar gel immunodiffusion (AGID) test has been used for detecting the precipitating antigen of capripoxvirus, but has the disadvantage that this antigen is shared by parapoxvirus. Agarose (1%) is prepared in borate buffer, pH 8.6, dissolved by heating, and 2 ml is poured on to a glass microscope slide (76 × 26 mm). When the agar has solidified, wells are cut to give a six-well rosette around a central well. Each well is 5 mm in diameter, with a distance of 7 mm between the middle of the central well and the middle of each peripheral well. The wells are filled as follows: 18 µl of the 10% lesion suspension is added to three of the peripheral wells, alternately with positive control antigen, and 18 µl of positive capripoxvirus control serum is added to the central well. The slides are placed in a humid chamber at room temperature for 48 hours, and examined for visible precipitation lines using a light box. The test material is positive if a precipitation line develops with the control serum that is confluent with that produced by the positive control antigen.

To prepare antigen for the AGID, one of two 125 cm² flasks of LT cells is infected with capripoxvirus, and harvested when there is 90% CPE (8–12 days). The flask is freeze–thawed twice, and the cells are shaken free of the flask. The contents are centrifuged at 4000 g for 15 minutes, most of the supernatant is decanted and stored, and the pellet is re-suspended in the remaining supernatant. The cells should be lysed using an ultrasonic probe for approximately 60 seconds. This homogenate is then centrifuged as before, the resulting supernatant being pooled with that already collected. The pooled supernatant is then added to an equal volume of saturated ammonium sulphate at pH 7.4 and left at 4°C for 1 hour. This solution is centrifuged at 4000 g for 15 minutes, and the precipitate is collected and resuspended in a small volume of 0.8% saline for use in the AGID test. The uninfected flask is processed in an identical manner throughout, to produce a tissue culture control antigen (Kitching et al., 1986).

**e) Enzyme-linked immunosorbent assay**

Following the cloning of the highly antigenic capripoxvirus structural protein P32, it is possible to use expressed recombinant antigen for the production of diagnostic reagents, including the raising of P32 monospecific polyclonal antiserum and the production of monoclonal antibodies (MAbs) (Carn, 1995). Using hyperimmune rabbit antiserum, raised by inoculation of rabbits with purified capripoxvirus, capripox antigen from biopsy suspensions or tissue culture supernatant can be trapped on an ELISA plate. The presence of the antigen can then be indicated using guinea-pig serum, raised against the group-specific structural protein P32, commercial horseradish-peroxidase-conjugated rabbit anti-guinea-pig immunoglobulin and a chromogen/substrate solution.

**f) Polymerase chain reaction (PCR)**

The conventional gel-based PCR method described below is a simple, fast and sensitive method for the detection of capripoxvirus genome in EDTA blood, biopsy, semen or tissue culture samples. However, it does not allow differentiation between LSD and sheep and goat pox viruses. Primers for the viral attachment protein gene and the viral fusion protein gene (Ireland & Binepal, 1998) are specific for all the strains within the genus Capripoxvirus. By the use of sequence and phylogenetic analysis; strains of virus can be identified (Le Goff et al., 2009). Virus isolates can also be characterised by comparing the genome fragments generated by HindIII digestion of their purified DNA (Black et al., 1986; Kitching et al., 1989). This technique has identified differences between isolates from the different species, but these are not consistent and there is evidence of the movement of strains between species and recombination between strains in the field (Gershon & Black, 1987; Gershon et al., 1989). More recently, quantitative real-time PCR methods has been described that are reported to be faster and have higher sensitivity (Bowden et al., 2008; Balinsky et al., 2008).
The LSD virus genome contains 156 putative genes (Tulman et al., 2001). An example of a published conventional gel-based PCR method is described below (Tuppurainen et al., 2005).

- **Test procedure**

  The extraction method described below can be replaced using commercially available DNA extraction kits.

  i) Freeze and thaw 200 µl of blood in EDTA, semen or tissue culture supernatant and suspend in 100 µl of lysis buffer containing 5 M guanidine thiocyanate, 50 mM potassium chloride, 10 mM Tris/HCl (pH 8); and 0.5 ml Tween 20.

  ii) Cut skin and other tissue samples into fine pieces using sterile scalpel blade and forceps. Grind with a pestle in a mortar. Suspend the tissue samples in 800 µl of the above mentioned lysis buffer.

  iii) Add 2 µl of proteinase K (20 mg/ml) to blood samples and 10 µl of proteinase K to tissue samples. Incubate at 56°C for 2 hours or over night, followed by heating at 100°C for 10 minutes. Add two volumes of ice cold ethanol (100%) and 1/10 of 3 M sodium acetate (pH 5.3). Place the samples at −20°C for 1 hour. Centrifuge again at 16,060 g for 15 minutes at 4°C and discard the supernatant. Wash the pellets with ice cold 70% ethanol (100 µl) and centrifuge at 16,060 g for 1 minute at 4°C. Discard the supernatant and dry the pellets thoroughly. Suspend the pellets in 30 µl of nuclease-free water and store immediately at −20°C (Tuppurainen et al., 2005).

  iv) The primers were developed from the viral attachment protein encoding gene. The size of the expected amplicon is 192 bp (Ireland & Binepal, 1998). The primers have the following gene sequences:

  - Forward primer 5'-TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT-3'
  - Reverse primer 5'- TAT- GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC-3'.

  v) DNA amplification is carried out in a final volume of 50 µl containing: 5 µl of 10 × PCR buffer, 1.5 µl of MgCl₂ (50 mM), 1 µl of dNTP (10 mM), 1 µl of forward primer, 1 µl of reverse primer, 1 µl of DNA template (~10 ng), 0.5 µl of Taq DNA polymerase and 39 µl of nuclease-free water. The volume of DNA template required may vary and the volume of nuclease-free water must be adjusted to the final volume of 50 µl.

  vi) Run the samples in a thermal cycler: first cycle: 2 minutes at 95°C , second cycle: 45 seconds at 95°C, 50 seconds at 50°C and 1 minute at 72°C. Repeat the second cycle 34 times. Last cycle: 2 minutes at 72°C and hold at 4°C until analysis.

  vii) Mix 10 µl of each sample with loading dye and load onto a 1.5% agarose gel in TAE buffer (Tris/acetate buffer containing EDTA). Load a parallel lane with a 100 bp DNA-marker ladder. Separate the products approximately 8–10 V/cm for 40–60 minutes and visualise.

2. Serological tests

   All the viruses in the Capripoxvirus genus share a common major antigen for neutralising antibodies and it is thus not possible to distinguish strains of capripoxvirus from cattle, sheep or goats using serological techniques.

   a) **Virus neutralisation**

      A test serum can either be titrated against a constant titre of capripoxvirus (100 TCID₅₀ [50% tissue culture infective dose]) or a standard virus strain can be titrated against a constant dilution of test serum in order to calculate a neutralisation index. Because of the variable sensitivity of tissue culture to capripoxvirus, and the consequent difficulty of ensuring the use of 100 TCID₅₀, the neutralisation index is the preferred method although it does require a larger volume of test sera. The test is described using 96-well flat-bottomed tissue-culture grade microtitre plates, but it can be performed equally well in tissue culture tubes with the appropriate changes to the volumes used, although it is more difficult to read an end-point in tubes. The use of Vero cells in the virus neutralisation test has been reported to give more consistent results.

      - **Test procedure**

        i) Test sera including a negative and a positive control are diluted 1/5 in Eagle's/HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) and inactivated at 56°C for 30 minutes.

        ii) Next, 50 µl of the first inactivated serum is added to columns 1 and 2, rows A to H of the microtitre plate. The second serum is placed in columns 3 and 4, the third in columns 5 and 6, the positive control
serum is placed in columns 7 and 8, the negative control serum is placed in columns 9 and 10, and 50 µl of Eagle’s/HEPES without serum is placed in columns 11 and 12 and to all wells in row H.

iii) A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue culture, with a titre of over $\log_{10} 6$ TCID$_{50}$ per ml is diluted in Eagle’s/HEPES in bijoux bottles to give a log dilution series of $\log_{10} 5.0; 4.0; 3.5; 3.0; 2.5; 2.0; 1.5$ TCID$_{50}$ per ml (equivalent to $\log_{10} 3.7; 2.7; 2.2; 1.7; 1.2; 0.7; 0.2$ TCID$_{50}$ per 50 µl).

iv) Starting with row G and the most diluted virus preparation, 50 µl of virus is added to each well in that row. This is repeated with each virus dilution, the highest titre virus dilution being placed in row A.

v) The plates are covered and incubated for 1 hour at 37°C.

vi) LT cells are prepared from pregrown monolayers as a suspension of $10^5$ cells/ml in Eagle’s medium containing antibiotics and 2% fetal calf serum. Following incubation of the microtitre plates, 100 µl of cell suspension is added to all the wells, except wells H11 and H12, which serve as control wells for the medium. The remaining wells of row H are cell and serum controls.

vii) The microtitre plates are covered and incubated at 37°C for 9 days.

viii) Using an inverted microscope, the monolayers are examined daily from day 4 for evidence of CPE. There should be no CPE in the cells of row H. Using the O240 KSGP vaccine strain of capripoxvirus, the final reading is taken on day 9, and the titre of virus in each duplicate titration is calculated by the Kärber method. If left longer, there is invariably a ‘breakthrough’ of virus in which virus that was at first neutralised appears to disassociate from the antibody.

ix) Interpretation of the results: The neutralisation index is the log titre difference between the titre of the virus in the negative serum and in the test serum. An index of $\geq 1.5$ is positive. The test can be made more sensitive if serum from the same animal is examined before and after infection. Because the immunity to capripox is predominantly cell mediated, a negative result, particularly following vaccination in which the response is necessarily mild, does not imply that the animal from which the serum was taken is not protected.

A constant-virus/varying-serum method has been described using serum dilutions in the range 1/5 to 1/500 and fetal calf muscle cells. Because these cells have a lower sensitivity to capripoxvirus than LT cells, the problem of virus ‘breakthrough’ is overcome.

Antibodies to capripoxvirus can be detected from day 2 after the onset of clinical signs. These remain detectable for about 7 months, but a significant rise in titre is usually seen between days 21 and 42.

b) Agar gel immunodiffusion

The AGID test cannot be recommended as a serological test for the diagnosis of LSD because of the cross-reaction with antibody to bovine papular stomatitis and pseudocowpox virus. A consequence of this cross-reaction is false-positive results. Lack of sensitivity of the test can also lead to false-negative results.

c) Indirect fluorescent antibody test

Capripoxvirus-infected tissue culture grown on flying cover-slips or tissue culture microscope slides can be used for the indirect fluorescent antibody test. Uninfected tissue culture control, and positive and negative control sera, should be included in the test. The infected and control cultures are fixed in acetone at –20°C for 10 minutes and stored at 4°C. Dilutions of test sera are made in PBS, starting at 1/20 or 1/40, and positives are identified using an anti-bovine gamma-globulin conjugated with fluorescein isothiocyanate. Antibody titres may exceed 1/1000 after infection. Sera may be screened at 1/50 and 1/500. Cross-reactions can occur with orf (contagious pustular dermatitis of sheep virus), bovine papular stomatitis and perhaps other poxviruses.

d) Western blot analysis

Western blotting of test sera against capripoxvirus-infected cell lysate provides a sensitive and specific system for the detection of antibody to capripoxvirus structural proteins, although the test is expensive and difficult to carry out.

Capripoxvirus-infected LT cells should be harvested when 90% CPE is seen, freeze–thawed three times, and the cellular debris pelleted by centrifugation. The supernatant should be decanted, and the proteins should be separated by SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis). A vertical discontinuous gel system, using a stacking gel made up of acrylamide 5% in Tris (125 mM), pH 6.8, and SDS (0.1%), and a resolving gel made up of acrylamide (10–12.5%) in Tris (560 mM), pH 8.7, and SDS (0.1%), is recommended for use with a glycine running buffer containing Tris (250 mM), glycerine (2 M), and SDS (0.1%). Samples of supernatant should be prepared by boiling for 5 minutes with an appropriate lysis.
buffer prior to loading. Alternatively, purified virus or recombinant antigens may replace tissue-culture-derived antigen.

Molecular weight markers should be run concurrently with the protein samples. The separated proteins in the SDS/PAGE gel should be transferred electrophoretically to a nitrocellulose membrane (NCM). After transfer, the NCM is rinsed thoroughly in PBS and blocked in 3% bovine serum albumin (BSA) in PBS, or 5% skimmed milk powder in PBS, on a rotating shaker at 4°C overnight. The NCM can then be separated into strips by employing a commercial apparatus to allow the concurrent testing of multiple serum samples, or may be cut into strips and each strip incubated separately thereafter. The NCM is washed thoroughly with five changes of PBS for 5 minutes on a rotating shaker, and then incubated at room temperature on the shaker for 1.5 hours, with the appropriate serum at a dilution of 1/50 in blocking buffer (3% BSA and 0.05% Tween 20 in PBS; or 5% milk powder and 0.05% Tween 20 in PBS). The membrane is then washed and incubated (in blocking buffer) with anti-species immunoglobulin horseradish-peroxidase-conjugated immunoglobulins at a dilution determined by titration. After further incubation at room temperature for 1.5 hours, the membrane is washed and a solution of diaminobenzidin tetrahydrochloride (10 mg in 50 ml of 50 mm Tris/HCl, pH 7.5, and 20 µl of 30% [v/v] hydrogen peroxide) is added. This is then incubated for approximately 3–7 minutes at room temperature on a shaker with constant observation, and the reaction is stopped by washing in PBS before excessive background colour is seen. A positive and negative control serum should be used on each occasion.

Positive test samples and the positive control will produce a pattern consistent with reaction to proteins of molecular weights 67, 32, 26, 19 and 17 kDa – the major structural proteins of capripoxvirus – whereas negative serum samples will not react with this pattern. Hyperimmune serum prepared against parapoxvirus (bovine papular stomatitis, pseudocowpox) will react with some of the capripoxvirus proteins, but not the 32 kDa protein that is specific for capripoxvirus.

e) Enzyme-linked immunosorbent assay

An ELISA for the detection of antibodies against capripox virus has been developed using the expressed structural P32 protein (Cam et al., 1994; Heine et al., 1999). More recently, an ELISA based on recombinant capripox antigens (Bowden et al., 2009) and on inactivated, purified whole sheep pox virus (Babiuk et al., 2009) have been documented. Currently, no validated ELISA for the detection of antibodies against capripoxviruses is commercially available.

C. REQUIREMENTS FOR VACCINES

1. Background

a) Rationale and intended use of the product

Four live attenuated strains of capripoxvirus have been used as vaccines specifically for the control of LSD (Brenner et al., 2006; Capstick & Coakley, 1961; Carn, 1993): a strain of Kenyan sheep and goat pox virus passaged 18 times in LT or fetal calf muscle cells, Yugoslavian RM 65 sheep pox strain, Romanian sheep pox strain and lumpy skin disease virus strain from South Africa, passaged 60 times in lamb kidney cells and 20 times on the chorioallantoic membrane of embryonated chicken eggs. All strains of capripoxvirus examined so far, whether of bovine, ovine or caprine origin, share a major neutralising site, so that animals recovered from infection with one strain are resistant to infection with any other strain. Consequently, it is possible to protect cattle against LSD using strains of capripoxvirus derived from sheep or goats (Coakley & Capstick, 1961). In 1989 and 1990 the Romanian strain of sheep pox vaccine was used to help control the LSD outbreak in Egypt (Michael et al., 1996). However, it is essential to carry out controlled trials, particularly using the most susceptible breeds in peak lactation, prior to introducing a vaccine strain not usually used in cattle. Protection following vaccination is probably lifelong, although as immunity wanes, local capripoxvirus replication will occur at the site of inoculation, but the virus will not become generalised. All strains of capripoxvirus used routinely as vaccines can produce a large local reaction at the site of inoculation in Bos taurus breeds (Davies, 1991), which some stock owners find unacceptable. This has discouraged the use of vaccine even though the consequences of an outbreak of LSD are invariably more severe.

2. Outline of production and minimum requirements for conventional vaccines

General requirements set for the facilities used for the production of vaccines and for the documentation and record keeping throughout the whole manufacturing process are described in Chapter 1.1.6 Principles of veterinary vaccine production. The documentation should include standard operating procedures (SOP) for the method of manufacture and each step for the testing of cells and reagents used in the process, each batch and the final product.
a) Characteristics of the seed

i) Biological characteristics

Each seed strain of capripoxvirus used for vaccine production must be accompanied by records clearly and accurately describing its origin, isolation and tissue culture or animal passage history.

A quantity of master seed vaccine virus should be prepared, frozen or desiccated and stored at low temperatures such as –40°C or –80°C in order to provide a consistent working seed for regular vaccine production. The virus should be cultured in primary or secondary LT cells of wool sheep origin for maximum yield. Vero cells may also be used.

Each seed strain must be safe to use in all breeds of cattle for which it is intended, including young and pregnant animals. It must also be non-transmissible, remain attenuated after further tissue culture passage, and provide complete protection against challenge with virulent field strains for a minimum of 1 year. It must produce a minimum clinical reaction in all breeds of cattle when given by the recommended route.

The necessary safety and potency tests are described in Section C.2.b.iv Final product batch tests.

ii) Quality criteria (sterility, purity, freedom from extraneous agents)

Each master seed must be tested to ensure its identity and shown to be free from adventitious viruses, in particular pestiviruses, such as border disease and bovine viral diarrhoea virus, and free from contamination with bacteria, fungi and/or mycoplasmas.

The general procedures for sterility/purity tests are described in chapter 1.1.7.

b) Method of manufacture

The method of manufacture should be documented as the Outline of Production.

i) Procedure

Vaccine batches are produced on fresh monolayers of secondary LT cells. A vial of seed virus is reconstituted with GMEM or other appropriate medium and inoculated onto an LT monolayer that has been previously washed with warm PBS, and allowed to absorb for 15 minutes at 37°C before being overlaid with additional GMEM. Cells should be harvested after 4–6 days when they exhibit 50–70% CPE for maximum viral infectivity, or earlier if CPE is extensive and they appear ready to detach. The culture is freeze–thawed three times, and the suspension is recovered and centrifuged at 600 g for 20 minutes. Before harvest, the culture should be examined for any evidence of nonspecific CPE, medium cloudiness or change in medium pH. A second passage may be required to produce sufficient virus for a production batch (to produce enough for 10^6 doses, the yield from five 175 cm^2 flasks is required).

The procedure is repeated and the harvests (consisting of clarified supernatants) from individually numbered flasks are each mixed separately with an equal volume of sterile, chilled 5% lactalbumin hydrolysate and 10% sucrose (dissolved in double-distilled water or appropriate balanced salt solution), and transferred to individually numbered bottles for storage at –20°C. Prior to storage, 0.2 ml is removed from each bottle for sterility control. An additional 0.2 ml is removed; 2 ml pools composed of 0.2 ml samples taken from ten bottles are used for virus titration. A written record of all the procedures must be kept for all vaccine batches.

ii) Requirements for substrates and media

The specification and source of all ingredients used in the manufacturing procedure should be documented and the freedom of extraneous agents: bacteria, fungi, mycoplasma and viruses should be tested. The detailed testing procedure is described in chapter 1.1.7. The use of antibiotics must meet the requirements of the licensing authority.

iii) In-process control

Cells: Records of the source of the master cell stocks should be maintained. The highest and lowest passage numbers of the cells that can be used for vaccine production must be indicated in the outline of the production. In case primary lamb testis cells are used, cells should be obtained from the testis of a healthy young lamb from a scrapie-free flock of a wool sheep breed. During cultivation, cells must be observed for any evidence of CPE, and for normal morphology (predominantly fibroblastic). They can usually be passaged successfully up to ten times. When used for vaccine production, uninfected control cultures should be grown in parallel and maintained for at least one additional passage for further observation. They should be checked for the presence of noncytopathic strains of pestiviruses such as bovine viral diarrhoea or border disease viruses by immunofluorescence or immunoperoxidase or by other appropriate techniques. If possible, master cell stocks should be prepared and screened
prior to vaccine production, and a stock stored in sterile DMSO (dimethyl sulphoxide) in liquid nitrogen (1–2 ml aliquots containing 20 × 10^6 cells/ml).

**Serum:** Bovine serum used in the growth or maintenance medium must be tested free from antibody to capripoxvirus and contamination with pestivirus or any other viruses, extraneous bacteria, mycoplasma or fungi.

**Medium:** must be tested free from antibody to capripoxvirus and contamination with pestivirus or any other extraneous viruses, bacteria, mycoplasma or fungi.

**Virus:** Seed virus and final vaccine must be titrated in tissue culture tubes or microtitre plates. The minimum recommended field dose of the Kenyan and South African vaccines is log_{10} 3.5 TCID_{50}, although the minimum protective dose is log_{10} 2.0 TCID_{50}. Capripoxvirus is highly susceptible to inactivation by sunlight, and allowance should be made for loss of activity in the field. The recommended field dose of the Romanian sheep pox vaccine for cattle is log_{10} 2.5 sheep infective doses (SID_{50}), and the recommended dose for cattle of the RM65-adapted strain of Romanian sheep pox vaccine is log_{10} 3 TCID_{50} (Coakley & Capstick, 1961). Vaccine samples must be examined for the presence of adventitious viruses including cytopathic and noncytopathic strains of pestivirus, and should be mixed with a high-titre capripoxvirus-immune serum that has previously tested negative for antibodies to pestiviruses, to prevent the vaccine virus itself from interfering with the test. The vaccine can be held at –20°C until all sterility tests and titrations have been completed, at which time it should be freeze-dried. A further titration is carried out on five randomly chosen vials of the freeze-dried preparation to confirm the titre.

iv) **Final product batch tests**

**Sterility/purity**

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

**Safety and efficacy**

The efficacy and safety studies should be demonstrated by statistically valid vaccination–challenge studies using seronegative young European dairy cattle breeds. Six cattle of known susceptibility to LSD are placed in a high containment level large animal unit and serum samples are collected. Five randomly chosen vials of the freeze-dried vaccine are reconstituted in sterile PBS and pooled. Overdose studies: two cattle are inoculated with 10 times the field dose of the vaccine, the remaining vaccine is diluted with sterile PBS and two cattle are inoculated subcutaneously with the recommended field dose. The remaining two cattle are control animals. The animals are clinically examined daily and rectal temperatures are recorded. On day 21 after vaccination, the six animals are again serum sampled and challenged with a known virulent capripoxvirus strain by intradermal inoculation. (The challenge virus solution should also be tested free from extraneous viruses that can contaminate the vaccine.) The clinical response is recorded during the following 14 days. Control animals should develop the typical clinical signs of LSD, whereas there should be no local or systemic reaction in the vaccinated other than a delayed-type hypersensitivity reaction, which should disappear after 4 days. Serum samples are again collected on day 30 after vaccination. The day 21 serum samples are examined for seroconversion to selected viral diseases that could have contaminated the vaccine, and the days 0 and 30 samples are compared to confirm the absence of antibody to pestivirus. Because of the variable response in cattle to LSD challenge, generalised disease may not be seen in the control animals, although there should be a large local reaction.

The fully reconstituted vaccine is also tested in mice and guinea-pigs. Two guinea-pigs are inoculated intramuscularly with 0.5 ml into the hind leg, and two guinea-pigs and six mice are inoculated intraperitoneally with 0.5 ml and 0.1 ml, respectively. Two guinea-pigs and four mice are kept as uninoculated controls. The animals are observed for 3 weeks, humanely killed and a post-mortem examination is carried out. There should be no evidence of pathology caused by the vaccine.

**Batch potency**

Potency tests in cattle must be undertaken for vaccine strains of capripoxvirus if the minimum immunising dose is not known. This is usually carried out by comparing the titre of a virulent challenge virus on the flanks of vaccinated and control animals. Following vaccination, the flanks of at least three animals and three controls are shaved of hair. Log_{10} dilutions of the challenge virus are prepared in sterile PBS and six dilutions are inoculated intradermally (0.1 ml per inoculum) along the length of the flank; four replicates of each dilution are inoculated down the flank. An oedematous swelling will develop at possibly all 24 inoculation sites on the control animals, although preferably there will be little or no reaction at the four sites of the most dilute inocula. The vaccinated animals should develop an initial hypersensitivity reaction at sites of inoculation within 24 hours, which should quickly subside. Small areas of necrosis may develop at the inoculation site of the most concentrated challenge virus. The titre of the challenge virus is calculated for the vaccinated and control animals; a difference of log titre >log_{10} 2.5 is taken as evidence of protection.
c) Requirements for authorisation

i) Safety requirements

Target and non-target animal safety
The vaccine must be safe to use in all breeds of cattle for which it is intended, including young and pregnant animals. It must also be non-transmissible and remain attenuated after further tissue culture passage.

Safety tests should be carried out on the final product of each batch as described in Section C.2.b.iv Final product batch tests.

The safety of the vaccine in non-target animals must have been demonstrated using mice and guinea-pigs as described in Section C.2.b.iv Final product batch tests. There should be no evidence of pathology caused by the vaccine.

Reversion-to-virulence for attenuated/live vaccines
The selected final vaccine should not revert to virulence during further passages in target animals.

Environmental consideration
Attenuated vaccine should not be able to perpetuate autonomously in a cattle population. Strains of LSD virus are not a hazard to human health.

ii) Efficacy requirements

For animal production
The efficacy of the vaccine must be demonstrated in vaccination challenge experiments under laboratory conditions. Six cattle of known susceptibility to LSD are placed in a high containment level large animal unit. Five randomly chosen vials of the freeze-dried vaccine are reconstituted in sterile PBS and pooled. Four cattle are vaccinated with recommended dose using the recommended route. The remaining two cattle are control animals. On day 21 after vaccination, the six animals are challenged with a known virulent capripoxvirus strain by intradermal inoculation. The clinical response is recorded during the following 14 days. Control animals should develop the typical clinical signs of LSD, whereas there should be no local or systemic reaction in the vaccinates other than a delayed-type hypersensitivity reaction, which should disappear after 4 days. Because of the variable response in cattle to LSD challenge, generalised disease may not be seen in the control animals, although there should be a large local reaction.

Once the potency of the particular strain being used for vaccine production has been determined in terms of minimum dose required to provide immunity, it is not necessary to repeat this on the final product of each batch, provided the titre of virus present has been ascertained.

For control and eradication
Vaccination is the only effective way to control lumpy skin disease outbreaks in endemic countries. Unfortunately, currently no marker vaccines allowing the differentiation of infected from vaccinated animals are available.

Immunity to virulent field virus following vaccination lasts 2 years with the Kenyan strain and 3 years with the South African vaccine, and protection against generalised infection following intradermal challenge is effectively lifelong. The duration of immunity produced by other vaccine strains should be ascertained in cattle by undertaking controlled trials in an environment in which there is no possibility of field strains of capripoxvirus interfering with the results.

iii) Stability

All vaccines are initially given a shelf life of 24 months before expiry. Real-time stability studies are then conducted to confirm the appropriateness of the expiry date. Multiple batches of the vaccine should be re-titrated periodically throughout the shelf-life to determine the vaccine variability.

Properly freeze-dried preparations of LSD vaccine, particularly those that include a protectant, such as sucrose and lactalbumin hydrolysate, are stable for over 25 years when stored at –20°C and for 2–4 years when stored at 4°C. There is evidence that they are stable at higher temperatures, but no long-term controlled experiments have been reported. No preservatives other than a protectant, such as sucrose and lactalbumin hydrolysate, are required for the freeze-dried preparation.
3. Vaccines based on biotechnology

A new generation of capripox vaccines is being developed that uses the LSD virus as a vector for the expression and delivery of immuno-protective proteins of other ruminant pathogens with the potential for providing dual protection (Wallace & Viljoen, 2005). For example, an LSD virus-vectorized rinderpest construct can provide protection against both LSD and rinderpest in a single vaccination (Romero et al., 1993). Recently LSDV has been utilized as viral vector for rabies virus (Aspden et al., 2003) and Rift Valley fever virus genes (Wallace et al. 2006).

a) Vaccines available and their advantages

Currently, no new generation recombinant capripox vaccines are commercially available.

b) Special requirements for biotechnology vaccines, if any

Not applicable at present.

REFERENCES


* * *

**NB:** There are OIE Reference Laboratories for Lumpy skin disease (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for lumpy skin disease.
CHAPTER 2.4.15.

MALIGNANT CATARRHAL FEVER

SUMMARY

Malignant catarrhal fever (MCF) is an acute, generalised and usually fatal disease affecting many species of Artiodactyla. The disease has been most often described as affecting species of the subfamily Bovinae and family Cervidae, but is also recognised in domestic pigs as well as giraffe and species of antelope belonging to the subfamily Tragelaphinae. MCF is defined by the recognition of characteristic lymphoid cell accumulations in nonlymphoid organs, vasculitis and T-lymphocyte hyperplasia in lymphoid organs, the main cause of which is either of two gammaherpesviruses. The alcelaphine herpesvirus-1 (AIHV-1), which is maintained by inapparently infected wildebeest, causes the disease in cattle in regions of Africa and in a variety of ruminant species in zoological collections world-wide. Ovine herpesvirus-2 (OvHV-2), which is prevalent in all varieties of domestic sheep as a subclinical infection, is the cause of MCF in most regions of the world. This form of the disease is also known as sheep-associated MCF. In both forms of the disease, animals with clinical disease are not a source of infection as virus is only excreted by the natural hosts – wildebeest and sheep, respectively.

MCF usually appears sporadically and affects few animals, though both AIHV-1 and OvHV-2 can give rise to epizootics. There is a marked gradation in susceptibility to the OvHV-2 form of MCF ranging from the relatively resistant Bos taurus and B. indicus, through water buffalo, North American bison and many species of deer, to the extremely susceptible Père David’s deer, and Bali cattle. The disease may present a wide spectrum of clinical manifestations ranging from the acute form, when minimal changes are observed prior to death, to the more florid cases characterised by high fever, bilateral corneal opacity, profuse catarrhal discharges from the eye and nares, necrosis of the muzzle and erosion of the buccal epithelium. Infectivity from animals with the AIHV-1 form of MCF can be recovered only by employing techniques that retain the viability of host cells, while OvHV-2 has never been recovered from affected animals. Diagnosis is normally achieved by observing the characteristic histopathological changes, though detection of viral DNA in either form of the disease has become the preferred option.

Identification of the agent: AIHV-1 may be recovered from clinically affected animals using peripheral blood leukocytes or cell suspensions prepared from lymph nodes and spleen, but cell viability must be preserved during processing, as infectivity cannot be recovered from dead cells. Virus can also be recovered from wildebeest, either from peripheral blood leukocytes or from cell suspensions of other organs. Most monolayer cultures of ruminant origin are probably susceptible and develop cytopathic effect (CPE), although bovine thyroid cell cultures have been used extensively for recovery of virus. Primary isolates typically produce multinucleated CPE in which viral antigen can be identified by immunofluorescence or immunocytochemistry using suitable antisera or monoclonal antibodies. The OvHV-2 agent has never been identified formally, although lymphoblastoid cell lines propagated from affected animals contain OvHV-2-specific DNA. Both agents have been transmitted experimentally to rabbits and hamsters, which develop lesions characteristic of MCF.

Viral DNA has been detected in clinical material from cases of MCF caused by both AIHV-1 and OvHV-2 using the polymerase chain reaction, and this is becoming the method of choice for diagnosing the OvHV-2 form of the disease.

Serological tests: Infected wildebeest, the natural host, consistently develop antibody to AIHV-1, which can be detected in a variety of assays including virus neutralisation, immunoblotting, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence. However, the antibody response of clinically affected animals is limited, with no neutralising antibody developing, so that
detection relies on the use of immunofluorescence, ELISA or immunoblotting. Antibody to OvHV-2 has only been detected by using AIHV-1 as the source of antigen. Domestic sheep consistently have antibody that can be detected by immunofluorescence, ELISA or immunoblotting. While antibody often can be detected by immunofluorescence and ELISA in cattle with MCF, in more acutely affected animals, such as deer, antibody is not always present. The competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) appears to have a sensitivity and specificity that are equal to or better than the other tests, although a recently described ELISA gave good concordance with this test.

Requirements for vaccines and diagnostic biologicals: No vaccine has been developed for this disease.

A. INTRODUCTION

Malignant catarrhal fever (MCF) is a generally fatal disease of cattle and many other species of Artiodactyla, which occurs following infection with either alcelaphine herpesvirus-1 (AIHV-1) or ovine herpesvirus-2 (OvHV-2). Wildebeest (Connochaetes spp. of the subfamily Alcelaphinae), the natural hosts of AIHV-1, experience no clinical disease following infection. Likewise, infection of domestic sheep, the natural host of OvHV-2, has not been associated with any clinical reaction following natural infection, although experimentally, large doses of virus produced clinical signs of MCF, when inoculated into susceptible sheep (Li et al., 2005b). Disease caused by AIHV-1 is restricted to those areas of Africa where wildebeest are present and to zoological collections elsewhere, and has been referred to as wildebeest-associated MCF. The OvHV-2 form of the disease occurs world-wide wherever sheep husbandry is practised and has been described as sheep-associated (SA) MCF. Both forms of the disease may present a wide spectrum of clinical entities, though the characteristic histopathological changes are very similar in all cases. These two viruses belong to a subgroup of closely related ruminant rhadinoviruses that infect three subfamilies of Bovidae (Alcelaphinae, Hippotraginae and Caprinae); all probably have a potential to cause typical MCF. On rare occasions members of this group of viruses other than AIHV-1 and OvHV-2 have been identified as a cause of MCF.

• Clinical and pathological changes

The clinical signs of MCF are highly variable and range from peracute to chronic with, in general, the most obvious manifestations developing in the more protracted cases. In the peracute form, either no clinical signs are detected, or depression followed by diarrhoea and dysentery may develop for 12–24 hours prior to death. In general, the onset of signs is associated with the development of a high fever, increased serous lachrymation and nasal exudate, which progresses to profuse mucopurulent discharges. Animals may be inappetent and milk yields may drop. Characteristically, progressive bilateral corneal opacity develops, starting at the periphery. In some cases skin lesions appear (characterised by ulceration and exudation), which may form hardened scabs associated with necrosis of the epidermis, and are often restricted to the perineum, udder and teats. Salivation associated with hyperaemia may be an early sign, progressing to erosions of the tongue, hard palate, gums and, characteristically, the tips of the buccal papillae. Superficial lymph nodes may be enlarged and limb joints may be swollen.

Nervous signs such as hyperaesthesia, incoordination, nystagmus and head pressing may be present in the absence of other clinical signs or as part of a broader more characteristic syndrome.

There is a wide spectrum of susceptibility to OvHV-2-induced disease, ranging from Bos taurus and B. indicus, which are relatively resistant, through most species of deer, bison (Bison bison) and water buffalo (Bubalus bubalis), which are much more susceptible, to the extremely susceptible Bali cattle (Bos javanicus) and Père David’s deer (Elaphurus davidianus). The more resistant species tend to experience a more protracted infection and florid lesions, while in the more susceptible species the disease course tends to be shorter and the clinical signs less dramatic.

Reports from several countries, and in particular from Norway, that the disease affects domestic pigs have recently been confirmed (Loken et al., 1998). Signs are very similar to those seen in acutely affected cattle.

A mild form of the disease described in 1930 was regarded with some scepticism because the disease could be confirmed only by histological changes observed at post-mortem. However, recent investigations using molecular and serological methods would appear to confirm that a few infected animals may recover following mild or even quite severe clinical reactions (Michel et al., 1994). Some studies indicate that substantial numbers of animals may become infected without developing clinical disease.
• Pathology

Gross pathological changes reflect the severity of clinical signs, but are generally widespread and may involve most organ systems. Erosions and haemorrhages may be present throughout the gastrointestinal tract, and in the more acute cases can be associated with haemorrhagic intestinal contents. In general, lymph nodes are enlarged, although the extent of lymph node involvement varies within an animal. Lymph nodes can often be firm and white when cross-sectioned, while others, in particular submandibular and retropharyngeal, may be haemorrhagic and even necrotic. Catarrhal accumulations, erosions and the formation of a diphtheritic membrane are often observed in the respiratory tract.

Within the urinary tract characteristic echymotic haemorrhages of the epithelial lining of the bladder are often present, while the renal cortex may be affected with multiple raised white foci, each 1–5 mm in diameter and sometimes surrounded by a thin zone of haemorrhage.

Histological changes have been the basis for confirming cases of MCF and are characterised by epithelial degeneration, vasculitis, hyperplasia and necrosis of lymphoid organs, and widespread interstitial accumulations of lymphoid cells in nonlymphoid organs. Epithelial lesions may be present at all epithelial surfaces and are characterised by erosion and ulceration, frequently with subepithelial and intraepithelial lymphoid cell infiltration, which may be associated with vasculitis and haemorrhages.

Vasculitis is generally present and may be pronounced in the brain, affecting veins, arteries, arterioles and venules. It is characterised by lymphoid cell infiltration of the tunica adventitia and media, often associated with fibrinoid degeneration. In the lumen there may be ‘pavementing’ by lymphoid cells, and in severe cases, endothelial damage and subendothelial accumulations by lymphoid cells can sometimes lead to occlusion.

Lymph-node hyperplasia is characterised by an expansion of lymphoblastoid cells in the paracortex, while degenerative lesions are generally associated with the follicles. Oedema with lymphoid inflammation often affects the perinodal tissue.

The interstitial accumulation of lymphoid cells in nonlymphoid organs, in particular the renal cortex and periportal areas of the liver, is typical, and in the case of the kidney may be very extensive. In the brain there may be a nonsuppurative meningoencephalitis with lymphocytic perivascular cuffing and a marked increase in the cellularity of the cerebrospinal fluid.

The macroscopic lesions observed in the cornea are reflected histologically by lymphoid cell infiltration originating in the limbus and progressing centrally, with oedema and erosion developing in the more advanced cases. Vasculitis, hypopyon and iridocyclitis also may be present.

The pathological features of MCF irrespective of the agent involved are essentially similar. However, apart from histological examination, the methods available for diagnosing AlHV-1- and OvHV-2-induced disease are very different and are thus considered separately.

B. DIAGNOSTIC TECHNIQUES

B1. Alcelaphine herpesvirus-1

This form of the disease occurs in the cattle-raising regions of eastern Africa where pastoralists use areas grazed by wildebeest, and in southern Africa in areas where wildebeest and cattle are grazed together. The disease, however, can also affect a variety of other ruminant species in zoological collections world-wide and so, apart from antelope of the subfamilies Alcelaphinae and Hippotraginae, it is advisable to regard all ruminants as susceptible. Most laboratory-based tests have relied on one attenuated isolate (WC11) that has been subjected to many laboratory passages as a source of viral antigen and DNA (Plowright et al., 1960). The full nucleotide sequence of the virulent low passage virus (C500) is now available and will form the basis of further studies of this virus (Enser et al., 1997).

1. Identification of the agent

• Clinically affected animals

a) Isolation

The striking feature of AlHV-1-induced MCF is the lack of detectable viral antigen or herpesvirus-specific cytology within lesions. Confirmation of infection thus relies on virus recovery. Generally, infectivity is strictly cell associated and thus isolation can be achieved only from cell suspensions either of peripheral blood leukocytes, lymph nodes or other affected tissues. Cell suspensions are prepared in tissue culture fluid,
approximately $5 \times 10^6$ cells/ml, and inoculated into preformed monolayer cell cultures. Bovine thyroid cells have been used extensively, but most primary and low passage monolayer cell cultures of ruminant origin will probably provide a suitable cell substrate for isolating the virus. Following 36–48 hours’ incubation, culture medium should be changed and monolayers should be examined microscopically ($\times40$) for evidence of cytopathic effects (CPE). These appear characteristic ally as multinucleate foci within the monolayers, which then progressively retract forming dense bodies with cytoplasmic processes that may detach. This is followed by regrowth of normal monolayers. A CPE may take up to 21 days to become visible and is seldom present before day 7. Infectivity at this stage tends to be largely cell associated and thus any further passage or storage must employ methods that ensure that cell viability is retained. Specificity of the isolate should be determined using specific antisera or monoclonal antibodies (MAbs) in fluorescence or immunocytochemical tests.

b) Viral DNA

Characteristically, very little viral DNA can be detected within affected tissues, hence it is necessary to amplify the viral genome either by conventional culture or the polymerase chain reaction (PCR).

The full sequence of the C500 isolate has been published permitting the design of primers for PCR reactions from conserved regions of the genome. The polymerase gene sequence has been employed for phylogenetic comparison of AlHV-1 and related viruses (Li et al., 2005a).

- Natural hosts

It is almost certain that all free-living wildebeest are infected with AlHV-1 by 6 months of age, virus having been spread as an intense epizootic during the perinatal period. The species Connochaetes taurinus taurinus, C.t. albojubatus and C. gnu are all assumed to be infected with the same virus. Infection also appears to persist in most groups of wildebeest held in zoological collections. However, it is possible that infection may be absent in animals that have been isolated during calf-hood or that live in small groups. Natural infection has been successfully demonstrated by in-situ hybridisation on lung sections from C.t. taurinus calves in South Africa (Michel et al., 1997).

Following infection there is a brief period when virus is excreted in a cell-free form and can be isolated from nasal swabs. Virus can also be isolated from blood leukocytes at this time, but in older animals this is less likely to be successful unless the animal is immunosuppressed either through stress or pharmacological intervention. In addition, virus may be isolated by establishing cultures of tissues from apparently normal animals, and this has been achieved in monolayer cultures of both kidney and thyroid cells from adult animals.

Other large antelope of the subfamilies Alcelaphinae and Hippotraginae are also infected with antigenically closely related gammaherpesviruses, but there is no evidence that they can spread to other species and cause MCF, except rarely in captive populations.

2. Serological tests

- Clinically affected animals

The antibody response of clinically affected animals is limited, with no neutralising antibody developing. Antibody in clinical cases can be demonstrated consistently by immunofluorescence or the immunoperoxidase test (IPT) using WC11-infected cell cultures as substrate. A competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) was first developed for detecting antibody to OvHV-2 (Li et al., 1994) using an MAb (15-A) that targets an epitope that appears to be conserved among all MCF viruses and is probably also applicable to AlHV-1 infected animals.

- Natural hosts

Antibody appears to develop consistently in wildebeest following infection and can be identified by neutralisation assays using the cell-free isolate WC11, or by immunofluorescence, again using the WC11 isolate and antibovine IgG, which has been shown to react with wildebeest IgG. The Minnesota MCF virus strain, which is indistinguishable from the WC11 strain of AlHV-1, is used for CI-ELISA antigen production.

There has been no attempt so far to standardise the indirect fluorescent antibody (IFA) test and the IPT, but the two methods below are given as examples. The CI-ELISA is available as a commercial kit

a) Indirect fluorescent antibody test

The IFA is less specific than virus neutralisation (VN); it can be used to demonstrate several varieties of ‘early’ and ‘late’ antigens in AlHV-1-infected cell monolayers. Antibodies reacting in the IFA test or the IPT
develop in cattle and experimentally infected rabbits during the incubation period, and later in the clinical course of the disease, though cross-reactions with some other bovine herpesviruses, as well as OvHV-2, reduce the differential diagnostic value. Detection of such cross-reacting antibodies can sometimes be useful in supporting a diagnosis of SA-MCF.

- Preparation of fixed slides

Inoculate nearly or newly confluent cell cultures (see Section B1.2.c) with AIHV-1 (strain WC11). Uninoculated control cultures should be processed in parallel. At about 4 days – when the first signs of CPE are expected to appear but before overt CPE is visible – treat the cultures as follows: discard the medium, wash with PBS, remove the cells with trypsin–versene solution, spin down cells at approximate 800 g for 5 minutes, discard the supernatant fluid, and resuspend the cells in 10 ml of phosphate buffered saline (PBS) for each 800 ml plastic bottle of cell culture.

Make test spots of the cell suspension on two wells of a polytetrafluoroethylene-coated multiwell slide; air-dry and fix in acetone. Stain the spots with positive standard serum and conjugated anti-IgG to the appropriate species. Examine the incidence of positive and negative cells under a fluorescent microscope. Adjust the cell suspension by adding noninfected cells and/or PBS to give a suitable concentration that will form a single layer of cells when spotted on to the slide, with clearly defined positive cells among a background of negative cells.

Spot the adjusted positive cell suspension and the control negative suspensions on to multiwell slides in the desired pattern, and air-dry. Fix in acetone for 10 minutes. Rinse, dry and store over silica gel in a sealed container at –70°C.

An alternative procedure, which is easier to evaluate, is to prepare monolayers of infected and noninfected cells in Leighton tubes or chamber slides. The cell monolayers are infected with from 150 to 200 TCID\textsubscript{50} (50% tissue culture infective dose) of virus that has been diluted in cell culture medium. The infected and noninfected slides are fixed in acetone and stored, as above, at –70°C.

- Test procedure

i) Rehydrate the slides for 5 minutes with PBS, rinse in distilled water and air-dry.

ii) Dilute sera 1/20 in PBS. Samples that give high background staining may be retested at higher dilutions. Apply diluted fluids to one MCF virus-positive cell spot and one negative control spot for each sample. Include positive and negative serum controls. Ideally, the test should be validated by titrating the control positive to determine its end-point.

iii) Incubate at 37°C for 30 minutes in a humid chamber.

iv) Drain the fluids from the spots. Wash the slides in two changes of PBS, for 5 minutes each.

v) Wash in PBS for 1 hour with stirring, and then air-dry the slides.

vi) Apply rabbit anti-bovine IgG fluorescein isothiocyanate (FITC) conjugate at a predetermined working dilution.

vii) Incubate at 37°C for 20 minutes, drain the slides, and wash twice in PBS for 10 minutes each.

viii) Counterstain in Evans blue 1/10\textsuperscript{4} for 30 seconds, and wash with PBS for 2 minutes. Dip in distilled water, dry and mount in PBS/glycerol (50/50).

ix) Examine by fluorescence microscopy for specific binding of antibody to the infected cells.

b) Immunoperoxidase test

A dilution of bovine turbinate (BT) cell-cultured AIHV-1 containing approximately 10\textsuperscript{3} TCID\textsubscript{50} is made in a freshly trypsinised suspension of BT cells and seeded into Leighton tubes containing glass cover-slips, 1.6 ml per tube, or four-chambered slides, 1.0 ml per chamber.

Observe the cell cultures at 4–6 days for CPE and fix the cultures with acetone when signs of CPE begin. Remove the plastic chambers, but not the gaskets, from the slide chambers before fixation, and use acetone (e.g. UltimAR) that will not degrade the gasket. Store the fixed cells at –70°C.

- Test procedure

i) Prepare IPT diluent (21.0 g NaCl and 0.5 ml Tween 20 added to 1 litre of 0.01 M PBS, pH 7.2) and washing fluid (0.5 ml Tween 20 added to 1 litre of 0.01 M PBS, pH 7.2).

ii) Dilute the serum to be tested 1/20 in IPT diluent and overlay 150–200 µl on to a fixed virus-infected cover-slip or slide chamber.
iii) Incubate the cover-slip in a humid chamber at 37°C for 30 minutes.
iv) Dip the cover-slip three times in washing fluid.
v) Overlay 150–200 µl of diluted (1/5000 in IPT diluent) peroxidase-labelled anti-bovine IgG on to the cover-slip or slide chamber.
vi) Incubate the cover-slip or slide chamber in a humid chamber at 37°C for 30 minutes.
vi) Dip the cover-slip three times in washing fluid.

viii) Dilute the AEC substrate (3-amino-9-ethylcarbazole) in distilled water (5 ml of distilled water, 2 drops buffer, 2 drops hydrogen peroxide, and 3 drops AEC) and apply to the cover-slip or slide chamber.
ix) Incubate in a humid chamber at 37°C for 8–10 minutes.
x) Dip the cover-slip in distilled water, air-dry, and mount on a glass slide. Slide chambers are read dry.
xi) The slide is read on a light microscope. The presence of a reddish-brown colour in the nuclei of the infected cells indicates a positive reaction.

c) Virus neutralisation
Tests have been developed for detecting antibodies to AIHV-1 in both naturally infected reservoir and indicator hosts. The first of these is a VN test using cell-free virus of the WC11 strain, and another uses a hartebeest isolate (AIHV-2). AIHV-1 and AIHV-2 have cross-reactive antigens and therefore either strain can be used in the test. The test is laborious, but can be performed in microtitre plates using low passage cells or cell lines. The main applications have been in studying the range and extent of natural gammaherpes viruses infection in wildlife, captive species in zoos and, to a lesser extent, sheep populations. It has also been useful in attempts to develop vaccines, all of which have had limited success. The VN test is of no value as a diagnostic test in clinically affected animals as no VN antibody develops in clinically susceptible species.

AIHV-1 stock (strain WC11) is grown in primary or secondary cell cultures of bovine kidney, bovine thyroid, low passage bovine testis, or another permissive cell type. The virus is stored in aliquots at –70°C. The stock is titrated to determine the dilution that will give 100 TCID_{50} in 25 µl under the conditions of the test.

• Test procedure

i) Inactivate the sera for 30 minutes in a water bath at 56°C.
ii) Make doubling dilutions of test sera in cell culture medium from 1/2 to 1/16 using a 96-well flat-bottomed cell-culture grade microtitre plate, four wells per dilution and 25 µl volumes per well. Positive and negative control sera are also included in the test. No standard sera are available, but internal positive standards should be prepared and titrated in an appropriate range.
iii) Add 25 µl per well of WC11 virus stock at a dilution in culture medium calculated to provide 100 TCID_{50} per well.
iv) Incubate for 1 hour at 37°C. The residual virus stock is also incubated.
v) Back titrate the residual virus in four tenfold dilution steps, using 25 µl per well and at least four wells per dilution.
vi) Add 50 µl per well of bovine kidney cell suspension at 3 × 10^5 cells/ml.
vii) Incubate the plates in a humidified CO_2 atmosphere at 37°C for 7–10 days.
viii) Read the plates microscopically for CPE. Validate the test by checking the back titration of virus (which should give a value of 100 TCID_{50} with a permissible range 30–300) and the control sera. The standard positive serum should give a titre within 0.3 log_{10} units of its predetermined mean.
ix) The test serum results are determined by the Spearman–Kärber method as the dilution of serum that neutralised the virus in 50% of the wells.
x) A negative serum should give no neutralisation at the lowest dilution tested (1/2 equivalent to a dilution of 1/4 at the neutralisation stage).

d) Competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA)
A CI-ELISA was first developed for detecting antibody to OvHV-2 (Li et al., 1994) using a MAb (15-A) that targets an epitope on a complex of glycoproteins that appears to be conserved among all MCF viruses. The MAb was raised against the Minnesota isolate of virus, which is indistinguishable from the WC11 strain of
Chapter 2.4.15. – Malignant catarrhal fever

AlHV-1. The test has been employed to detect antibody in serum of wild and domestic ruminants in North America and antibody to the following pathogenic viruses has been detected: AlHV-1, AlHV-2, OvHV-2, CpHV-2 and the herpesvirus of unknown origin observed to cause classic MCF in white-tailed deer, as well as the MCF-group viruses not yet reported to be pathogenic, such as those carried by the oryx, muskox, and others. The test has recently been reformatted to increase sensitivity (Hsu et al., 1990). This change was made to enable the detection antibody in newly infected lambs and animals in the acute stage of the disease, which were sometimes not detected in the previous format. The CI-ELISA has the advantage of being faster and more efficient than the IFA or IPT. Additional validation data will become available as its use is expanded to more laboratories in other parts of the world.

The complete reagent set for the CI-ELISA, including pre-coated plates, labelled MAb and control sera, is commercially available. For laboratories wishing to prepare their own antigen-coated plates, the following protocol is provided. Immuno 4 ELISA plates (Dynatech Lab, Chantilly, Virginia) are coated at 4°C (39°F) for 18–20 hours with 50 µl of a solution containing 0.2 µg per well of semi-purified MCF viral antigens (Minnesota or WC11 isolates of AlHV-1) in 50 mM carbonate/bicarbonate buffer (pH 9.0). The coated plates are blocked at room temperature (21–25°C, 70–77°F) for 2 hours with 0.05 M PBS containing 2% sucrose, 0.1 M glycine, 0.5% bovine serum albumin and 0.44% NaCl (pH 7.2). After blocking, wells are emptied and the plates are then dried in a low humidity environment at 37°C for 18 hours, sealed in plastic bags with desiccant, and stored at 4°C (39°F) (Li et al., 2001). MAb 15-A is conjugated with horseradish peroxidase by the VMRD, Inc. using a standard periodate method.

• Test procedure
  i) Dilute positive and negative controls and test samples (either serum or plasma) 1/5 with dilution buffer (PBS containing 0.1% Tween 20, pH 7.2).
  ii) Add 50 µl of diluted test or control samples to the antigen-coated plate (four wells for negative control and two wells for positive control). Leave well A1 empty and for use as a blank for the plate reader.
  iii) Cover the plate with parafilm and incubate for 60 minutes at room temperature, (21–25°C, 70–77°F).
  iv) Using a wash bottle, wash the plate three times with wash buffer (same as dilution buffer: PBS containing 0.1% Tween 20, pH 7.2).
  v) Prepare fresh 1 × antibody-peroxidase conjugate by diluting one part of the 100 × conjugate with 99 parts of dilution buffer.
  vi) Add 50 µl of diluted antibody-peroxidase conjugate to each sample well. Cover the plate with parafilm and incubate for 60 minutes at room temperature (21–25°C, 70–77°F).
  vii) Wash the plate with wash buffer three times.
  viii) Add 100 µl of substrate solution (TMB Microwell, BioFX Laboratories, Owings Mills, Maryland) to each sample well. Incubate for 60 minutes at room temperature (21–25°C; 70–77°F). Do not remove the solution from the wells.
  ix) Add 100 µl of stop solution (0.18 M sulphuric acid) to each well. Do not remove the solution from the wells.
  x) Read the optical densities (OD) on an ELISA plate reader at 450 nm.
  xi) Calculating % inhibition:
      \[ \frac{100 - \text{Sample OD (average)} \times 100}{\text{Mean negative control OD}} = \% \text{Inhibition} \]
  xii) Interpreting the results: If a test sample yields equal to or greater than 25% inhibition, it is considered positive. If a test sample yields less than 25% inhibition, it is considered negative.
  xiii) Test validation: The mean OD of the negative control must fall between 0.40 and 2.10. The mean of the positive control must yield greater than 25% inhibition.

B2. Ovine herpesvirus-2

This form of the disease occurs world-wide in cattle and other species, normally appearing sporadically and affecting only one or a few animals. However, on occasion, incidents occur in which several animals become affected, and this appears to be associated with certain sheep flocks that may continue to transmit disease for a number of years. The disease can also spread and cause substantial losses in North American Bison (Bison bison), red deer (Cervus elaphus), other deer species and water buffalo (Bubalus bubalis) and even more readily to Pére David’s deer (Elaphurus davidianus) and Bali cattle (Bos javanicus). OvHV-2 is also responsible for causing MCF in zoological collections, where disease has been reported in a variety of species including giraffe.
Disease in pigs has been reported from several countries, but is most frequently recognised in Norway where incidents involving several animals regularly occur.

Diagnosis based on clinical signs and gross pathological examination cannot be relied on as these can be extremely variable. Histological examination of a variety of tissues including, by preference, kidney, liver, urinary bladder, buccal epithelium, cornea/conjunctiva and brain, has been the only method of reaching a more certain diagnosis. However, detection of antibody to the virus and/or viral DNA can now also be attempted and are rapidly becoming the methods of choice.

It must be emphasised that the viral cause of SA-MCF cannot be reliably isolated and evidence for OvHV-2 relies on: (a) the presence of antibody in sera of all domestic sheep that cross-reacts with AIHV-1 antigens in the IFA test and immunoblots (Hart et al., 2007), but not in neutralisation assays; (b) the development of antibody that cross-reacts with AIHV-1 in the IFA test and CI ELISA in most cattle with SA-MCF and in all experimentally infected hamsters; (c) the detection and cloning of DNA from lymphoblastoid cell lines derived from natural cases of SA-MCF that cross-hybridises with, but is distinct from, AlHV-1 DNA; (d) the detection by PCR of amplicons unique to OvHV-2 in peripheral blood and affected tissues.

1. Identification of the agent

   • Clinically affected animals

Attempts to recover the disease-causing virus from clinical cases have failed consistently. There are, however, several reports of the recovery of different viral agents from clinical cases, none of which has established any causal relationship; their isolation is certainly fortuitous or due to laboratory contamination. However, lymphoblastoid cell lines have been generated from affected cattle and deer, some of which transmit MCF following inoculation into experimental animals (Reid et al., 1989). Such cell lines contain viral sequences that hybridise with clones of AIHV-1 DNA (Bridgen & Reid, 1991). A virus sequence was cloned from such a cell line that coded for a tegument protein that was distinct from AIHV-1. Subsequently the whole length of the viral genome has been cloned and the nucleotide sequence determined (Hart et al., 2007). Primers were identified within this sequence that were suitable for use in the PCR, and a sensitive protocol was designed in which a fragment of 422 base pairs (bp) is amplified initially, followed by amplification of a truncated internal fragment of 238 bp. It has been proven that this test is able to detect as few as 35 viral genome equivalents and that no product is amplified from AIHV-1 or other bovid herpesviruses (Baxter et al., 1993). This PCR is thus both highly specific and sensitive for OvHV-2 and has been employed world-wide in studies of the disease in clinically affected animals and the natural host. It is emerging as a robust test that can be employed to detect viral DNA in peripheral blood leukocytes of clinically affected animals as well as fresh tissues and paraffin-embedded samples collected at post-mortem. The use of magnetic particles to purify DNA prior to amplification has been reported to be an additional improvement to the test, but is yet to be evaluated. A quantitative fluorogenic PCR assay for OvHV-2 has also been established and validated using the semi-nested PCR (Baxter et al., 1993) as a gold standard (Hussy et al., 2001) and is likely to have valuable future application.

While early studies indicated that infection of MCF-susceptible species would normally result in death, some prospective studies in high incidence herds of animals suggest that animals may become infected without developing a clinical response. Factors that predispose animals to infection and development of disease are not understood and it is likely to be a complex interaction of environmental, host factors and the infecting virus. That MHC class 11a polymorphism may contribute to resistance of American bison as suggested in one study (Traul et al., 2007) is of interest and should be further examined.

   • Polymerase chain reaction

Extraction of DNA from clinical material is performed according to the protocol defined in an appropriate extraction kit (e.g. Quiagen DNeasy Tissue Kit). Amplification reactions are performed in 50 µl volumes containing not more than 2 µg test DNA in 10 mM Tris HCl, pH 8.3, 50 mM KCl, 2 mM MgCl2, 0.01% (v/v) gelatine, 10% (v/v) dimethyl sulfoxide (DMSO), 200 µm dATP, dCTP, dGTP and dTTP (Pharmacia), 1 µM of each primer and 2 units Taq DNA-polymerase overlaid with 50 µl mineral oil (Sigma) to prevent evaporation.

The programme consists of a precycle at 99°C for 3 minutes, after which dNTP and enzyme mix are added. This is followed by 25 cycles of 94°C for 20 seconds, 60°C for 30 seconds and 72°C for 30 seconds. A 2 µl aliquot of the primary amplification product, specified by the primer pair 556/755, is transferred directly to a new reaction mixture and amplified using the primer pairs 556/555 under identical conditions for a further 25 cycles with a final extension at 72°C for 5 minutes.

Final amplification products (10 µl) are analysed directly by 1.8% agarose gel electrophoresis and ethidium bromide fluorescence. With each batch of test samples a known positive control and distilled water are also amplified and analysed.
Chapter 2.4.15. – Malignant catarrhal fever

• Natural hosts

The domestic sheep is the natural host of OvHV-2 and probably all sheep populations are infected with the virus in the absence of any clinical response. Studies of the dynamics of infection within sheep flocks have however, generated conflicting results with some suggesting productive infection occurs in the first weeks of a lamb’s life while others suggest infection of most lambs does not occur until 3 months of age with excretion of infectious virus occurring between 5 and 6 months (Li et al., 2004). There is also evidence that some lambs may become infected in utero while other studies suggest that removal of lambs from their dams during the first week permits the establishment of virus-free animals. There may therefore be considerable variation in the dynamics of infection in different flocks. However, circumstantial evidence of the occurrence of MCF in susceptible species does suggest that the perinatal sheep flock is the principal source of infection, but that periodic recrudescence of infection may occur in sheep of all ages.

Factors that predispose to virus shedding and transmission to MCF-susceptible hosts remain speculative.

In addition to domestic sheep, domestic goats and other members of the subfamily Caprinae have antibody that reacts with AIHV-1 in a similar pattern to sheep serum. This implies that these species are infected with viruses similar to OvHV-2, and some goats have been found to be positive to an OvHV-2 PCR, though their potential role in causing MCF would appear to be very limited.

2. Serological tests

Antibody to OvHV-2 has only been detected using AIHV-1 as the source of antigen. Antibody to AIHV-1 can be detected in 70–80% of clinically affected cattle by IFA or IPT procedures, but may not be present in affected deer or animals that develop acute or peracute disease. Antibody is detected by IFA using tissue culture cells infected with AIHV-1. Cell monolayers grown on cover-slips exhibiting 10–50% CPE are harvested, washed, fixed in acetone and used in the assay. Cover-slips are mounted with DPX, the side containing the cells facing uppermost, on microscope slides and treated with 10% normal horse serum before progressing with a conventional IFA test. The IPT procedure can be carried out as for AIHV-1. The only virus of cattle that has been reported to cross-react with AIHV-1 is bovine herpesvirus-4 (BHV-4). Thus the negative control for this test should be similarly infected monolayers of BHV-4. Sera are only considered to be positive when foci show characteristic intranuclear distribution of antigen with little or no cytoplasmic staining being detected in the AIHV-1-infected cells and no reaction in the BHV-4-infected cells. Sera that react to antigens of both viruses are considered to be inconclusive. A CI-ELISA has been developed for detecting antibody to OvHV-2 (Li et al., 1994) using a MAb (15-A) raised against the so-called Minnesota isolate of virus, which is indistinguishable from AIHV-1. The test has been employed to detect antibody in serum of wild and domestic ruminants in North America and appears to have some merit (Section B1.2.d) (Li et al., 2001). In a study on the reaction of sheep serum to the structural proteins of AIHV-1 in immunoblots, the reactivity of different sera varied strikingly, indicating that individual sheep responded differently with regard to antibody recognition of cross-reacting epitopes of AIHV-1.

B3. Control

Control at present relies on segregating natural hosts from susceptible species, the extent to which this is enforced depending on the species involved. With AIHV-1, it would appear that MCF-affected animals never or rarely transmit infection, hence it is only the natural hosts that can act as a source of infection. Wildebeest would appear to be relatively efficient transmitters of infection to most other categories of ruminant, and hence their segregation in mixed collections is important. Likewise, pastoralists must ensure that cattle are entirely segregated from the vicinity of wildebeest and pastures recently grazed by them, particularly around the time of wildebeest calving.

With OvHV-2, the requirement to segregate sheep depends on the susceptibility of the species involved. Thus with Père David’s deer and Bali cattle, strict separation and avoidance of contact through fomites must be ensured. Equally, with bison and farmed deer every reasonable effort must be taken to segregate the management of sheep, although fallow deer (Dama dama) appear to be more resistant to MCF. Cattle only rarely develop SA-MCF, and thus are generally managed with sheep without taking precautions to guard against disease transmission. However, if multiple cases do occur, it is essential to segregate the sheep flock as far as possible from cattle. As such flocks may continue to be sources of infection for some years, disposal of these flocks for slaughter should be considered.

Virus also appears to have been transmitted over substantial distances thus it is not possible to define the distance that sheep should be segregated.

The possibility that very long incubation periods may occur, up to 9 months, further necessitates a guarded prognosis when advising on the control of such outbreaks.
C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Numerous attempts to produce a protective vaccine against the AlHV-1 form of the disease have met with disappointing results. However, recent trials which have focussed on stimulating high titres of neutralising antibody in nasal secretions of cattle have produced encouraging results and should be the target for further research.

As OvHV-2 cannot be successfully propagated in the laboratory no attempts at developing a vaccine have been attempted.

REFERENCES


CHAPTER 2.4.16.
THEILERIOSIS

SUMMARY

Tick-transmitted Theileria parasites of cattle are a major constraint to the improvement of the livestock industry in large parts of the Old World\(^1\). Theileria annulata and T. parva, the most economically important species, are responsible for mortality and losses in production. Bovine theileriosis is generally controlled by the use of acaricides to kill ticks, but this method is not sustainable. Acaricides are expensive, they cause environmental damage, and over time ticks develop resistance to them requiring newer acaricides to be developed. More sustainable and reliable methods for the control of theileriosis that deploy a combination of strategic tick control and vaccination are desirable. However, these are yet to be successfully applied on a large scale in endemic areas.

Identification of the agent: Diagnosis of a variety of disease syndromes caused by the parasites is principally based on clinical signs, knowledge of disease and vector distribution, and identification of parasites in Giemsa-stained blood and lymph node smears. The presence of multinucleate intra-cytoplasmic and free schizonts, in lymph node biopsy smears, is a characteristic diagnostic feature of acute infections with T. parva and T. annulata. Animals infected with T. parva show enlarged lymph nodes, fever, a gradually increasing respiratory rate, dyspnoea and occasional diarrhoea. Post-mortem lesions observed are pulmonary oedema with froth in the trachea, enlargement of lymph nodes and spleen, haemorrhages in internal organs, abomasal erosions, the presence of parasitised lymphocytes and lympho-proliferative infiltrations in visceral tissues. The gross pathology caused by schizonts of T. annulata resembles that of T. parva, while the piroplasm stages may also be pathogenic, causing anaemia and jaundice.

Serological tests: The most widely used diagnostic test for Theileria species is the indirect fluorescent antibody (IFA) test. For the IFA test, both schizont and piroplasm antigens may be prepared on slides or in suspension and preserved by freezing at \(\leq -20^\circ\text{C}\), except in the case of the piroplasm suspension, which is stored at 4°C. Test sera are diluted with bovine lymphocyte lysate and incubated with the antigen in suspension, and anti-bovine immunoglobulin conjugate is then added. Using the test as described, the fluorescence is specific for the causative agent. The IFA test is sensitive, fairly specific, and usually easy to perform. However, because of the problems of cross-reactivity among some Theileria species, the test has limitations for large-scale surveys in areas where species distribution overlaps. The IFA test for T. parva, does not distinguish among the different immunogenic stocks. The new indirect enzyme-linked immunosorbent assays for T. parva, and T. mutans, based on recombinant parasite-specific antigens, have demonstrated higher sensitivity and specificity and have largely replaced the IFA tests previously used in Africa. In addition, newer molecular diagnostic tests, particularly those based on the polymerase chain reaction and reverse line blot hybridisation are proving to be powerful tools for characterising parasite polymorphisms, defining population genetics and generating epidemiological data.

Requirements for vaccines and diagnostic biologicals: Reliable vaccines of known efficacy have been developed for T. parva and T. annulata. For T. annulata, the vaccine is prepared from schizont-infected cell lines that have been isolated from cattle and attenuated during in-vitro culture. The vaccine must remain frozen until shortly before administration. Vaccination against T. parva is based on a method of infection and treatment in which cattle are given a subcutaneous dose of tick-derived sporozoites and a simultaneous treatment with a long-acting tetracycline formulation. This treatment results in a mild or inapparent East Coast fever reaction followed by recovery. Recovered

\(^1\) In this chapter, the term 'New World' refers to the Americas and the term 'Old World' refers to Europe, Africa and Asia.
animals demonstrate a robust immunity to homologous challenge, which usually lasts for the lifetime of an animal. Immunisation of animals with a stock(s) engendering a broad-spectrum immunity is desirable to cover a range of immunological T. parva strains that exist in the field. Immunised animals usually become carriers of the immunising parasite stock. Safety precautions must be taken in the preparation and handling of T. parva vaccines to protect the workers and to avoid contamination of the stabilates. Consideration should also be given to the risk of introducing new isolates into an area where they may then become established through a carrier state.

A. INTRODUCTION

Theileriae are obligate intracellular protozoan parasites that infect both wild and domestic Bovidae throughout much of the world (some species also infect small ruminants). They are transmitted by ixodid ticks, and have complex life cycles in both vertebrate and invertebrate hosts. There are six identified Theileria spp. that infect cattle; the two most pathogenic and economically important are T. parva and T. annulata. Theileria parva causes in 13 countries in sub-Saharan Africa causing East Coast fever (ECF). Corridor disease and January disease. Theileria annulata, the cause of tropical theileriosis, occurs in large parts of the Mediterranean coast of North Africa, extending to northern Sudan, and southern Europe. South-eastern Europe, the near and Middle East, India, China and Central Asia are also affected. Endemic regions of T. annulata and T. parva do not overlap. Theileria taurotragi and T. mutans generally cause no disease or mild disease, and T. velifera is non-pathogenic. These latter three parasites are mainly found in Africa, and overlap in their distribution complicating the epidemiology of theileriosis in cattle. The parasite group referred to as T. sergenti/T. buffeli/T. orientalis complex is now thought to consist of two species – T. sergenti, occurring in the Far East, and T. buffeli/T. orientalis (referred to as T. buffeli) with a global distribution (Fujisaki et al., 1994).

Most T. parva stocks produce a carrier state in recovered cattle, and studies using DNA markers for parasite strains have shown that T. parva carrier animals are a source of infection and can be transmitted naturally by ticks in the field (R. Bishop, R. Skilton, D. Odongo and S. Morzaria, unpublished data). The severity of ECF may vary depending on factors such as the virulence of the parasite strain, sporozoite infection rates in ticks and genetic background of infected animals. Indigenous cattle in East Coast fever-endemic areas are often observed to experience mild disease or subclinical infection, while introduced indigenous or exotic cattle usually develop severe disease.

The most practical and widely used method for the control of theileriosis is the chemical control of ticks with acaricides. However, tick control practices are not always fully effective due to a number of factors including development of acaricide resistance, the high cost of acaricides, poor management of tick control, and illegal cattle movement in many countries. Vaccination using attenuated schizont-infected cell lines has been widely used for T. annulata, while for T. parva control, infection and treatment using tick-derived sporozoites and tetracycline is being implemented in a number of countries in eastern, central and southern Africa.

Chemotherapeutic agents such as parvaquone, buparvaquone and halofuginone are available to treat T. parva and T. annulata infections. Treatments with these agents do not completely eradicate theilerial infections leading to the development of carrier states in their hosts.

The immune response to these parasites is complicated. Cell-mediated immunity is the most important protective response in T. parva and T. annulata. In T. parva, the principal protective responses are mediated through the bovine major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes. Theileria annulata schizonts inhabit macrophages and B cells. Innate and adaptive immune responses cooperate to protect cattle against T. annulata theileriosis. Infection of macrophages with T. annulata activates the release of cytokines, initiating an immune response and helping to present parasite antigen to CD4+ T cells. The CD4+ T cells produce interferon-γ (IFN-γ), which activates non-infected macrophages to synthesise tumour necrosis factor α (TNF-α) and nitric oxide (NO), which destroy schizont- and piroplasm-infected cells. B cells produce antibody that along with NO kill extracellular merozoites and intracellular piroplasms. On the other hand overproduction of cytokines, in particular TNF-α, by macrophages generates many of the clinical signs and pathological lesions that characterise T. annulata theileriosis and the outcome of the infection depends upon the fine balance between protective and pathological properties of the immune system.

B. DIAGNOSTIC TECHNIQUES

Diagnosis of acute theileriosis is based on clinical signs, knowledge of disease, and vector distribution as well as examination of Giemsa-stained blood, lymph node and tissue impression smears. Theileria parva and T. annulata are diagnosed by the detection of schizonts in white blood cells or piroplasms in erythrocytes. The schizontic stage follows the schizont stage and, in both T. parva and T. annulata, it is usually less pathogenic and is thus often found in recovering or less acute cases.
1. Identification of the agent (a prescribed test for international trade)

Multinucleate intralymphocytic and extracellular schizonts can be found in Giemsa-stained biopsy smears of lymph nodes, and is a characteristic diagnostic feature of acute infections with *T. parva* and *T. annulata*. Both intracellular and free-lying schizonts may be detected, the latter having been released from parasitised cells during preparation of the smears. Schizonts are transitory in *T. mutans* and the *T. sergenti–* *T. buffeli–* *T. orientalis* group, in which the piroplasm stage may be pathogenic. *Theileria taurotragi* schizonts are not readily detected in Giemsa-stained blood smears. A veil to the side of the piroplasm may distinguish *T. velifera*. The schizonts of *T. mutans*, if detected, are distinct from *T. parva*, having larger, flattened, and irregular nuclear particles. The piroplasms (intra-erythrocytic stage) of *T. parva*, *T. annulata* and *T. mutans* are similar, but those of *T. annulata* and *T. mutans* are generally larger and may be seen to divide. However, for practical purposes schizonts and piroplasms of different theilerias are difficult to discriminate in Giemsa-stained smears.

The schizont is the pathogenic stage of *T. parva* and *T. annulata*. It initially causes a lymphoproliferative, and later a lymphodestructive disease. The infected animal shows enlargement of the lymph nodes, fever, a gradually increasing respiratory rate, dyspnoea and/or diarrhoea. The most common post-mortem lesions are enlarged lymph nodes, a markedly enlarged spleen, pulmonary oedema, froth in the trachea, erosions and ulceration of the abomasum, and enteritis with necrosis of Peyer’s patches. Lymphoid tissues become enlarged in the initial stages of the disease, but then atrophy if the animal survives into the chronic stages of the disease. When examined histologically, infiltrations of immature lymphocytes are present in lung, kidney, brain, liver, spleen, and lymph nodes. Schizont-parasitised cells may be found in impression smears from all tissues: lung, spleen, kidney and lymph node smears are particularly useful for demonstrating schizonts. In longer standing cases, foci of lymphocytic infiltrations in kidneys appear as infarcts. In animals that recover, occasional relapses occur. A nervous syndrome called ‘turning sickness’ is sometimes observed in *T. parva*-endemic areas, and is considered to be associated with the presence of intravascular and extravascular aggregations of schizont-infected lymphocytes, causing thrombosis and ischaemic necrosis throughout the brain.

In *T. annulata*, both the schizont and piroplasm stages may be pathogenic. Schizonts are scarce in the peripheral blood of acutely sick animals and their presence in blood smears indicates a poor prognosis. However, schizonts can be easily detected in smears from lymph nodes, spleen and liver tissues obtained by needle biopsy of these organs. The gross pathology caused by schizonts of *T. annulata* resembles that of *T. parva*, while anaemia and jaundice are features of both schizont and piroplasm pathology. Pathogenic strains of *T. mutans* also cause anaemia, as can strains from Japan and Korea referred to as *T. sergenti*.

Piroplasms of most species of *Theileria* may persist for months or years in recovered animals, and may be detected intermittently in subsequent examinations. However, negative results of microscopic examination of blood films do not exclude latent infection. Relapse parasitaemia can be induced with some *Theileria* species by splenectomy. Piroplasms are also seen in prepared smears at post-mortem, but the parasites appear shrunken and their cytoplasm is barely visible.

2. Serological tests

- The indirect fluorescent antibody test (a prescribed test for international trade)

The indirect fluorescent antibody (IFA) test is the most widely used diagnostic test for *Theileria* spp.

- Preparation of schizont antigen
  - Schizont antigen slides

The antigens used for the IFA test are intracytoplasmic schizonts derived from infected lymphoblastoid cell lines for *T. parva* and from infected macrophage cell lines for *T. annulata*.

Cultures of 200 ml to 1 litre of either *T. parva* or *T. annulata* schizont-infected cells containing $10^6$ cells/ml of which at least 90% of the cells are infected, are centrifuged at 200 $g$ for 20 minutes at 4°C. The supernatant fluid is removed and the cell pellet is resuspended in 100 ml of cold (4°C) phosphate buffered saline (PBS), pH 7.2–7.4, and centrifuged as before. This washing procedure is repeated three times, and after the final wash the cell pellet is resuspended in PBS (approximately 100 ml) to give a final concentration of $10^7$ cells/ml.

Thin layers of the cell suspension are spread on Teflon-coated multipot slides, or on ordinary slides using TEXPEN or nail varnish for separation. The smears should give between 50 and 80 intact cells per field view when examined under a ×40 objective lens. The antigens are distributed on to the slides using immunoglobulin G (IgG) at a 1:100 dilution (for use in IFA testing).

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2 Obtainable from, for example, Bellco Glass, Vineland, New Jersey, United States of America or Glaxo-Wellcome, United Kingdom.

3 Obtainable from TWmark-tex, Roseland, N.J. 07068, USA.
using multichannel or a 100-µl pipette. By dispensing and immediately sucking up the schizont suspension, a monolayer of schizonts remains in each well. This is performed for each enclosure until the volume is exhausted. With this method, approximately 600 good quality slides containing a total of 6000 individual antigen spots can be obtained. The slides are air-dried, fixed in acetone for 10 minutes, individually wrapped in tissue paper and then in groups of five in aluminium foil, and stored in airtight, waterproof plastic containers at either −20°C or −70°C. The antigens keep for at least 1 year at −20°C and longer at −70°C.

ii) Schizont antigen in suspension

First, 500 ml of *T. parva* or *T. annulata*-infected cells containing \(10^6\) cells/ml are centrifuged at 200 g for 10 minutes at 4°C, and the cell pellet obtained is washed twice in 100 ml of cold PBS. The viability of the cells is determined by eosin or trypan blue exclusion (it should be greater than 90%). The cells are resuspended at \(10^7\)/ml in cold saline. To this volume, two volumes of a cold fixative solution containing 80% acetone and 0.1% formaldehyde (0.25% formalin) in PBS are added drop by drop while the cell suspension is stirred gently and continuously. The cell suspension is kept at −20°C and allowed to fix for 24 hours. The fixed cells are then washed three times in cold saline and centrifuged at 200 g for 20 minutes at 4°C. After the last wash, the cells are resuspended at \(10^7\)/ml saline. The fixed cells are distributed in aliquots of 0.5 ml. The antigen is stable at 4°C with 0.2% sodium azide as preservative for 2 weeks, and keeps indefinitely at −20°C. This method can also be used to prepare schizont antigen for *T. taurotragi* (J. Katende, A. Musoke and S. Morzaria, unpublished data).

- Preparation of piroplasm antigen
  
  i) Piroplasm antigen slides

  The piroplasm stage of *Theileria* spp. cannot be maintained in culture, therefore the piroplasm antigen must be prepared from the blood of infected animals. Experimental infections are induced by infecting cattle subcutaneously with sporozoites, or using ticks infected with *T. parva*, *T. annulata* or *T. taurotragi*. Infection with *T. annulata* is invariably produced by inoculation of blood drawn from cattle with acute theileriosis. Splenectomy of the recipient cattle prior to the infection considerably increases the piroplasm parasitaemia in red blood cells (RBC). Peak parasitaemias are of short duration and if animals survive the disease the percentage of infected RBC decreases considerably in a few days. Infections with the parasite group referred to as *T. sergenti/T. buffeli/T. orientalis*, *T. mutans* or *T. velfera* are usually induced by inoculating splenectomised cattle intravenously with blood from a carrier animal, or with a blood stabilate, or by application of infected ticks. When the piroplasm parasitaemia is 50% or higher, 100 ml of the infected blood is collected from the jugular vein in a heparinised or ethylene diamine tetra-acetic acid (EDTA) vacutainer, and gently mixed with 2 litres of PBS. The mixture is centrifuged at 500 g for 10 minutes at 4°C; the plasma and buffy coat are removed, the RBC are again resuspended in 2 litres of PBS, and the centrifugation step is repeated. It is important to remove the buffy coat after each wash. This washing procedure is repeated four times. After the final wash, an aliquot of the packed RBC is used to make doubling dilutions in PBS, and a 5 µl drop of each dilution is placed on slides. The dried spots are fixed in methanol and stained with Giemsa’s stain, and the concentration of RBC is examined using a light microscope. The dilution that gives a single layer of RBC spread uniformly on the spot is then selected for large-scale preparation of piroplasm antigen slides. Approximately 10,000 antigen slides (100,000 antigen spots) can be prepared from 100 ml of infected blood. The antigen smears are allowed to dry at room temperature before fixing in cold (4°C) acetone for 10 minutes. The fixed smears can be stored as for the schizont antigen slides, and kept for similar periods.

  ii) Piroplasm antigen suspension

  An alternative method of preparing antigens to that described above is available, and has been tested for *T. parva*. In this procedure, 100 ml of blood are taken from an animal with a high piroplasm parasitaemia and prepared as described previously, and the packed cell volume is adjusted to 5% in PBS.

  One volume of the RBC suspension is added to two volumes of the fixative (see above schizont antigen in suspension) while stirring. The cells are allowed to fix at −20°C for 24 hours. The fixed cells are then washed three times with PBS and centrifuged at 1000 g for 30 minutes. The deposit is resuspended to the original volume of blood with PBS containing 0.2% sodium azide, and distributed in aliquots of 0.5 ml.

  The piroplasm antigen is stable at 4°C when preserved with 0.2% sodium azide for a period of at least 3 years.

- Standardisation of antigen

  Schizont or piroplasm antigen suspensions are mixed on a rotor mixer and titrated in PBS by doubling dilution starting from undiluted through to 1/16. The dilution giving a cell distribution of approximately 50–80 schizont-infected cells or 150–200 infected RBC per field view when examined under a ×40 objective
lens is recommended for use for that batch of antigen. Using this dilution, test antigen smears are prepared on slides. These antigen smears plus the antigen slides previously frozen (and thawed before use) are tested against a range of dilutions of a panel of known strong, intermediate and weak positive and negative control sera. If the positive control sera titrate to their known titres and the negative control sera give no fluorescence, the antigen is used in the routine IFA test.

Both types of antigen preparations, acetone-fixed smears stored at either –20°C or –70°C, and antigens fixed in suspension and stored at either 4°C or –20°C, are used routinely in many laboratories. The sensitivity of both types of antigen is comparable. In laboratories where adequate low temperature storage facilities and a reliable supply of electricity are available, the antigen slides can be used. However, such antigens can only be transported on dry ice or in liquid nitrogen. Antigens fixed in suspension have the advantage over antigen slides in that the initial method of preparation is simpler and quicker. A large batch of this antigen can be stored in one container, and aliquots may be taken out as necessary from which fresh smears are prepared for the IFA test. The need for a large storage facility is thereby avoided. The antigens fixed in suspension can also be stored at 4°C and can be safely transported at room temperature without loss of antigenicity.

- **Preparation of bovine lymphocyte lysate**

  A lymphocyte lysate is prepared according to the method described by Goddeeris et al. (1982), for use in tests with antigens of *T. parva* in suspension. Briefly, a 3-month-old calf is splenectomised and maintained in a tick-free environment. To exclude the possibility of latent theilerial infections, Giemsa-stained blood smears are examined daily for a period of 4 weeks for parasites. The parasite-free animal is killed and the thymus and all the accessible lymph nodes are removed. These tissues are sliced into small pieces in cold PBS containing 0.45% EDTA as anticoagulant. Cells are teased out of the tissue, separated from the debris by passing through a muslin cloth, and washed three times with PBS/EDTA by centrifugation at 200 g for 20 minutes at 4°C. The washed lymphocytes are resuspended in PBS without EDTA, to give a final concentration of 5 × 10^7 cells/ml. The cells are disrupted by sonication in 100-ml aliquots on ice for 5 minutes using the 3/8 probe. The sonicated material is centrifuged at 1000 g for 30 minutes at 4°C, and the supernatant, adjusted to 10 mg protein/ml, is stored at –20°C in 4-ml aliquots.

- **Test procedure**

  1. **With schizont or piroplasm slide antigen**
     i) Remove antigen slides from freezer and allow to thaw for 30 minutes at 4°C and then for 30 minutes at room temperature.
     ii) Inactivate the sera to be tested for 30 minutes in a water bath at 56°C.
     iii) Unpack the slides and label the numbers of the sera tested.
     iv) Prepare 1/40 and 1/80 dilutions of sera to be tested. Validated positive and negative sera are included with each test as controls. Further doubling dilutions can be made if end-point antibody titres are desired.
     v) Transfer 25 μl of each serum dilution to a spot of antigen.
     vi) Incubate in a humid chamber for 30 minutes at room temperature.
     vii) Remove the serum samples from the antigen wells by washing with PBS and rinse by immersing in two consecutive staining jars containing PBS for 10 minutes each time.
     viii) Distribute to each well 20 μl of diluted anti-bovine immunoglobulin fluorescein isothiocyanate conjugate at appropriate dilution (generally, dilutions recommended by manufacturers are suitable; however, minor adjustments may be necessary for optimal results). Incorporate Evans blue into the conjugate at a final dilution of 1/10,000 as a counterstain and incubate in a humid chamber for 30 minutes at room temperature.
     ix) Repeat step vii and mount with a cover-slip in a drop of PBS/glycerol (50% volumes of each).
     x) Read the slides under a fluorescent microscope equipped with epi-Koem illumination (100 W mercury lamp), UV filter block, ×6.3 eyepieces and Phaco FL 40/1.3 oil objective lens.

  2. **With schizont antigen stored in suspension**
     i) Thaw frozen antigen at room temperature.
     ii) Distribute the antigen suspension on the spots of multispot slides, using multichannel or a 100-µl pipette. By dispensing and immediately sucking up the suspension a monolayer of schizont-infected cells remains on each well.
     iii) Allow slides to dry at room temperature or 37°C.
iv) Dilute test and control sera 1/40 in lymphocyte lysate (195 μl lymphocyte lysate + 5 μl serum).

v) Proceed as described in v to x in the test procedure with slide antigen.

With piroplasm antigen stored in suspension

i) Resuspend piroplasm antigen (stored at 4°C) by agitation and disperse RBC by passing the suspension through a 25-gauge needle to break the clumps.

ii) Dilute the antigen to previously standardised dilutions (see preparation of piroplasm antigen).

iii) Allow slides to dry at room temperature or 37°C.

iv) Proceed as described in iv and v in the test procedure with schizont antigen in suspension.

• Characteristics of the indirect fluorescent test

The incorporation of Evans blue provides a good contrast, enabling good differentiation of non-infected cells from the infected ones under the fluorescent microscope. Mounting the slides in 50% glycerol, at pH 8.0, reduces the rapid fading of FITC and makes photography of the preparation possible. Once prepared, slides are stable and can be read for up to 72 hours after preparation when kept at 4°C in the dark.

The sensitivity of the IFA test depends upon the period elapsed from infection. Following infection with sporozoites, antibodies to *T. parva* and *T. annulata* are first detected between days 10 and 14 using the schizont antigen. Using the piroplasma antigen, antibodies are first detected between days 15 and 21. Antibodies last for a variable period of time after recovery, depending on such factors as the establishment of a carrier state, chemotherapeutic intervention, and presence or absence of a rechallenge. Following recovery from infection with *T. parva* or *T. annulata* theileriosis, high levels of antibody are generally detected for 30–60 days. The antibody levels gradually decline and low antibody titres are still detectable 4–6 months after recovery. Later, antibody may become undetectable at a serum dilution of 1/40, but may persist for more than 1 year following a single challenge. In ECF endemic regions, the seroprevalence in cattle population fluctuates considerably depending on the level and regularity of challenge. In an epidemiological study with *T. parva* the overall diagnostic sensitivity of the IFA test has been evaluated as 55% at a cut off titre 1/40 and 28% at cut off 1/160. The specificity of the test for the two cut off points was 86% and 95% respectively (Billiouw *et al*., 2005).

The IFA test is useful for identifying herds that contain carriers of *T. annulata*, but is not always sufficiently sensitive to detect all infected individuals. Both schizont and merozoite (piroplasm) IFA antigens have failed to detect antibody in some animals despite carrying patent infection with piroplasms (Darghouth *et al*., 1996).

In *T. mutans* infections induced by sporozoite inoculation, antibodies are first detected between days 10 and 15 after the appearance of piroplasms. Low titres are detectable for at least 12–24 months.

The *T. parva* IFA test is highly sensitive for detection of antibodies in an epidemiological situation where only one species of *Theileria* exists. However, if the test is used to detect antibodies where mixed infections of *Theileria* occur, the specificity of the test needs to be carefully evaluated. For example, *T. annulata* and *T. parva* cross-react, although these cross-reactions are four- to six-fold lower than with the homologous sera. The cross-reactivity between the two species has little practical significance as the geographical distribution of these two parasites does not overlap. In the IFA test such cross-reactivity does not occur between *T. parva* and *T. mutans* or between *T. annulata* and *T. mutans*. There is a low level of cross-reactivity between *T. parva* and *T. taurotragi*, reducing the specificity of these two tests in many parts of sub-Saharan Africa where their distribution overlaps.

A panel of monoclonal antibodies (MAs) detecting various epitopes on the polymorphic immunodominant antigen of the *T. parva* schizont stage has been generated. This panel can be used in the IFA test using the schizont-infected lymphoblastoid cells (see footnote 2) to detect differences between certain stocks of *T. parva* and between *T. parva* and other theilerial species. This test has been deployed as one of the several characterisation tools to differentiate various stocks of *T. parva*, and for quality control during sporozoite stabilate preparation (Bishop *et al*., 1994).

• Future tests for *Theileria* diagnosis

The IFA test is easy to perform and provides adequate sensitivity and specificity for use in the field for detection of prior infection with *T. parva* and *T. annulata* infections under experimental situations and in a defined epidemiological environment where only one theilerial species is present. The IFA test has limitations for large-scale serological surveys due to its reduced specificity in field situations where several *Theileria* species co-exist. There is a need for tests that are more specific, easy to interpret, and robust
enough to be used in field conditions. Serological tests based on the enzyme-linked immunosorbent assays (ELISA) are being used increasingly for the detection of parasite-specific antibodies. ELISAs have been successfully adapted for the detection of antibodies to *T. annulata* (Gray et al., 1980), and has been shown to detect antibodies for a longer period of time than the IFA (Kachani et al., 1992; 1996). An ELISA for *T. mutans* has also been described (Katende et al., 1990). Two MAbs specific for *T. mutans* have been used in the ELISA system for the detection of antibodies and antigens in acute, subacute and chronic infections. The test is more specific and sensitive than the IFA test. However, the tests now most widely used for *T. parva* and *T. mutans* are indirect ELISAs based on parasite-specific antigens, PIM and p32 respectively. These tests have been extensively evaluated in the laboratory and the field, and are now being used in large parts of Africa. The antigens being used in these tests are expressed in *Escherichia coli* using pGEX as the expression vector (Morzaria et al., 1999a; Musoke et al., 1994). The expressed products are fusion proteins with glutathione S transferase, and are directly coated on to ELISA plates. These ELISAs provide higher (over 95%) sensitivity and specificity than the IFA tests (Morzaria et al., 1999a; Musoke et al., 1994) and are soon expected to be available commercially.

A range of probes is available to detect all the *Theileria* species that are known to infect cattle and are based on ribosomal RNA gene sequences (Alisopp et al., 1993; Bishop et al., 1995). DNA probes specific for *T. parva* (Alisopp & Allisopp, 1988; Conrad et al., 1987; Morzaria et al., 1999a) and *T. mutans* (Morzaria et al., 1989), have also been developed. The technology of the polymerase chain reaction (PCR) is available to amplify minute quantities of parasite DNA one million-fold, thereby greatly increasing the sensitivity of the DNA probes (Alisopp et al., 1989). A specific PCR was developed to test whole blood samples from *T. annulata*-carrier cattle (D’Oliveira et al., 1995). A reverse line blot (RLB) assay based on hybridisation of PCR products to specific oligonucleotide probes immobilised on a membrane for simultaneous detection of different *Theileria* species has been introduced (Gubbels et al., 1999). It is hoped that a combination of ELISA, PCR and DNA probes will greatly enhance our present capacity to identify infected animals, thus making possible accurate surveys of *Theileria* species. Eventually, the aim would be to develop these technologies for the diagnosis of all the vector-borne diseases.

PCR amplification of the p33/34 genes of the *T. sergenti/T. buffeli/T. orientalis* complex followed by restriction enzyme analysis can be used to differentiate *T. sergenti* from *T. buffeli*/*T. orientalis* (Kawazu et al., 1992).

### C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

#### C1. Cell culture vaccines for *Theileria annulata*

Vaccination against *T. parva* and *T. annulata* has been attempted since the causal organisms were first recognised early in the last century. However, reliable live vaccines of known potency are a more recent development. The most widely used are attenuated schizont cell culture vaccines against *T. annulata*. The procedures for production and safety testing have been described (Food and Agriculture Organization of the United Nations (FAO), 1984; Hashemi-Fesharki, 1988; Pipano, 1989b), and the vaccine is used in Israel, Iran, Turkey, Spain, India, northern Africa, central Asia and the People’s Republic of China.

Despite the fact that vaccination with the cell culture vaccine against *T. annulata* has been available for more than three decades and has shown to be effective under field conditions, the use of this vaccine has been limited. The concern about the introduction of vaccine-derived parasites into the field tick population has led to individual countries developing vaccines from local isolates (Morisson & Mc Keever, 2006). Some attenuated cell lines have lost the ability to differentiate to erythrocytic merozoites (piroplasms) when inoculated to cattle and in one instance, *Hyalomma* nymphs fed on vaccinated cattle did not become infected (Kachani et al., 2004a). However in most cases the loss of differentiation is based on macroscopic examination of blood films from vaccine inoculated cattle. This drawback, the difficulties in standardisation of the antigenic composition of the cultured parasites and the need of a cold chain for distribution of the vaccine to the field are limiting factors in commercialisation of this vaccine (Morisson & Mc Keever, 2006).

1. **Seed management**
   
   a) **Characteristics of the seed**

   Primary cultures of *T. annulata*-infected cells may be established from trypsinised lymph nodes, liver, or spleen taken aseptically from an infected animal after death, or from the buffy coat of heparinised peripheral blood separated on a density gradient (Ficoll Hypaque), or by lymphocytes harvested from lymph node biopsy material using a plastic syringe method (Brown, 1979; FAO, 1984).

   Seed cultures are prepared from cryopreserved cell lines that have been isolated from cattle and attenuated as described below. Vaccines should be produced from a seed culture (master seed) that has been passed
less than 30 times, because there is some uncertainty about the immunogenic stability of these cultures in long-term passage.

b) Method of culture

The infected cells are cultured initially in Eagle’s minimal essential medium (MEM) or Leibovitz L15 medium supplemented with 20% calf serum and containing penicillin (100 units/ml), streptomycin (50 µg/ml), and mycostatin (75 units/ml) in 25-ml plastic screw-cap tissue-culture flasks. An alternative medium is RPMI 1640 with 10% fetal calf serum, 2 mM glutamine, penicillin and streptomycin, and is usually used with established cultures. Medium is replenished every 3–4 days. The presence of bright refractile cells free in the medium (on examination using a phase-contrast or inverted microscope) is indicative of infected cell growth. The cultures may establish as a monolayer or in suspension. Passage is effected by decanting the medium, adding 0.025% EDTA (versene) for 15 minutes to monolayer cultures, dispersing the cells, then counting and dispensing according to flask size. Approximately 10^6 cells are introduced into a 25 cm^2 flask, and the same seed rate in 100–200 ml is used in larger flasks. The general culture technique is as described by Brown (1979).

Serum is essential for maintenance of these cultures, and is obtained either from calves up to the age of 6 months, or from commercial sources, and is tested for toxicity through three passages in an established cell line before use.

c) Attenuation of virulence

Attenuation of *T. annulata* schizonts is achieved by prolonged growth and passage in culture (Pipano, 1989b). The loss of parasite virulence appears to be due to a change in parasite gene expression. Attenuation is assessed by the inoculation of the culture into susceptible calves every 20–30 passages. A sample of culture should be cryopreserved every ten passages in case of accidental loss or contamination. Complete attenuation is achieved when cultures do not cause fever or detectable schizonts and piroplasms in susceptible cattle. An attenuated culture will reliably infect cattle at 10^5 cells and induce a serological reaction, and will not produce disease at 10^9 cells. Cultures may be cryopreserved using either dimethyl sulphoxide (DMSO) or glycerol. Two methods of storing and delivering the vaccine are described below.

2. Method of manufacture

Before starting to produce vaccine, seed material with known characteristics is required (Pipano, 1997). Three types of seed material are distinguished:

**Master seed:** Schizont-infected cells from a specific passage that have been selected and permanently stored and from which all other passages are derived. The master seed should consist of a single uniform batch of seed that has been mixed and filled into containers as one batch. As *T. annulata* schizont infected cells are used for the manufacturing process, the master seed also represents the master cell stock (see Chapter 1.1.6 Principles of veterinary vaccine production). To prepare a master seed, schizont-infected cells that have proved to be safe for cattle are propagated to obtain in a single culture passage approximately 5 × 10^8 cells. The cells are cryopreserved in about 100 cryotubes each containing 5 × 10^6 cells. A viability check of the master seed should be performed once the master seed has been cryopreserved for at least 24 hours by reviving one of the cryotubes.

**Working seed:** Schizont-infected cells at a passage level between the master seed and the production seed. To prepare a working seed, the contents of a single cryotube of master seed are transferred to a 10 ml centrifuge tube containing 8 ml complete medium. The tube is centrifuged at 600 g for 15 minutes at 4°C and the pellet is transferred into a 75 cm^2 culture flask containing 15–20 ml medium. The medium is replaced the next day, and 4 days later the cells are dispersed and subcultured in larger vessels. After 5–6 subcultivations, a sufficient number of infected cells is available to start the production run.

**Production seed:** Schizont-infected cells from a specific passage level are used without further propagation for the preparation of a batch of vaccine. The production seed is obtained by propagating large numbers of cells in monolayer or suspension cultures. Monolayer cultures are grown in flasks, 150 cm^2 to 175 cm^2, which usually provide an average of from 7 × 10^7 to 8 × 10^7 cells per vessel. About 80 ml of complete medium per flask is required. In a roller bottle culture system, 1.2–1.5 × 10^8 cells can be obtained in a conventional roller bottle (700 cm^2) containing 100–120 ml of medium. To obtain optimal yield of cells, stationary cultures or roller bottles cultures are incubated for 6–7 days with culture media as described previously, see Section C1.1.b.

The schizont-infected cells from all vessels are harvested and pooled together and the total number is computed. Alternatively, about 20% of the cells may be seeded again to prepare another batch of vaccine. Several batches of vaccine can be produced using a portion of the production seed as working seed. As prolonged cultivation may
generate alteration in the futures of the schizonts, such as immunogenic capacity, after several batches, subsequent vaccine is produced by making fresh production seed from the master seed.

Schizont-infected cells are mixed with DMSO at a final concentration of 7% or glycerol at a final concentration of 10%, and dispersed in 1.8-ml aliquots into 2-ml plastic vials, each vial containing ten doses of concentrated vaccine. As DMSO immediately penetrates the cell membranes, the time spent in dispensing the vaccine into the vials should be as short as possible. When glycerol is used, an equilibration time of 30–40 minutes is required before freezing the vaccine. There is no consensus on how many schizont-infected cells should constitute one dose of the vaccine. A recommended practical approach is to prepare doses of $10^5$–$10^7$ infected cells in order to counteract variable environmental conditions in the field. However, considerable protection against sporozoite-induced infection has been achieved by vaccination with $10^5$ infected cells (Kachani et al., 2004b).

The vaccine is frozen by introducing the vials in an ultracold deep freezer (–70°C) and 24 hours later transferred to liquid nitrogen containers. Alternatively vials can be introduced in gas phase liquid nitrogen for 3 hours and then immersed in the liquid nitrogen for storage (Pipano, 1989b). Vaccine is transported to the field in liquid nitrogen, and diluted 1/10 in isotonic buffered saline before aseptic withdrawal. For dilution of vaccine frozen with glycerol, isotonic buffered saline should contain 10% glycerol in order to avoid osmotic damage to the schizonts. The vaccine is administered subcutaneously within 30 minutes of thawing (Pipano, 1977).

The vaccination regimen in Iran consists of inoculation of two doses of vaccine prepared from two different stocks, 30–60 days apart (Hashemi-Fesharki, 1988). A fresh culture vaccine is used in Morocco, usually at a tenfold lower dose ($10^4$ schizont-infected cells) (Kachani et al., 2004b). However, there are problems with quality control of vaccines with short shelf life.

**Safety precautions**

*Theileria annulata* shizonts are not hazardous for humans or contagious for animals, therefore the main purpose in designing a vaccine production facility is to prevent contamination of the product by extraneous organisms.

### 3. Batch control

In Israel the schizont vaccines are tested using a documented procedure (Pipano, 1989a) before release.

The frozen vaccine has a practically unlimited shelf life. Usually, the schizont vaccine is produced in small individual batches (3–5 thousand doses), which makes the full testing of each batch impractical for economic reasons. It is recommended therefore that the first batch of vaccine produced from a master seed be tested for safety, efficacy, potency and sterility, while each subsequent batch be tested for sterility and potency only. This recommendation is based on the fact that once the cultured schizonts become attenuated, no reversion to virulence has ever been observed during further cultivation. As far as efficacy is concerned, no obvious alteration of the immunogenic properties has been observed during the limited number (20–30) of passages involved in producing the actual vaccine.

**a) Safety**

*Freedom from properties causing undue local or systemic reactions:* for testing the safety of the master seed, two to four susceptible calves, of the most sensitive stock available, are inoculated with a tenfold greater dose than is recommended for immunisation. This dose should not produce clinical signs beyond a transient rise in temperature. With completely attenuated master seed, no schizonts or piroplasms will be seen in lymph node and liver smears or in blood films. However, different breeds of cattle may show different sensitivities to the vaccine. This should be borne in mind when vaccine from a partially attenuated master seed is to be administered to high-grade cattle stocks.

Following a successful test for safety of a sample, all subsequent batches produced from the same master seed can be released without further testing for safety. However, if parasites are detected in the blood or tissues of vaccinated field animals, or if clinical signs develop following the inoculation of the vaccine, the batch or a parallel batch, from the same master seed, should be retested for safety.

**b) Efficacy**

*Capacity to protect against naturally transmitted theileriosis:* The batch of experimental vaccine used for the safety test can also be used for testing efficacy of the culture-derived anti-theilerial vaccine. Three or four calves are vaccinated with a conventional dose of vaccine and 6 weeks later; the vaccinated calves and the same number of unvaccinated calves are infected with sporozoites of *T. annulata*. Infection can be induced by live adult ticks issued from *T. annulata*-infected preimaginal stages or by inoculation of stabibrate prepared from macerated infected ticks (for techniques see Section C2.1). Experience shows that inoculation of stabibrate (macerated ticks) generally induces a more severe response than an equivalent number of live,
infected ticks allowed to feed on the cattle. However in the long run, the results obtained by challenge with stabilitate appear to be more reproducible than those obtained with different batches of live ticks.

There are no internationally agreed standards for the size of a challenge dose used in testing the efficacy of *T. annulata* culture-derived vaccine. Five to ten female and the same number of infected, unfed male *Hyalomma* ticks have been used for infection of cattle. Alternatively, stabilitate equivalent to 2–4 macerated ticks inoculated subcutaneously in the neck area will invariably produce acute theileriosis. The responses to the challenge infection of the vaccinated and unvaccinated control calves are monitored using the following parameters: duration and severity of pyrexia, rate of schizont-infected cells in lymph node or liver biopsy smears, rate of piroplasm infected erythrocytes in the blood films, decrease in white and red blood cell counts, and severity of clinical manifestations such as anorexia, depression and recumbency.

The results of the efficacy test depends on factors such as immunological characteristics of the *T. annulata* isolate grown and attenuated in culture, the virulence and dose of the field isolate used for challenge, the species of infected ticks used to produce sporozoites. Research studies (Pipano, 1989b) show that calves vaccinated with stabilitate vaccine may exhibit an apparently near total protection or show a low level parasitaemia, accompanied by mild fever and insignificant alteration of the remaining parameters from their pre-vaccination values following a potentially lethal homologous challenge. A lesser degree of protection has been exhibited when cattle vaccinated with stabilitate vaccine were challenged with tick-derived parasites from a geographically remote area. In contrast, in most of the trials the non-vaccinated control calves have exhibited a high level of parasitaemia and pancytopenia accompanied by severe clinical manifestations. In the absence of specific medication, the majority of the control animals have succumbed to the infection (Pipano, 1989b). Controversial results about the length of immunity engendered by vaccination with the cell culture vaccine have been obtained. Periods of from more than 48 months (Stepanova & Zablotskii, 1989) to less than 13 months (Ouelli et al., 2004) have been reported.

Field observations have also been used for evaluation of the efficacy of anti-theilerial vaccines (Pipano, 1989a; Stepanova & Zablotskii, 1989). Susceptible indigenous cattle as well as high-grade exotic breeds were protected against clinical theileriosis and death in pastures on which nonvaccinated cattle succumbed to theileriosis. As completely attenuated schizont vaccine does not yield piroplasms, the presence of this theileriosis stage in vaccinated cattle showing no clinical signs is considered to be the result of unapparent tick-induced infection.

c) **Potency**

*Viability of schizont-infected cells:* The potency test is conducted by quantitative in-vitro methods. Frozen vaccine remains stable during the storage period, even for long periods, but some loss of viability occurs during the freezing and thawing processes. Viability should be tested under conditions as similar as possible to those obtained when the vaccine is used in the field. For this reason, vaccine should be thawed and the diluted suspension of schizont-infected cells should be left at ambient temperature for 60 minutes before performing the viability tests. A simple test for evaluating viability of the infected cells is nigrosin dye exclusion counting (Wathanga et al., 1986). Vaccine that, after being thawed and diluted and left at room temperature for 1 hour, still contains 50% or more live cells can be released for use although in most cases 80–90% of live cells are found.

Viability of the schizonts is also reflected by the plating efficiency of the schizont-infected cells (Wathanga et al., 1986), as only cells containing viable schizonts multiply in culture. For this purpose, the thawed, diluted vaccine is transferred from the bottle to a centrifuge tube. A sample for counting is taken and the suspension is centrifuged for 15 minutes at 600 g. Meanwhile, the total number of cells (live and dead) is determined in order to ascertain that the frozen vaccine had the necessary initial concentration of cells. After centrifugation, the supernatant is discarded and the cells are resuspended to the original volume using complete culture medium. Serial tenfold dilutions of cells in complete medium are performed in sterile 10 ml tubes so that the last two dilutions contain 5 × 10⁵ and 5 cells per ml. Twelve replicates of 200 µl from each of the last two dilutions are introduced into a 96-well culture plate. The plates are incubated at 37°C in a 5% CO₂ atmosphere and cultures are checked with an inverted microscope 6 and 9 days after seeding. The number of wells theoretically containing 1 cell each in which growth is observed is counted. Vaccine showing a plating efficiency <2 (cells) are adequate for field use.

d) **Sterility**

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

e) **Method of use**

The frozen vaccine is viably preserved in large liquid nitrogen refrigerators at production facility and transported to farms in smaller liquid nitrogen containers. Field centres for storage and supply of vaccine can...
be set up in theileriosis-enzootic areas. The basic equipment required for field application of frozen vaccine includes a wide-mouthed jar for preparing a 40°C water bath, a thermometer for measuring the temperature of water, long forceps, face shield and temperature-resistant gloves. Application of the frozen vaccine to field cattle begins by donning the face shield and temperature-resistant gloves. The required numbers of vials are withdrawn with the forceps from the canister of the liquid nitrogen refrigerator. When withdrawing the vials, the canister should be kept as deep as possible in the neck of the refrigerator to avoid quick warming of the remaining vials. Each withdrawn vial should be checked in order to ascertain that liquid nitrogen has not leaked inside. The liquid nitrogen does not alter the vaccine, but may cause the vial to explode when introduced in the water bath. Such a vial should be held at ambient temperature for 1–2 minutes to allow the nitrogen to escape and then processed in the usual way. Leaking of liquid nitrogen into a vial containing frozen vaccine has raised questions to about the sterility of the frozen vaccine. However the system has been used for decades with no significant problem observed. The vaccine is administered subcutaneously within 30 minutes of thawing (Pipano, 1977).

These vaccines produce no adverse effects in healthy cattle. However, animals with existing infections, particularly viral infections, may not tolerate vaccination well. The administration of a viral vaccine, such as for foot and mouth disease, during the immunisation period (reaction period) is not recommended as the immune response may be compromised (Hashemi-Fesharki, 1988). In Iran, it is not recommended to vaccinate cows that are over 5 months pregnant, although studies in pregnant cattle with the vaccine stocks used in Israel found no effect on pregnancy (Pipano, 1989a). The immunity engendered is long lasting.

In general, cattle should be immunised in the first few months of life, and tick challenge under natural conditions reinforces the immunity. Although antigenically different strains of *T. annulata* have been identified (Pipano, 1977), it is generally considered that there is sufficient cross-protection among strains to provide adequate protection against field challenge as observed in Israel. In the vast infected areas of central Asia, a single stock has proved immunologically effective in 1.5 million cattle (Dolan, 1989; Wathanga et al., 1986). However, as described previously, two stocks are used routinely in Iran (Hashemi-Fesharki, 1988).

C2. Immunisation of cattle against *Theileria parva* by the infection and treatment method

Vaccination against *T. parva* is based on a method of infection and treatment in which an aliquot of viable sporozoites is inoculated subcutaneously, and the animals are simultaneously treated with a formulation of a long-acting tetracycline (Radley, 1981). Tetracyclines reduce the severity of the infection, and the resulting mild infection is usually controlled by the host’s immune response, so that a carrier state is achieved. There are always risks associated with the use of live parasites for immunisation, however, with appropriate quality control and careful determination of a safe and effective immunising dose, the method can and is being used successfully in the field. This method has also been applied effectively for *T. annulata*, but cell culture vaccination, which is not practical for immunisation against *T. parva*, is preferred. Some *T. parva* stocks have been shown to infect cattle reliably without inducing disease, and these can be used without tetracycline treatment. One such stabilate is being applied in the field and offers considerable advantages over potentially lethal stabilate infections and savings in the cost of vaccination. However, different stabilates of these stocks can produce severe disease in cattle, emphasising the importance of a carefully controlled immunising dose.

1. Stabilate preparation

For consistency in immunisation in field, it is essential that tick-derived sporozoite stabilates of an immunising stock are prepared from a fully characterised ‘working seed stabilate’. The ‘working seed stabilate’ should be derived directly from the reference ‘master seed stabilate’, which is available in suitable quantity for future preparation of immunising stabilates. Immunising stabilates can be prepared according to a proposed set of standards (Morzaria et al., 1999b).

Infection is established, with the working seed stabilate of *T. parva*, by inoculation of healthy cattle serologically negative for tick-borne diseases. During the parasitaemic phase of the ensuing disease reaction, clean laboratory-raised nymphs of *Rhipicephalus appendiculatus* are fed on the animals, and the engorged infected ticks are collected. The resultant adult ticks, within 3 weeks to 4 months after moultling, are applied in ear-bags to healthy rabbits. About 600 ticks are applied to each ear and unattached ticks are removed after 24 hours. After 4 days, the ticks are removed and samples (usually 60 ticks) taken to determine infection rates in dissected salivary glands. The remaining ticks are counted into batches of approximately 1000. An estimate of the total number of ticks can be obtained by counting and weighing a given number of ticks and then weighing the total number of ticks. The ticks are washed in a sieve under fast flowing tap water and may be surface disinfected in 1% benzalkonium chloride, or in 70% alcohol, and then rinsed again in distilled water.
The ticks are placed (~1000) in heavy glass specimen jars or plastic beakers, and 50 ml MEM with Hank’s or Earle’s salts and 3.5% bovine plasma albumin (BPA) is added. The jars are kept on ice, and the ticks are ground using a tissue homogeniser (for instance Silverson LR2) for 2 minutes using a large aperture disintegrating head, and for 3 minutes using a small aperture head (emulsol screen). The ground-up tick material is made up to 50 ml for every 1000 ticks, then centrifuged at 50 g for 5 minutes, and the supernatant is harvested. An equal volume of cold 15% glycerol in MEM/BPA is added dropwise while the tick material is maintained chilled on ice and stirred by a magnetic stirrer. The final volume will contain sporozoites from the equivalent of ten ticks/ml. The number of tick-equivalents/ml can be adjusted if parasite infection rates in a particular tick batch were either very high or very low. The final concentration of glycerol in the sporozoite stabilate is 7.5%.

The ground-up tick material is then dispensed into glass vials by syringe or pipette for small total volumes, or by automatic syringe for larger volumes. Alternatively, artificial insemination equipment, as used to dispense semen, has been used with pre-labelled plastic straws. This latter system is ideal for large volume stabilates, and colour coding and labelling provide additional check on the identity of the immunising stabilate. An equilibration time of 30–45 minutes should be allowed for small-volume stabilates before they are placed in a deep freezer (~70°C). Once frozen, the stabilate may be transferred to permanent storage in liquid nitrogen taking care not to allow any significant increase in temperature during transfer.

The evaluation of the number of acini infected with T. parva in dissected tick salivary glands, before grinding, is a useful indicator of the level of infection but does not take into account the variable loss of viability during stabilate preparation caused by the intensity of grinding and the freeze–thaw processes. Furthermore, the state of maturation of the sporozoites is difficult to estimate by histological examination of the tick salivary glands. Therefore, the infectivity of the stabilate is determined by inoculation of a standard dose of 1.0 ml into susceptible cattle. The contents of 2–4 randomly selected tubes is mixed and then titrated in cattle, and its infectivity and lethality at different dilutions are established for use in immunisation. As the response of cattle to the infection and treatment method is dependent upon their susceptibility to the infection, it is important to titrate stabilates in cattle of the same type as those to be immunised. The sensitivity to tetracyclines is also determined, essentially to provide a dose of stabilate that is controlled, preferably by a single dose of long-acting tetracycline administered at the same time as inoculation. The immunising dose should induce a very mild or unapparent infection (Anon, 1989), and the animal should develop a serological titre and be immune to lethal homologous challenge. Should a single treatment with tetracycline fail to suppress the infection in all cattle, then either a lower dose of the immunising stabilate or two treatments of tetracycline (on days 0 and 4) may be used. A single dose of 30 mg/kg long-acting oxytetracycline has been found to be effective in field immunisations, when used with an appropriate stabilate dilution. An alternative method that has been used involves stabilate infection and treatment with parvaquone at 20 mg/kg on day 8 (depending on the stabilate). This method can be applied where tetracyclines are not reliable, but it requires that the animal be handled more than once. A single treatment with buparvaquone at 2.5 mg/kg at the time of infection has also been shown to be effective with stabilate infections that were not controlled with a single treatment at 20 mg/kg of a long-acting formulation of tetracycline.

Once the procedure which results in a safe and effective immunising dose is established, it must be adhered to strictly in the field, or breakdown of immunisation may occur. It is also important that the stabilate dilution and drug/dose regimen be determined in the most susceptible cattle in which it is likely to be used. The infection and treatment method is usually applied using long-acting tetracycline, and it is recommended that the tetracycline be administered first, in case an animal escapes having received stabilate only.

2. Safety precautions

At a meeting in Malawi in 1988, the following recommendations on safety in the preparation, handling and delivery of T. parva infection and treatment vaccines were adopted (Anon, 1989).

a) Field collection of ticks

It is important that well characterised laboratory strains of Rhipicephalus appendiculatus be used during preparation of immunising stabilates.

If field ticks are collected for experimental purposes, then consideration should be given to the possible hazard to humans from pathogens present in these ticks. The most important pathogen that has been recognised is Crimean–Congo haemorrhagic fever virus, usually associated with ticks of the genus Hyalomma and widely prevalent within the geographical distribution of R. appendiculatus. Those handling field tick collections should, therefore, be made aware of potential hazards. Ticks of Hyalomma species generally should not be removed from hosts; engorged or partially engorged ticks should not be crushed between the fingers. If removed, ticks should be handled with a forceps.

b) Tick-handling facilities

The handling of field-collected ticks in the laboratory must be controlled in order to avoid accidental attachment to personnel. Field-collected ticks should be fed on rabbits and cattle in isolation facilities.
Animals on which laboratory-infected or field-collected ticks have fed should be destroyed. Following engorgement of field-collected ticks on laboratory animals, aliquots should be homogenised and tested for extraneous human pathogens by inoculation in baby hamster kidney (BHK) and Vero cells. The effects of these inoculations should be studied through three passages. Any unused ticks should be destroyed by chemical means or by incineration.

c) Stabilate preparation

Care should be taken during the preparation of sporozoite stabilates to avoid aerosol infection of personnel with extraneous pathogens when ticks are being ground. Those grinding ticks should be educated in the potential hazards involved; access to areas where ticks are homogenised should be restricted to specified and informed personnel; personnel should wear protective clothing, including gloves and masks; and tick grinding should be carried out in a microbiological safety cabinet (see Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

3. Purity of stabilates

Both ticks and experimental mammals are potential sources of contamination of stabilates with extraneous pathogens. In both cases, potential contaminants include Ehrlichia bovis, bovine Borrelia sp., orbiviruses, bunyaviruses, and others. Field-collected ticks should therefore not be used for the preparation of stabilates to be used for immunisation. Well characterised and pathogen-free laboratory colonies of ticks should be used for this purpose. Only healthy cattle and rabbits, free from tick-borne parasites, should be used for tick feeding. Stabilates should be prepared under aseptic conditions. In some circumstances, the use of antibiotics at concentrations appropriate for tissue culture may be indicated. Prepared stabilates should be subjected to routine tests for any viral infections in BHK and Vero cells (as above). Stabilates should be subjected to routine characterisation in vivo, which should involve infectivity testing in intact susceptible cattle, sensitivity to tetracyclines and other anti-thélerial drugs, and cross-immunity studies. A characterised ‘working seed stabilate’ should be prepared to ensure the purity of the T. parva stocks in the daughter immunising stabilate.

During stabilate preparation care must also be taken to avoid extraneous contamination of the stock being used with other T. parva stocks. Quality assurance procedures must be enforced, for example for the handling of infected ticks, and the rules should be adhered to rigidly. Tick unit facilities should allow for strict separation of infected and uninfected ticks. Tick unit personnel should use separate overalls for each batch of ticks used in stabilate preparation, and the overalls should be sterilised daily. Simultaneous work on many different stocks should be avoided. Stabilate storage systems should incorporate clear labelling of each stabilate tube or straw.

Quality control checks on the stabilate should determine the similarity to the parent seed stock and also detect any extraneous T. parva contamination.

4. Vaccination risks

The introduction of an immunising stock into an area/country from which it does not originate may result in that parasite, or a component parasite(s) of that stock, becoming established through a carrier state in cattle and transmission by ticks. The long-term effect of the introduction of new (and potentially lethal) parasites on the disease epidemiology should be considered before introduction, and should be monitored carefully following immunisation.

The characterisation of parasites in target populations should be carried out before immunisation, and at intervals following immunisation. At present the characterisation of parasite stocks with reference to vaccination relies primarily on immunisation and cross-challenge experiments in cattle. However a number of methods for characterising parasite stocks in vitro have been attempted in laboratories possessing a high degree of expertise. Preliminary studies have shown that parasite stocks that differ in MAAb profile may not cross-protect, whereas stocks showing similar profiles give cross-protection (Irvin & Morrison, 1987). However, in more recent experiments using other T. parva stocks, this observation has been proven to be wrong. Another method to detect antigenic differences has used T cell clones specific for parasitised cell lines, as T cell responses are believed to be important in mediating immunity against T. parva (Irvin & Morrison, 1987). Currently there are no in vitro assays that correlate with protection in vivo. Statistically derived disease reaction index, based on parasitological, clinical and haematological measurements, was proposed for characterising levels of infectivity and virulence of different parasite stocks and assessing the impact of control intervention against theileriosis (Rowlands et al., 2000).

5. Vaccination strategy

Unlike T. annulata, where a considerable cross-protection is observed among different strains in the field, a more complex situation exists for T. parva. Two strategies are used to try to overcome this antigenic complexity. A
A combination of three stocks, which provides a broad spectrum of protection, has been tested in a number of countries. A large volume of a trivalent stabilate was prepared for the FAO by the International Livestock Research Institute (ILRI) between 1998 and 2000. This stabilate was prepared to the latest proposed standards and is used safely and effectively in Tanzania. A further batch is being prepared at ILRI with increasing demand for the infection and treatment method of immunisation in _T. parva_-endemic areas in sub-Saharan Africa. If an immunising stabilate fails to protect against a ‘breakthrough stock’, this should be isolated, characterised, tested and considered for use, either alone, or as an addition to the current immunising stabilate. Another strategy is to prepare stabilates of national or local stocks for use within defined areas. This latter strategy is more costly in time and resources, but it avoids, to some extent, the introduction of new stocks into an area. With movement of cattle, there is a risk of the introduction of different stocks into an area, which may breakthrough the immunity provided by the local stock. Therefore the use of local or introduced stocks for immunisation needs to be carefully evaluated.

The infection and treatment method of immunisation is effective provided the appropriate quality assurance measures are enforced. In the longer term, the attendant delivery problems and the risk of induction of carrier states and disease transmission, emphasise the need for the identification of protective antigens for development of subunit vaccines.

REFERENCES


Chapter 2.4.16. — Theileriosis


Chapter 2.4.16. – Theileriosis


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**NB:** There is an OIE Reference Laboratory for Theileriosis (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Theileriosis.
CHAPTER 2.4.17.

TRICHOMONOSIS

SUMMARY

Bovine venereal trichomonosis is caused by Tritrichomonas foetus, a flagellate protozoan parasite. It is world-wide in distribution and at one time was of major economic importance as a cause of abortion and infertility, especially in dairy cattle. The widespread use of artificial insemination in many areas of the world has contributed to reduced prevalence. Nevertheless, trichomonosis is still of importance in herds where artificial insemination is not used.

Transmission of the disease is primarily by coitus, but mechanical transmission by insemination instruments or by gynaecological examination can occur. The organism can survive in whole or diluted semen at 5°C. Bulls are the main reservoir of the disease as they tend to be long-term carriers, whereas most cows clear the infection spontaneously. For these reasons samples from bulls are usually preferred for diagnosing and controlling the disease in herds.

Identification of the agent: Tritrichomonas foetus is a flagellate, pyriform protozoan parasite, approximately 8–18 µm long and 4–9 µm wide, with three anterior and one posterior flagellae and an undulating membrane. The organisms move with a jerky, rolling motion and are seen in culture tests of preputial samples of infected bulls and vaginal washings or cervico-vaginal mucus of infected cows, or sometimes in aborted fetuses. Tritrichomonas foetus can be cultured in vitro, and may be viewed in a wet mount or stained slide. The standard diagnostic method for bulls involves the appropriate collection, examination and culture of smegma from the prepuce and penis. Smegma can be collected by a variety of means including preputial lavage or scraping the preputial cavity and glans penis at the level of the fornix with a dry insemination pipette. A number of in-vitro culture media exist, but a commercially available field culture test kit allows for trichomonad growth and direct microscopic examination.

Alternative tests: Bovine trichomonosis may also be detected by polymerase chain reaction amplification. In the past, an agglutination test using mucus collected from the cervix and an antigen made from cultured organisms has been used as a herd test. Similarly, an intradermal test using a trichloracetic acid precipitate of the organism has been used in herds.

Requirements for vaccines: A partially efficacious, killed whole-cell vaccine is commercially available as either a monovalent, or part of a polyvalent vaccine containing Campylobacter and Leptospira.

A. INTRODUCTION

Bovine venereal trichomonosiosis is caused by the flagellate protozoan parasite, Tritrichomonas foetus. The normal hosts of T. foetus are cattle (Bos taurus, B. indicus). Non-pathogenic species of trichomonads occur in the intestine of cattle; T. suis of pigs is indistinguishable morphologically, serologically and, with modern molecular analysis, genetically from T. foetus (Felleisen, 1997; Soulsby, 1982). Further genetic characterisation is required to determine the taxonomic status of isolates from cattle and pigs.

Tritrichomonas foetus is pyriform, 8–18 µm long and 4–9 µm wide, with three anterior and one posterior flagellae, and an undulating membrane. Live organisms move with a jerky, rolling motion, and can be detected by light microscopy. Phase-contrast dark-field microscopy or other methods must be used to observe the details needed for identification. Detailed morphological descriptions, including electron microscopy studies, have been published (Warton & Honigberg, 1979). It is important to differentiate T. foetus from other contaminant flagellated protozoa that may be present in samples from the bovine reproductive tract (BonDurant et al., 1999; Campero et al., 2003; Parker et al., 2003b; Taylor et al., 1994). Under phase contrast illumination, the number of flagellae observed is
an important characteristic as this can help to differentiate *T. foetus* from some bovine flagellates that appear similar. A staining technique has been described that can be used to more clearly observe the morphology and facilitate a definitive identification (Lun & Gajadhar, 1999).

*Trichomonas foetus* multiplies by longitudinal binary fission; sexual reproduction is not known to occur, and environmentally resistant stages of the parasite have not been observed. However, *T. foetus* may be cultured *in vitro* using a commercially available kit or an in-house prepared medium such as Diamond’s medium (Diamond, 1983). The organism may also be detected by molecular methods such as polymerase chain reaction (PCR) (Campero et al., 2003; Felleisen et al., 1998).

In a few early studies, three serotypes were recognised based on agglutination (Skirrow & BonDurrant, 1988): the ‘belfast’ strain, reportedly predominated in Europe, Africa and the USA (Gregory et al., 1990); the ‘brisbane’ strain in Australia (Elder, 1964); and the ‘manley’ strain, which has been reported in only a few outbreaks (Skirrow & BonDurrant, 1988). Further work needs to be done in the area of comparing the growth characteristics, genetic and antigenic variation and pathogenesis of isolates of *T. foetus* from different areas before ‘strain’ and ‘serotype’ designations can be reliably established.

Transmission of infection occurs by coitus, by artificial insemination, or by gynaecological examination of cows. The site of infection in bulls is primarily the preputial cavity (BonDurant, 1997; Parsonson et al., 1974), and little or no clinical manifestation occurs. For bulls older than 3–4 years, spontaneous recovery rarely occurs, resulting in a permanent source of infection in herds. In bulls under 3–4 years old, infection may be transient.

*Trichomonas foetus* is present in small numbers in the preputial cavity of bulls, with some concentration in the fornix and around the glans penis (Hammond & Bartlett, 1943). Chronically infected bulls show no gross lesions. In the infected cow, the initial lesion is a vaginitis, which can be followed in animals that become pregnant by invasion of the cervix and uterus. Various sequelae can result, including a placentitis leading to early abortion (1–16 weeks), uterine discharge, and pyometra. In some cases, despite infection, pregnancy is not terminated by abortion and a normal, full-term calf is born. On a herd basis, cows may, following infection, exhibit irregular oestrous cycles, uterine discharge, pyometra, or early abortion (BonDurant, 1997; Fitzgerald, 1986; Skirrow & BonDurrant, 1988). Cows usually clear their infection and generally become immune, at least for that breeding season (BonDurant, 1997; Fitzgerald, 1986; Soulsby, 1982).

### B. DIAGNOSTIC TECHNIQUES

#### 1. Identification of the agent

##### a) Agent identification by direct examination or culture (the prescribed test for international trade)

A tentative diagnosis of trichomonosis as a cause of reproductive failure in a herd is based on the clinical history, signs of early abortion, repeated returns to service, or irregular oestrous cycles. Confirmation of infection depends on the demonstration of organisms in placental fluid, stomach contents of the aborted fetus, uterine washings, pyometra discharge, vaginal mucus or preputial smegma. In infected herds, the most reliable material for diagnosis is either preputial or vaginal washings or scrapings (European Union, 1988; Kittel et al., 1998; Mukhufhi et al., 2003; Parker et al., 1999; Schonmann et al., 1994).

The number of organisms varies in different situations. They are numerous in the aborted fetus, in the uterus several days after abortion, and, in recently infected cows, they are plentiful in the vaginal mucus 12–20 days after infection. In the infected bull, *T. foetus* organisms are present on the mucosa of the prepuce and penis, apparently not invading the submucosal tissues. It is generally recommended to allow at least 1 week to pass after the last service before taking a preputial sample.

- **Sample collection**

A number of techniques for collecting preputial samples from bulls or vaginal samples from cows have been described. It is important to avoid faecal contamination, as this may introduce intestinal protozoa that may be confused with *T. foetus* (Taylor et al., 1994). Contamination of samples should be minimised by removal of extraneous material and soiled hair from around the preputial orifice or vulva; however, cleansing of the area, particularly with disinfectants, is to be avoided, as this may reduce diagnostic sensitivity. Samples can be collected from bulls by scraping the preputial and penile mucosa with an artificial insemination pipette (Parker et al., 1997; Schonmann et al., 1994) or brush (Ostrowski et al., 1974; Parker et al., 1999), by preputial lavage (Schonmann et al., 1994) or by washing the artificial vagina after semen collection (Gregory et al., 1990). The latter technique is not recommended as its sensitivity may be lower (Gregory et al., 1990). Samples from cows are collected by washing the vagina, or by scraping the cervix with an artificial insemination pipette or brush (Kittel et al., 1998; Mancebo et al., 1995).
Where samples must be submitted to a laboratory and cannot be delivered within 24 hours, a transport medium containing antibiotics should be used (e.g. a thioglycollate broth media with antibiotics [Bryan et al., 1999; Thomas et al., 1990] or the field culture plastic pouch). During transportation, the organisms should be protected from exposure to daylight and extremes of temperature, which should remain above 5°C and below 38°C (Bryan et al., 1999).

- Culture

Where organisms are too few to allow for direct detection and accurate identification, cultures should be prepared. Culture of *T. foetus* is usually required because, in most cases, the number of organisms is not large enough to make a positive diagnosis by direct examination. Several media can be used. Diamond’s trichomonad medium or commercial culture kits are the media of choice (Bryan et al., 1999; Eaglesome & Garcia, 1992; Parker et al., 2003a; Ribeiro, 1990). However, other culture media that may be used include Clausen’s and Oxoid’s media (Eaglesome & Garcia, 1992). Inoculation of samples into culture media should be done as soon as possible after collection. For samples collected by preputial wash it is necessary to process the sample by centrifugation. The sediment is then inoculated into culture media. Some protocols recommend direct viewing of the aspirate or sediment before inoculation but this does not increase diagnostic sensitivity. It is also important to make sure that the culture media are used before their established expiry date, as many media are not stable. The quality of the water used is important and an antifungal can be added to the media to control yeast growth.

Initial detection of organisms can be done by light microscopy, on a wet mount slide prepared directly from the sample or culture, or through the plastic wall of the InPouch™ kit (see footnote 1) using the specially provided plastic clip. The motile organisms may be seen under a standard compound microscope using a magnification of 100 or more. An inverted microscope may be useful for examining tubes containing culture medium. Culture media should be examined microscopically at intervals from day 1 to day 7 after inoculation (Bryan et al., 1999; Lun et al., 2000). The organisms may be identified on the basis of characteristic morphological features. The pear-shaped organisms have three anterior and one posterior flagellae and an undulating membrane that extends nearly to the posterior end of the cell. They also have an axostyle that usually extends beyond the posterior end of the cell. Phase-contrast microscopy is very valuable in revealing these features or a rapid-staining procedure may also be used (Lun & Gajadhar, 1999). Both these techniques work best when relatively high numbers of organisms are present, especially the staining technique.

- Culture procedures
  - Modified Diamond’s medium

Glassware used for culture should be washed in distilled water (avoiding the use of detergents). The modified Diamond’s medium consists of: 2 g trypticase peptone, 1 g yeast extract, 0.5 g maltose, 0.1 g L-cysteine hydrochloride, and 0.02 g L-ascorbic acid and is made up with 90 ml distilled water containing 0.08 g each of K₂HPO₄ and KH₂PO₄, and adjusted to pH 7.2–7.4 with sodium hydroxide or hydrochloric acid. Following the addition of 0.05 g agar, the medium is autoclaved for 10 minutes at 121°C, allowed to cool to 49°C, and then 10 ml inactivated bovine serum (inactivated by heating to 56°C for 30 minutes), 100,000 units crystalline penicillin C and 0.1 g streptomycin sulphate are added aseptically. The medium is aseptically dispensed in 10 ml aliquots into sterile 16 × 125 mm screw-top vials and refrigerated at 4°C until use. Media should be cultured for up to 7 days, samples being examined at daily intervals (BonDurant, 1997; Lun et al., 2000). The incorporation of agar into the medium confines contaminating organisms largely to the upper portion of the culture medium, while helping to maintain microaerophillic conditions at the bottom where the trichomonads occur in largest numbers.

- Commercial culture test kit

Where a combination of convenience and sensitivity is required, the culture kit (see footnote 2) may be used (BonDurant, 1997; Borchardt et al., 1992; Parker et al., 2003a; Schonmann et al., 1994; Thomas et al., 1990). The kit consists of a clear flexible plastic pouch with two chambers. The upper chamber contains special medium into which the sample is introduced. Field samples for direct inoculation into the culture pouch would normally be collected by the preputial scraping technique (BonDurant, 1997; Schonmann et al., 1994). Samples collected by preputial washing require centrifugation before introduction of the sediment into the upper chamber. Following mixing, the medium is forced into the lower chamber, and the pouch is then sealed and incubated at 37°C. Microscopic examination for trichomonads can be done directly through the plastic pouch (Borchardt et al., 1992). Diagnostic results with samples from bulls using either Diamond’s medium or the field kit have shown that the two methods give comparable results but there are some advantages (in convenience and in test results) with the kit (Borchardt et al., 1992; Bryan et al., 1999; Kittel et al., 1998; Parker et al., 2003a; Schonmann et al., 1994).
• **Overall sensitivity and specificity of the culture and identification test**

Any estimate of the diagnostic sensitivity and specificity of the culture and identification test will be dependent on the efficacy of sample collection, handling and processing, as well as the composition and quality of the culture medium. In bulls, the sensitivity of the InPouch™ TF kit has been estimated to be 92% (95% confidence interval, 94–96%) (Parker et al., 1999; 2003a). Estimates for Diamond’s and related media have been variable, possibly due to variation in composition and preparation, but range from 78% to 99% (Parker et al., 2003a; Skirrow & BonDurant, 1988). Until recently, it has been assumed that the specificity of the culture test was 100%, but this is likely to be an overestimation.

Not every sample taken from a particular bull, known to be infected, will necessarily give a positive culture result. Even with optimum conditions of sampling, transport, culture and identification, more than one negative sample should be obtained before there is reasonable assurance that the animal is uninfected. To estimate the probability that an animal is uninfected, negative predictive values should be calculated using an estimate of diagnostic test sensitivity and the animal’s pretest probability of infection (Parker et al., 1999). The infection in females is usually cleared within 90–95 days, so it may be difficult to isolate organisms from animals in the late stages of their infection. In experimentally infected young cows, using the InPouch™ TF method of culture, an apparent sensitivity of 86% was achieved through a 10-week period after infection (Kittel et al., 1998).

The diagnosis of abortion induced by *T. foetus* may be relatively easy where an aborted fetus is recovered, because of the large number of organisms demonstrable in the fetal abomasal contents or placental fluids. Additionally, immunohistochemical techniques and DNA methods can be used to demonstrate tissue-invasive *T. foetus* organisms in aborted fetuses.

**b) Polymerase chain reaction**

Molecular-based techniques that use PCR technology have been developed for the identification of *T. foetus* (Campero et al., 2003; Cobo et al., 2007; Felleisen et al., 1997; 1998; Parker et al., 2001). A PCR diagnostic test offers a number of potential advantages, including increased analytical sensitivity, faster diagnostic turnaround time, and the fact that the organisms in the collected sample are not required to be viable. A real-time PCR has been described (McMillen & Lew, 2006) that described a low detection limit, however insufficient field samples were tested to adequate evaluate its performance. A diagnostic PCR assay includes both a specific extraction technique and DNA amplification using PCR techniques with specific primers. The sensitivity and specificity of the assay will be affected by the choice of extraction, choice of PCR conditions and the choice of primers. PCR assays are capable of detecting very low numbers of parasites from laboratory cultures of the organism with no preputial material present and in the presence of preputial material (Felleisen et al., 1998; Parker et al., 2001). However, in the presence of preputial material, a higher number of parasites is required to yield a positive PCR result; this is most likely due to inhibition by components of the preputial smegma. Several DNA extraction techniques have been described (Felleisen et al., 1998; Huby-Chilton et al., 2009; Parker et al., 2001) and typically, the sensitivity of the diagnostic test will be influenced by the efficiency of the extraction method and the procedures to overcome contaminating inhibitors. Diagnostic specificity of the PCR test depends in part, on the specificity of the primers. A set of primers based on the 5.8s rRNA sequence demonstrated good diagnostic specificity in samples from negative animals (TFR3 and TFR4; Felleisen et al., 1998) and are the primer set most frequently cited in published literature. These primers do, however produce amplification products from some closely related flagellates (*Trichomonas suis*, *T. mobilensis* and a trichomonad from cats) that are indistinguishable from those of *T. foetus* (Felleisen et al., 1998; Gookin et al., 2002). These species also cannot be differentiated by microscopy and it is possible that some of these organisms are synonymous with *T. foetus*. These primers can be used to differentiate between *T. foetus* and a non-*T. foetus* trichomonad sometimes found in preputial samples (BonDurant et al., 1999; Campero et al., 2003; Parker et al., 2003b).

The diagnostic sensitivity and specificity of these assays have yet to be determined in an adequate sample of positive and negative animals, although research to date suggests good specificity (Campero et al., 2003; Rhyan et al., 1995). The diagnostic sensitivity of PCR tests has been estimated to be similar to that of the InPouch™ TF culture kit (Campero et al., 2003) but with very few animals. PCR techniques are an attractive alternative to microscopy in that they have a faster turnaround time, and they also allow the detection of dead organisms. The validation of PCR techniques should be continued and a large number of known positive and negative samples should be tested. DNA-based techniques have potential as an ancillary or primary test (BonDurant et al., 1999; Campero et al., 2003; Felleisen et al., 1998; Mukhufhi et al., 2003; Parker et al., 2001) and play a key role in differentiating trichomonad protozoa recovered from bovine samples from the reproductive tract.

Several different approaches have been taken to continue on from earlier work that used the one set of primers (TFR3 and TFR4; [Felleisen et al., 1998]) specifically diagnose *T. foetus*. The use of two sets of primers together, one set amplifying DNA from the trichomonad group (TFR1 and TFR2; [Felleisen, 1997]) and one set specific to *T. foetus* (TFR3 and TFR4; [Felleisen et al., 1998]) to differentiate between...
organisms considered to be faecal contaminants of the bovine reproductive system and *T. foetus* (Campero et al., 2003). Alternatively, the generic primers (TFR1 and TFR2; [Felleisen, 1997]) were used to amplify DNA and then different protozoal species were differentiated using RFLP analysis (Hayes et al., 2003). In a third study, another set of primers was designed to amplify different sized amplicons from trichomonad protozoa, allowing different species to be distinguished (Grahn et al., 2005). Recently, single strand conformation polymorphism (SSCP) analysis demonstrated efficiency in distinguishing bovine *T. foetus* from other trichomonads (Huby-Chilton et al., 2009). PCR on pooled aliquots of sedimented samples has shown evidence of enhanced diagnostic efficiency (Kennedy et al., 2009). It has also been demonstrated that a PCR assay can be used to detect *T. foetus* DNA in formalin fixed endometrial and aborted fetal tissue (BonDurant et al., 2003).

c) **Immunological tests**

Several immunological tests have been used in the past or have been recently developed for the diagnosis of bovine trichomonosis (Kerr & Robertson, 1941; Pierce, 1949; Rhyan et al., 1999; Soto & Parm, 1989). However, they are limited in use and are not recommended for the detection of *T. foetus* in individual animals. In the 1940s, mucus agglutination tests and intradermal diagnostic tests were developed, but problems with sensitivity and specificity restrict their usefulness. Other immunological tests based on the antigen-trapping enzyme-linked immunosorbent assay (ELISA) have been developed (BonDurant, 1997; Gault et al., 1995). Immunohistochemical techniques using monoclonal antibodies have been shown to reveal *T. foetus* organisms in formalin-fixed tissues (Rhyan et al., 1995).

- **Immunohistochemistry on tissues**

There are no specific macroscopic or microscopic lesions in the aborted fetus, and identification of the organisms is necessary for diagnosis. An immunohistochemical technique using a monoclonal antibody (MAB) to detect *T. foetus* in formalin-fixed paraffin-embedded placenta and fetal lungs from bovine abortions has been reported (Rhyan et al., 1995). Immunohistochemical staining is done using a commercially available labelled streptavidin/biotin system and an MAB (34.7C4.4) to *T. foetus*. In the procedure, deparaffinised 4 µm sections are incubated with the MAb following blocking with non-immune goat serum. After three rinses in buffer, the sections are incubated with biotinylated goat anti-mouse and anti-rabbit immunoglobulin for 30 minutes at 37°C. Following three additional rinses in buffer, peroxidase-labelled streptavidin is applied for 30 minutes at 37°C, and the enzyme activity is diluted with 3% AEC (3-amin-9-ethylcarbazole) in N,N dimethylformamide. Sections are counterstained with Gill II haematoxylin for 3 minutes, rinsed, and blued in buffer for 1 minute. This method has been used to diagnose abortions caused by *T. foetus*.

C. **REQUIREMENTS FOR VACCINES**

Whole cell vaccines for cows have been shown to offer protection and are available commercially (Corbeil, 1994) as either a monovalent vaccine or part of a polyvalent vaccine also containing *Campylobacter* and *Leptospira* spp. (BonDurant, 1997). These products have shown efficacy in the female but not in the bull (BonDurant et al., 1993).

One example of a method of whole cell vaccine production is by growing *T. foetus* (culture VMC-84) in modified Diamond’s medium (Corbeil, 1994) and freezing the culture at −20°C for 60 minutes. After thawing, a suspension of $5 \times 10^7$ organisms/ml in phosphate buffered saline is added to the CL-vaccine.

**REFERENCES**


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1 DAKO Corporation, Carpinteria, California, USA


Chapter 2.4.17. — Trichomonosis


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CHAPTER 2.4.18.

TRYPANOSOMOSIS
(tsetse-transmitted)

SUMMARY

Definition of the disease: Tsetse-transmitted trypanosomosis\(^1\) is a disease complex caused by several species of protozoan parasites of the genus Trypanosoma, mainly transmitted cyclically by the genus Glossina (tsetse flies), but also transmitted mechanically by several biting flies (tabanids, stomoxes, etc.). The disease can affect various species of mammals but, from an economic point of view, tsetse-transmitted trypanosomosis, is particularly important in cattle. It is mainly caused by Trypanosoma congolense, T. vivax and, to a lesser extent, T. brucei brucei.

Description of disease: Tsetse-transmitted trypanosomosis is a classically acute or chronic disease that causes intermittent fever and is accompanied by anaemia, oedema, lacrimation, enlarged lymph nodes, abortion, decreased fertility, loss of appetite and weight, leading to early death in acute forms or to digestive and/or nervous signs with emaciation and eventually death in chronic forms.

Identification of the agent: Several parasite detection techniques can be used, including the microscopic examination of the wet and stained thick or thin blood films. Diagnostic sensitivity is increased significantly by concentrating the parasites prior to examination in combination with a phase-contrast or dark-ground microscope. The parasite concentration techniques have the added advantage that the packed cell volume, and hence the level of anaemia, can be determined at the individual animal and/or herd level. A highly specific and more sensitive test, used in an increasing number of laboratories, is the polymerase chain reaction (PCR), which can identify parasites at the genus, species or subspecies level, depending on the cases.

Serological tests: Two trypanosomal antibody detection tests, the indirect fluorescent antibody test and the antibody-detection enzyme-linked immunosorbent assay (ELISA), are routinely used for the detection of antibodies in cattle. They have high sensitivity and specificity but can only be used for the presumptive diagnosis of trypanosomosis. The antibody-detection ELISA, in particular, lends itself to automation and will allow a high degree of standardisation when recombinant antigens have been developed and validated. However, they are at the present time carried out with native soluble antigens of trypanosomes grown in rodents with satisfying sensitivity and specificity.

Requirements for vaccines and diagnostic biologicals: No vaccines are in use at the present time. Diagnostic biological products produced from cultured parasites are available and in use for the processing of the indirect fluorescent antibody test and the indirect-ELISA.

A. INTRODUCTION

Trypanosomes are flagellate protozoans that inhabit the blood plasma, the lymph and various tissues of their hosts. The genus Trypanosoma belongs to the protozoan branch, order Kinetoplastida, family Trypanosomatidae.

\(^1\) Note on nomenclature of parasitic diseases: The World Association for Advances in Veterinary Parasitology has recommended a 'Standardised Nomenclature of Animal Parasitic Diseases' (Kassai T., Cordero del Campillo M., Euzeby J., Gaafar S., Hepe Th. & Himonas C.A. [1988]. Vet. Parasitol., 29, 299–326). In principle, the disease name is constructed by adding the suffix ‘-osis’ to the stem of the name of the parasite taxon. This terminology has been followed in this Terrestrial Manual, and ‘trypanosomosis’ therefore replaces the old term of ‘trypanosomiasis’.
Tsetse-transmitted trypanosomes belong to the salivarian section, subgenus Nannomonas for \textit{T. congoense}, Duttonella for \textit{T. vivax}, and Trypanozoon for \textit{T. brucei} ssp.

Tsetse-transmitted trypanosomosis is a disease complex caused by several of these species, mainly transmitted cyclically by the genus \textit{Glossina} (tsetse flies), but also mechanically by biting flies. Tsetse infest 10 million square kilometres and affect 37 countries, mostly in Africa, where it is known as ‘nagana’. The disease infects various species of mammals but, from an economic point of view, tsetse-transmitted trypanosomosis is particularly important in cattle (also referred as tsetse-fly disease in southern Africa). It is mainly caused by \textit{Trypanosoma congoense}, \textit{T. vivax} and, to a lesser extent, \textit{T. brucei brucei}. \textit{Trypanosoma uniforme}, and \textit{T. simiae} are other, less common tsetse-transmitted species. \textit{Trypanosoma vivax} is also transmitted mechanically by biting flies, among which tabanids and stomoxes are presumed to be the most important, as exemplified by its presence in South and Central America, but also as observed in some areas of Africa free or cleared of tsetse (Ethiopia, Chad, etc.). Tsetse-transmitted trypanosomosis can affect camels and is a natural barrier preventing the introduction of this mammalian species into the southern Sahel region of West Africa. Horses are also highly sensitive. Very rare human cases have been observed caused by animal \textit{Trypanosoma} species. However, tsetse-transmitted trypanosomosis also affects humans, causing sleeping sickness, through infection with either \textit{T. brucei gambiense} or \textit{T. brucei rhodesiense}. A large range of wild and domestic animals can act as reservoirs of these human parasites; particular care must be taken for people handling biological material that can contain infective human parasites, for example in livestock.

Clinical signs of tsetse-transmitted trypanosomosis may include intermittent fever, oedema, abortion, decreased fertility and emaciation. Anaemia usually develops in affected animals and is followed by loss of body condition, reduced productivity and often mortality. Post-mortem signs may include emaciation, enlarged lymph nodes, enlarged liver and spleen, excessive fluid in the body cavities, and petechial haemorrhages. In animals that died during the chronic phase of the disease, the lymphoid organs are usually no longer enlarged and severe myocarditis is a common finding. Neither clinical nor post-mortem signs of tsetse-transmitted trypanosomosis are pathognomonic. Therefore, diagnosis must rely on direct techniques that confirm the presence of trypanosomes either by microscopic visualisation or by indirect serological techniques or by polymerase chain reaction (PCR). Clinically, trypanosomosis can be confused with babesioses, anaplasmosis, theileriosis, hemonchosis and even ehrlichioses, rabies or plant intoxications. Differential diagnosis is oriented by clinical observations, evolution, epidemiological context, but it is essentially based on laboratory diagnosis.

**B. DIAGNOSTIC TECHNIQUES**

A variety of diagnostic tests are available (Toure, 1976) and researchers are still trying to improve existing tests and to develop new ones. Current diagnostic tests vary in their sensitivity and specificity, the ease with which they can be applied and their cost (Paris et al., 1982). The choice of a particular test will be guided by economic principles and the availability of expertise, but especially by the diagnostic requirement. For example, different degrees of sensitivity and specificity are applied to the confirmation of the infection in an individual animal as compared to the detection of infection at a herd level. Similarly, the diagnostic test(s) to establish the parasitological prevalence of trypanosomosis are different from those required to establish the presence or absence of the disease in an area. Reliable diagnosis may be achieved by combining appropriate diagnostic tests. Reliable interpretation of results from diagnostic tests will depend on test validity as well as on proper sample selection/collection, the sample size, and the way the diagnostic tests are conducted.

1. Identification of the agent

Parasite detection techniques are highly specific, but their sensitivity is relatively low (i.e. the proportion of false-negative results recorded is high). Sensitivity is especially low when results are considered at the individual animal level rather than the herd level. Sensitivity is highly variable during the course of the infection: (i) in the early phase, the sensitivity is high as parasites are actively multiplying in the blood in the absence of immunological control; (ii) during the chronic phase the sensitivity is low as, due to the immune response of the host, parasites are scanty and rarely seen in the blood; (iii) finally the sensitivity is almost nil in healthy carriers, where parasites are never seen. At the population level these variations mean that parasite detection techniques are highly sensitive during epizootic outbreaks (when most of the animals are in the early stages of infection), and are of low or very low sensitivity in enzootic areas (most of the animals are in the chronic stages of infection), especially during subclinical phases of the infection (healthy carriers). Due to this low sensitivity, the apparent parasitological prevalence of trypanosomosis is a little or much lower than the true parasitological prevalence. The low diagnostic sensitivity also makes it difficult to detect trypanosomosis when present at low parasitological prevalence and it is impossible to establish the absence of the disease with a high degree of confidence. Moreover, in areas where trypanocidal drugs are used extensively, parasites may not be detected.

Several parasite detection techniques are available, each with varying sensitivity. The choice will depend on the laboratory facilities available and the aim of the diagnosis.
• Direct examination techniques

The simplest techniques are examination of wet, thick or thin films of fresh blood, usually obtained from the ear vein, jugular vein or the tail. Amongst the direct examination techniques, stained thin blood films are generally regarded as more specific but less sensitive than the other two. The actual specificity and sensitivity of these techniques is directly dependent on the volume of blood actually examined and the skill and experience of the microscopist.

a) Wet blood films

These are made by placing a droplet of blood (about 2 µl) on a clean microscope slide and covering with a cover-slip (22 × 22 mm). The blood is examined microscopically at ×400 total magnification with condenser aperture, phase-contrast or interference contrast. Approximately 50–100 fields are examined. Trypanosomes can be recognised by their movement among the red blood cells (RBCs).

The method is simple, inexpensive and gives immediate results. Depending on the trypanosome size and movements a presumptive diagnosis can be made of the trypanosome species. Final confirmation of the species is made by the examination of the stained preparation.

The diagnostic sensitivity of the method is generally low but depends on the examiner’s experience and the level of parasitaemia. Sensitivity can be improved significantly by lysing the RBCs before examination using a haemolytic agent such as sodium dodecyl sulfate (SDS).

b) Thick blood films

These are made by placing a drop of blood (5–10 µl) on a clean microscope slide and spreading it over an area of approximately 2 cm in diameter, using the corner of another slide. The thickness of the resultant film should be such that, when dry, the figures on a wristwatch dial can just be read through it. The film is dried thoroughly by rapidly waving in the air and, without fixation, is dehaemoglobinised by immersion in distilled water for a few seconds and dried before staining. The dry smear should be kept dry and protected from dust, heat, flies and other insects. It is stained for 30 minutes with 4% diluted Giemsa stain in phosphate buffered saline, pH 7.2. Staining time and stain dilution may vary with stain and individual technique. Therefore, it is important to start with the manufacturer’s directions and to vary staining time and stain concentration to obtain the optimal result. The stained smear is then washed with buffered water and examined at ×500 to ×1000 total magnification.

The method is simple and relatively inexpensive, but results are delayed because of the staining process. Trypanosomes are easily recognised by their general morphology, but may be damaged during the staining process. This may make it difficult to identify the species.

c) Thin blood smear films

Thin blood smears are made by placing a small drop of blood (about 5 µl), for example from a microhaematocrit capillary tube, on a clean microscope slide approximately 20 mm from one end (allowing for space to apply the thick smear) and spreading with the edge of another slide. This slide is placed at an angle of approximately 30° to the first slide and drawn back to make contact with the blood droplet. The blood is allowed to run along the edge of the spreader, which is then pushed to the other end of the slide in a fairly rapid but smooth motion. If the correct amount of blood is used, the slide should be covered with a film of blood with no surplus before the end of the slide is reached, and the smear should take the shape of a bullet. Ideally, thin films should be prepared so that the RBCs are fairly close to each other but not overlapping. The slide is dried quickly by waving in the air and protected from dust, flies and other insects. The slide is fixed for 3 minutes in methanol, and stained as for thick blood smears. After staining, the slide is washed gently under tap water and allowed to dry. A variation of this method is to fix in methanol for 2 minutes, apply May–Grünwald stain for 2 minutes, then add an equal volume of buffered water, pH 7.2, leave for a further 8 minutes and drain off. Approximately 50–100 fields of the stained thin smear are examined, with a ×50 or ×100 oil-immersion objective lens, before the specimen is considered to be negative. Even after a trypanosome has been detected, approximately 20 extra fields are investigated to determine if more than one species is present. The sharp extremity of the smear must be extensively explored as, because of their capillary properties, trypanosomes may be concentrated at this place (especially true for large species like *T. brucei* and *T. vivax*).

The technique described above can also be used for biopsy samples of lymph obtained from punctured lymph nodes.

Usually, both a thin and thick smear is made from the same sample. Thick smears contain more blood than thin smears and, hence, have a higher diagnostic sensitivity. Thin smears on the other hand allow
**Trypanosoma** species identification. Trypanosome species can be identified by the following morphological characteristics:

**Trypanosoma vivax**: 20–27 µm long, undulating membrane is not obvious, free flagellum present at the anterior end, posterior end rounded, kinetoplast large and terminal.

**Trypanosoma brucei** is a polymorphic trypanosome species. Two distinctly different forms can be distinguished, i.e. a long slender form and a short stumpy form. Often, intermediate forms, possessing characteristics of both the slender and stumpy forms, are observed. The cytoplasm often contains basophilic granules in stained specimens.

- **Trypanosoma brucei** (long slender form): 17–30 µm long and about 2.8 µm wide, undulating membrane is conspicuous, free flagellum present at the anterior end, posterior end pointed, kinetoplast small and subterminal.

- **Trypanosoma brucei** (short stumpy form): 17–22 µm long and about 3.5 µm wide, undulating membrane is conspicuous, free flagellum absent, posterior end pointed, kinetoplast small and subterminal.

**Trypanosoma congolense**: 8–25 µm (small species), undulating membrane not obvious, free flagellum absent, posterior end rounded, kinetoplast is medium sized and terminal, often laterally positioned. Although *T. congolense* is considered to be monomorphus, a degree of morphological variation is sometimes observed. Within *T. congolense*, different types or subgroups exist (savannah, forest, kilifi, tsavo) that have a different pathogenicity (Bengaly et al., 2002). However, these types can only be distinguished using PCR.

**Trypanosoma theileri**: (large species), typically 60–70 µm but individual organisms can range from 19 to 120 µm (Levine, 1985; Matthews, 1979), undulating membrane is conspicuous, long free flagellum present, posterior end pointed, kinetoplast is large and positioned near the nucleus and in a marginal position. *Trypanosoma theileri* is normally nonpathogenic, but its presence can confuse the parasitological diagnosis. In Western Europe, *T. theleiri* is the only trypanosome species occurring in cattle.

- **Parasite concentration techniques**

The probability of detecting trypanosomes in a sample from an infected animal depends largely on the amount of blood examined and the level of parasitaemia. The amount of blood examined with direct examination techniques is low and parasites are often very scanty in the blood of an infected animal. Both of these factors contribute to the low sensitivity of direct examination techniques. Sensitivity can be improved by increasing the volume of blood to be examined and by concentrating the trypanosomes.

**a) Microhaematocrit centrifugation technique (Woo method)**

The microhaematocrit centrifugation technique, or the Woo method (1970), is widely used for the diagnosis of animal trypanosomosis. It is based on the separation of the different components of the blood sample depending on their specific gravity. The method is as follows:

i) Fresh, usually ear vein blood (about 70 µl) is collected into heparinised capillary tubes (75 × 1.5 mm).

ii) One end of the capillary tube is sealed with cristaseal or by heating, ensuring that the column of blood is not charred by the flame.

iii) The sealed capillary tubes are placed in a microhaematocrit centrifuge with the sealed ends pointing towards the outside. To ensure good balance, the tubes are loaded symmetrically.

iv) The rotary cover is screwed on and the centrifuge lid is closed.

v) The capillary tubes are centrifuged at 9000 \( g \) for 5 minutes.

vi) A tube carrier is made from a slide on which two pieces of glass 25 × 10 × 1.2 mm have been fixed, 1.5 mm apart, to form a groove.

vii) The tube is placed in the groove, a cover-slip is placed on top and the interface is flooded with water. Alternatively, examination can be done without flooding the interface with water, but in such case, the light condenser must be placed in such a way that cells become refringent.

viii) The plasma/white blood cell interface (buffy coat) is examined by slowly rotating the tube. Trypanosome movement can first be detected using the ×10 objective lens with reduced condenser aperture; the trypanosomes can be seen more clearly using the ×40 objective lens preferably with a long working distance to allow adequate depth of focus through the capillary tube.
Chapter 2.4.18. – Trypanosomosis (tsetse-transmitted)

The microhaematocrit centrifugation technique is more sensitive than the direct examination techniques (Kratzer & Ondiek, 1989). In the case of T. vivax infections, the sensitivity of the Woo methods approaches 100% when the parasitaemia is >700 trypanosomes/ml blood. Sensitivity decreases to 50% when parasitaemia varies between 60 and 300 trypanosomes/ml blood. Trypanosomes become very difficult to detect when the parasitaemia is lower than 60 trypanosomes/ml blood (Desquesnes & Tresse, 1996). Identification of trypanosome species is difficult. As the specific gravity of T. congolense is similar to that of RBCs, parasites are often found below the buffy coat in the RBC layer. To improve the separation of RBCs and parasites, and increase the sensitivity for T. congolense, the specific gravity of RBCs can be increased by the addition of glycerol.

A modification of the Woo method is the quantitative buffy coat method (QBC) (Bailey & Smith, 1992). The method has been used for the diagnosis of T. b. gambiense infections; it is generally too expensive for the routine large-scale use in animal trypanosomosis surveys.

b) Dark-ground/phase-contrast buffy coat technique (Murray method)

The buffy coat technique or Murray method (Murray et al., 1977) represents an improved technique for the detection of trypanosomes and is widely used. It is carried out following steps i to v above, after which the capillary tube is cut, with a diamond tipped pencil, 1 mm below the buffy coat, to include the top layer of RBCs. The buffy coat and the uppermost layer RBCs are extruded on to a clean microscope slide (check that the buffy coat is not sticking to the capillary tube; it should be visible on the slide before covering it with a cover-slip [22 × 22 mm]). Approximately 200 fields of the preparation are examined for the presence of motile trypanosomes with a dark-ground or a phase-contrast microscope with a ×40 objective lens. Trypanosome species can be identified by reference to the following criteria:

Trypanosoma vivax: Large, extremely active, traverses the whole field very quickly, pausing occasionally.

Trypanosoma brucei: Various sizes, rapid movement in confined areas; undulating membrane traps the light into ‘pockets’ moving along the body.

Trypanosoma congolense: Small, sluggish, adheres to RBCs by anterior end.

Trypanosoma theileri: More than twice the size of pathogenic trypanosomes, tends to rotate; the posterior end is clearly visible, very long and sharp.

As with the microhaematocrit centrifugation technique, the buffy coat technique is more sensitive than direct examination techniques. The sensitivity of the buffy coat method can be improved by using the buffy coat double-centrifugation technique (Kratzer & Ondiek, 1989). A total amount of 1500–2000 µl of blood is centrifuged, after which the buffy coat is aspirated into a microhaematocrit capillary tube and centrifuged again. The buffy coat is examined. However, collection of the buffy coat after the initial centrifugation is a delicate step and results may vary from one technician to another.

Compared with the microhaematocrit centrifugation technique, the buffy coat technique has the added advantage that preparations can be fixed and stained for more accurate identification of species and for retention as a permanent record.

Both the microhaematocrit centrifugation and buffy coat techniques give direct results and can be used for screening large numbers of animals. They require specialised equipment and an electricity supply making the test more expensive compared with the examination of the wet blood film. However, this is compensated for by increased sensitivity. Both parasite concentration techniques rely on the detection of motile, live, trypanosomes. Because trypanosomes can lose their vigour and die rather quickly once the blood sample is drawn, samples collected in capillary tubes should be cooled immediately and not be allowed to overheat in the microhaematocrit centrifuge or on the microscope stage. Samples should be examined as soon as possible after collection, preferably within a couple of hours.

The microhaematocrit centrifugation and buffy coat techniques are particularly useful in that the packed cell volume (PCV) can be assessed at the same time. To determine the PCV after centrifugation, the microhaematocrit capillary tube (containing ear vein or jugular vein blood) is placed in a haemocrit reader. The length of the packed RBC column is expressed as a percentage of the total volume of blood. Measuring the PCV is useful for determining the degree of anaemia. Anaemia can be caused by factors other than tsetse-transmitted trypanosomosis. It remains, however, one of the most important indicators of trypanosomosis in cattle. As trypanosomosis is a herd problem, the PCV-profile of a herd is influenced by the number of trypanosome-infected animals and can be used to indicate differences in disease challenge. The average PCV is also influenced by the age and level of genetic susceptibility of cattle.
c) Anion exchange

The miniature anion-exchange chromatography technique (m-AECT) is widely used for the diagnosis of human sleeping sickness caused by *T. b. gambiense* (Lumsden *et al*., 1979). Blood is passed through a diethyl amino-ethyl (DEAE)-cellulose column equilibrated with a phosphate buffered saline (PBS) solution of an ionic strength suited to the blood of the animal species under examination. As the RBCs are more negatively charged than the trypanosomes, they are held in the column and the trypanosomes pass through with the eluate, which is collected, centrifuged to concentrate the trypanosomes and examined under the microscope.

Large volumes of blood can be examined from each animal and, therefore, the method has high sensitivity. However, the technique is cumbersome and is not suitable for the examination of a large number of animals because it is very expensive and time consuming.

d) In-vitro cultivation

A procedure for the in-vitro cultivation of *T. brucei* has been described, but success has been irregular over many years. Moreover, the method needs sophisticated equipment, produces results after a considerable delay and is certainly not suitable for large-scale use. A kit (KIVI) for in-vitro isolation of trypanosomes has proven to be promising in isolating and amplifying all species of *T. brucei* in humans, domestic and game animals (Truc *et al*., 1992). The test's value in isolating *T. congolense* and *T. vivax* is still unknown. As it is based on the cultivation of procyclic forms of trypanosomes, species differentiation is not possible (Komoin-Oka *et al*., 1994). It should be noted that cultivation is a highly efficient and sensitive method for the detection of tabanid-transmitted *T. theileri*, the prevalence of which is often found to be close to 100% using this technique. In the case of mixed infections, *T. theileri* easily overgrows *T. b. brucei* (Verloo *et al*., 2000).

- **Animal inoculation**

The subinoculation of blood into rodents, usually mice or rats, is particularly useful in revealing subpatent infections. The laboratory animals are injected intraperitoneally with 0.2–5 ml (depending on the size of the rodent) of freshly collected blood. Artificial immunosuppression of recipient animals by irradiation or drug treatment will greatly increase the chances of isolating the parasite. They are bled three times a week for at least 2 months. Collected blood is examined using the wet film method.

Animal inoculation is more sensitive than direct examination of the wet blood film. Nevertheless, the method is not practical; it is expensive and diagnosis is not immediate. The method is highly sensitive in detecting *T. b. brucei* infections (it is a technique of choice for detection of the non tsetse-transmitted animal trypanosome: *T. evansi*). However, some *T. congolense* strains are not easily transmitted and *T. vivax* rarely infects laboratory rodents. Also animal inoculation should be avoided as it raises serious animal welfare concerns.

- **Test to detect trypanosomal antigen**

Antigen-detection enzyme-linked immunosorbent assays (ELISA) for trypanosomosis have been described (Nantulya & Lindquist, 1989). Field evaluations of the tests have given inconsistent results (Desquesnes, 1997a; International Atomic Energy Agency [IAEA], 1993). Additional works have been done under controlled conditions, which led to the conclusion that the sensitivity and specificity of these tests are not suitable for the diagnosis of trypanosomosis (Desquesnes, 1996; 1997; 2004; Eisler *et al*., 1998).

- **DNA amplification tests**

A PCR method has been developed as a tool for the diagnosis of infections with African trypanosomes in humans and animals, as well as tsetse flies. Specific repetitive nuclear DNA sequences can be amplified for *T. vivax* and three types of *T. congolense* (Desquesnes, 1997a; Desquesnes & Davila, 2002; Masiga *et al*., 1992); however current primers for *T. vivax* seem to not be able to amplify some genotypes within this species. A common primer set is available for detection of the three *T. brucei* subspecies. The primer sets available for different trypanosome subgenus, species and types are referred to as follows: Trypanozoon subgenus – TBR1 and TBR2; *T. congolense* (savannah type) – TCN1 and TCN2; *T. congolense* (forest type) – TCF1 and TCF2; *T. congolense* (Kenya Coast type) – TCK1 and TCK2; and *T. vivax* – TVW1 and TVW2. Due to the multiplicity of these taxon-specific primers in tsetse flies or cattle, a full trypanosome species identification requires that five PCR test be carried out per sample, which considerably increases the cost of diagnosis. Recently PCR restriction fragment length polymorphism (RFLP) assays and ITS1 of ribosomal DNA amplification have been developed that allow the identification of all *Trypanosoma* species as single or mixed infections using one single test (Delespaux *et al*., 2003; Desquesnes & Davila, 2002; Desquesnes *et al*., 2001; Geysen *et al*., 1997); however these tests are not yet suitable for routine diagnosis. Loop-mediated isothermal amplification is also under development for trypanosome diagnosis (Kuboki *et al*., 2003).
Standard monovalent PCR amplifications are carried out in a reaction mixture containing Tris/HCl, MgCl₂, KCl, each of the four deoxyribonucleotide triphosphates, primers, DNA template and Taq DNA polymerase. Samples are incubated during several cycles at varying temperatures. The PCR products are electrophoresed through agarose. Gels are stained with ethidium bromide and visualised under UV light for the presence of specific weight products.

The procedure is extremely sensitive, but false-positive results may occur as a result of contamination of samples with other DNA. The test requires specialised equipment and highly trained personnel, so it is not suitable for use in many laboratories. False-negative results may occur when the parasitaemia is very low (< 1 trypanosome/ml of blood), which occurs frequently in chronic infections; they may also occur when the specificity of the primers is too high, so that not all isolates of a particular trypanosome species are recognised. Sample collection has been simplified by adapting the test using blood or buffy coats spotted on to filter paper (Geysen et al., 1997; Katakura et al., 1997). A large number of samples can be processed at one time, making it potentially suitable for large-scale surveys. However, at the moment, the cost of PCR analyses is prohibitive for the routine use of the test.

2. Serological tests

Several antibody detection techniques have been developed to detect trypanosomal antibodies for the diagnosis of animal trypanosomosis, with variable sensitivity and specificity. The methods of choice are the indirect fluorescent antibody test (IFAT) (Katende et al., 1987) and the trypanosomal antibody-detection ELISA (Hopkins et al., 1998; Luckins, 1977). The identification of major antigens of trypanosomes, and their production as recombinant molecules or synthetic peptides, should hopefully lead to the development of new tests based on the use of defined molecules. Thus, in the future, it may be possible to improve the specificity of serological tests to allow the detection of species-specific antibodies, and to reach a high level of standardisation that is currently not achieved by the use of total parasite extracts.

a) Indirect fluorescent antibody test

The original method for this test (Wilson, 1969) has been replaced by a new technique for the preparation of trypanosomal antigens (Katende et al., 1987), which involves fixation of live trypanosomes using a mixture of 80% cold acetone and 0.25% formalin in normal saline.

• Test procedure
  i) Prepare thin smears from heavily parasitaemic blood or from a trypanosome suspension. Air-dry and fix in acetone for 5 minutes.
  ii) Mark circles of 5 mm diameter on glass slides using nail varnish.
  iii) Using a pipette, place a test serum, diluted 1/40, in each circle, ensuring that the area in each circle is completely covered.
  iv) Incubate the antigen/test serum preparation at 37°C for 30 minutes in a humid chamber.
  v) Wash the preparation three times in PBS for 5 minutes each time at 4°C, with gentle agitation. Air-dry the slides.
  vi) Apply conjugate: rabbit or goat anti-bovine IgG (for tests on bovine sera) conjugated to fluorescein isothiocyanate.
  vii) Incubate and wash as above. Rinse in distilled water. Air-dry the slides.
  viii) Mount the slides in PBS or buffered glycerol and examine for fluorescence.

b) Antibody-detection enzyme-linked immunosorbent assay

The original antibody ELISA (Luckins, 1977) has recently been further developed for use in large-scale surveys of bovine trypanosomosis (Desquesnes, 1997b; Hopkins et al., 1998). Recommendations have been made that allow antigen production and standardisation of the test on a local basis (Desquesnes, 1997b; 2004; Greiner et al., 1997; Wright et al., 1993).

The standard antigen for trypanosomosis antibody tests is derived from bloodstream-form trypanosomes. Trypanosomes are purified by DEAE anion-exchange chromatography of parasites from whole blood of infected rats. Antigens are prepared as a soluble fraction with lysis (with the addition of anti-enzyme) using seven freeze–thaw cycles and centrifugation at 10,000 g for 30 minutes. Antigens obtained from in-vitro propagated procyclic trypanosome forms can also be used (Greiner et al., 1997). Soluble antigens can be stored at −80°C or −20°C for long and short periods, respectively, but they may also be lyophilised for conservation at room temperature. ELISAs using *T. congolense* or *T. vivax* precoated microtitre plates have
been developed that have the advantage that a standardised denatured antigen is used that can be stored for long periods at room temperature (Rebeski et al., 2000).

Both the IFAT and antibody-detection ELISA have been adapted for the analysis of blood samples collected on filter paper. Blood contained in one heparinised microhaematocrit centrifuge capillary tube is extruded on to a filter paper (Whatman® No. 4). Samples are air-dried out of direct sunlight and placed in a plastic bag with self-indicating silica gel desiccant. The bag is sealed and should be kept as cool as possible until specimens are refrigerated or frozen.

Each ELISA-microplate is run with strong positive, weak positive and negative reference sera, which are required to comply with pre-set values for quality assurance. The absorbance of each ELISA-sample tested is expressed as a percentage (percentage positivity: PP) of the strong positive reference standard (Wright et al., 1993), or the positive and negative reference standards (Desquesnes, 1997b); results are, therefore, quantifiable. The cut-off value is determined using known positive and negative field or experimental samples (Desquesnes, 1997b; 2004).

Both antibody-detection tests have high sensitivity and genus specificity. Their species specificity is generally low, but may be improved by using a standardised set of the three species-specific tests (Desquesnes, 2004). They detect immune responses to current and past infections and can, therefore, only provide a presumptive diagnosis of active infection. However, persistence of antibodies after a curative treatment or a self-cure is estimated to be on the average of 3–4 months in young and adult cattle infections (Desquesnes, 2004); although it might take up to 13 months before all antibodies have disappeared in some animals (Van den Bossche et al., 2000) consequently, proper sampling and knowledge of trypanocidal use will give more acute information.

Immunodiagnosis needs expensive, sophisticated equipment and expertise, which is not always available. It has to be performed in specialised laboratories and there is a substantial delay between the actual sampling and the availability of the results. Nevertheless, the antibody ELISA lends itself to a high degree of automation and standardisation. Sample collection and storage is made easy through the use of filter papers. All of these factors make the antibody ELISA a very useful test for large-scale surveys to determine the distribution of tsetse-transmitted trypanosomosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No vaccines are in use at the present time. The only requirement for diagnostic biologicals in livestock is to grow parasites in rodents to avoid the presence of livestock components in IFAT and ELISA biologics.

REFERENCES


Chapter 2.4.18. — Trypanosomosis (tsetse-transmitted)


Chapter 2.4.18. – Trypanosomosis (tsetse-transmitted)


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NB: There is an OIE Reference Laboratory for Trypanosomosis (see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).

Please contact the OIE Reference Laboratories for any further information on diagnostic tests and reagents for Trypanosomosis (tsetse-transmitted)