FOREWORD

The aims of this Terrestrial Manual are to facilitate international trade in animals and animal products and to contribute to the improvement of animal health services world-wide. By describing internationally agreed laboratory methods for disease diagnosis and requirements for the production and control of biological products (mainly vaccines), its objective is to harmonise these important elements of animal disease prevention, surveillance and control.

This ambitious objective required the cooperation of animal health specialists from many countries. The OIE, the World Organisation for Animal Health, is clearly one of the most appropriate organisations to undertake this task on a global level. The main activities of the organisation, which was established in 1924 and in 2004 comprised 166 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
3. To provide expertise and encourage international solidarity in the control of animal diseases
4. Within its mandate under the WTO 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 fifth edition includes new chapters on zoonotic infections reflecting the OIE’s increased involvement in public health issues. As a companion volume to the Terrestrial Animal Health Code, the Terrestrial Manual sets laboratory standards for all OIE Lists A and B diseases as well as several other diseases of global importance. It 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 International Committee of the OIE. Manuscripts were requested from specialists in each of the diseases or the other topics covered. 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 and the Consultant Technical Editor took all the resulting comments into consideration, often referring back to the authors for further help, before finalising the chapters. The final text has the approval of the International Committee of the OIE.

With the increasing content of the Terrestrial Manual it has been necessary for this edition to split it into two volumes. It is our sincere hope that it will prove even more useful to veterinary diagnosticians and vaccine manufacturers in all the OIE Member Countries.

Doctor Bernard Vallat  
Director General, OIE

Professor Steven Edwards  
President, OIE Biological Standards Commission

January 2004
ACKNOWLEDGEMENTS

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 B. 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. M. Truszczynski, Prof. S. Edwards, Dr B. Schmitt and Dr A. Golovko, and observers at the Biological Standards Commission’s meeting, Dr A. Diallo and Dr P. Wright, 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 xxxiii who contributed their invaluable time and expertise to write the chapters,

- The reviewers and OIE Reference Laboratory experts 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,

- Dr G.A. Cullen and Dr J.E. Pearson, Consultant Technical Editors of the *Terrestrial Manual*, who did a great deal, not only in editing and harmonising the contents, but also in providing missing information where required,

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

Dr Abdoulaye Bouna Niang
President of the OIE International Committee

January 2004
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INTRODUCTION
(How to use this Terrestrial Manual)

• Arrangement of the Terrestrial Manual and numbering system

Part I, the beginning of this Terrestrial Manual, contains ten introductory chapters that deal with a variety of general subjects of interest to veterinary laboratory diagnosticians. These chapters are intended to give a brief introduction to their subjects. They are to be regarded as background information rather than standards.

The main part of the Terrestrial Manual (Part 2) covers standards for diagnostic tests and vaccines for the diseases listed in the OIE Terrestrial Animal Health Code. They appear in the same order and use the same numbering system as that used in the Terrestrial Code, to facilitate cross-reference between the two books. List A contains the 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. List B contains the transmissible diseases that are considered to be of socio-economic and/or public health importance within countries and that are significant in the international trade of animals and animal products. List B is subdivided by animal host species.

The first four diseases in Section 2.10. are included in some individual species sections of List B, but these chapters cover several 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 included in Section 2.10.

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

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

• 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 disease of Lists A and B. ‘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 also be of some practical value in local situations or which may still be under development.
• List of OIE Reference Laboratories

A list of OIE Reference Laboratories is given in Part 3 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 reference laboratories.

The list of OIE Reference Laboratories will be updated by the International Committee of the OIE 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 disease of Lists A and B. 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.

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* Please refer to Terrestrial Manual chapters to verify which method is prescribed
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<td>2.6.6.</td>
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<td>Acariosis of bees</td>
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<td>European foulbrood</td>
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<td>Nosemosis of bees</td>
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<td>Varroosis</td>
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<td>2.10.1.</td>
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<td>2.10.4.</td>
<td>Mange</td>
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### List of tests for international trade

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<th>Chapter No.</th>
<th>Disease name</th>
<th>Prescribed tests</th>
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<td>2.10.5.</td>
<td>Border disease</td>
<td>Agent id.</td>
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<td>2.10.6.</td>
<td>Bovine viral diarrhoea</td>
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<td>2.10.7.</td>
<td>West Nile encephalitis</td>
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<td>Cryptosporidiosis</td>
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<td>2.10.10.</td>
<td>Hendra and Nipah virus diseases</td>
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<td>2.10.11.</td>
<td>Swine influenza</td>
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<td>2.10.12.</td>
<td>Toxoplasmosis</td>
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<td>2.10.13.</td>
<td>Verocytotoxigenic Escherichia coli</td>
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<td>2.10.14.</td>
<td>Listeria monocytogenes</td>
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**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

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

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</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</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 immunelectrophoresis</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>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 modified Tobie’s medium</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>
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<tr>
<td>GIT</td>
<td>Growth inhibition test</td>
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<tr>
<td>HA</td>
<td>Haemagglutination</td>
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<tr>
<td>HAD</td>
<td>Haemadsorption</td>
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<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
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<tr>
<td>HEP</td>
<td>High-egg-passage (virus)</td>
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<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid [buffer]</td>
</tr>
<tr>
<td>HI</td>
<td>Haemagglutination inhibition</td>
</tr>
<tr>
<td>HRPO</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IB</td>
<td>Immunoblot test</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>ID_{90}</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>
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<tr>
<td>LA</td>
<td>Latex agglutination</td>
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<tr>
<td>LD</td>
<td>Lethal dose</td>
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<tr>
<td>LEP</td>
<td>Low egg passage (virus)</td>
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<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MAT</td>
<td>Microscopic agglutination test</td>
</tr>
<tr>
<td>MCS</td>
<td>Master cell stock</td>
</tr>
<tr>
<td>MDT</td>
<td>Mean death time</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MSV</td>
<td>Master seed virus</td>
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<tr>
<td>NI</td>
<td>Neutralisation index</td>
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<tr>
<td>OGP</td>
<td>1-octyl-beta-D-glucopyranoside (buffer)</td>
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<tr>
<td>OPD</td>
<td>Orthophenyldiamine (buffer)</td>
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<tr>
<td>OPG</td>
<td>Oxalase-phenol-glycerin (preservative solution)</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAP</td>
<td>Peroxidase–antiperoxidase (staining procedure)</td>
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<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>
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<tr>
<td>PD</td>
<td>Protective dose</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>PPLO</td>
<td>Pleuropneumonia-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>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</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>
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<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>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>

1 American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, United States of America
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 wavelength that a sample absorbs and the absorbance is proportional to the amount of a particular analyte present.

• **Accuracy**

Level of agreement between a test value and the expected value for a reference standard of known activity or titre.

• **Assay**

Synonymous with test or test method, e.g. enzyme immunoassay or complement fixation test.

• **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 have 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:

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

• **Cross-reaction**

Detectable activity in a test method attributable to an analyte from or elicited by another organism that results in a false-positive reaction; assays of this nature have poor analytical specificity.

• **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 four parts, i.e. a 20% 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.

• **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**

Calibration of the same or similar test methods against an international standard reagent(s) and expression of quantitative or semi-quantitative test results normalised against working standard(s) incorporated in every test run.

• **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.

• **Interlaboratory 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, stock, seed)**

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

Attribute of a test method; may include analytical sensitivity and specificity, accuracy and precision, and/or diagnostic sensitivity and specificity.

• 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

Degree of dispersion of results for a repeatedly tested sample.

• Predictive value (negative)

Proportion of animals that test negative in an assay that are truly uninfected; predictive value is influenced by diagnostic sensitivity and specificity, as well as prevalence of infection.

• Predictive value (positive)

Proportion of animals that test positive in an assay that are truly infected; predictive value is influenced by diagnostic sensitivity and specificity, as well as 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 of 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 to provide consistent results when applied to aliquots of the same sample at 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)**

Smallest detectable amount of the analyte in question; 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 analytes other than that in question react in an assay; the higher the level of cross-reactions, the lower the analytical specificity.

• **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, for issue to National Laboratories.

  • **National Standard Reagents**
  Standard reagents calibrated by comparison with International Standard Reagents; prepared and distributed by a National Reference Laboratory, for issue to national laboratory networks.

  • **Working Standards (reagents)**
  Standard reagents calibrated by comparison with National Standard Reagents; included in routine diagnostic tests as controls 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. 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 modifies the text to take account of comments received. Once this review process is complete and the text is finalised, the Terrestrial Manual is presented to the OIE International Committee during its annual General Session for adoption before it is printed. 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:

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2.9.2. American foulbrood

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Laboratory of Zoophysiology, University of Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium.

2.9.3. European foulbrood

Dr B.V. Ball
Plant and Invertebrate Ecology Division, Rothamstead Research, Harpenden, Hertfordshire AL5 2JQ, UK.

2.9.4. Nosemosis of bees

Dr A. de Ruijter
Research Centre for Insect Pollination and Beekeeping, Ambrosiusweg 1, 5081 NV Hilvarenbeek, The Netherlands.

2.9.5. Varroosis

Dr W. Ritter
CVUA-Freiberg, Animal Health, Am Moosweihert 2, D79018 Freiberg, Germany.

2.9.6. Tropilaelaps infestation of honey bees (Tropilaelaps clareae, T. koenigerum)

Dr D. Sammataro
Carl Hayden Honey Bee Research Center, 2000 East Allen Road, Tucson, Arizona 85719-1596, USA.

2.10.1. Cysticercosis

Dr S. Lloyd
Department of Clinical Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK.

2.10.2. Bunyaviral diseases of animals excluding Rift Valley fever

Dr G.H. Gerdes
Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort 0110, South Africa.

2.10.3. Salmonellosis

Dr R. Davies
VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK.

2.10.4. Mange

Dr P. Bates
VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK.

2.10.5. Border disease

Dr P.F. Nettleton
Moredun Research Institute, International Research Centre, Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ, Scotland, UK.
2.10.6. Bovine viral diarrhoea  
Dr T. Drew  
VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK.

2.10.7. West Nile fever  
Dr E.N. Ostlund  
USDA, APHIS, National Veterinary Services Laboratories, P.O. Box 844, Ames, Iowa 50010, United States of America.

2.10.8. Campylobacterosis  
Dr Diane Newell  
VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK.

Dr W.F. Jacobs-Reitsma & Dr J.A. Wagenaar  
Animal Sciences Group, Wageningen UR, P.O. Box 65, 8200 AB Lelystad, The Netherlands.

2.10.9. Cryptosporidiosis  
Prof. H. Smith  
Scottish Parasite Diagnostic Laboratory, Stobhill General Hospital, Glasgow G21 3UW, UK.

2.10.10. Hendra and Nipah virus diseases  
Dr B.T. Eaton & Dr P. Daniels  
Australian Animal Health Laboratory, CSIRO, Livestock Industries, 5 Portarlington Road, Geelong, Victoria 3220, Australia.

Dr M. Narasiman  
Veterinary Research Institute, 59, Jalan Sultan Azlan Shah, 31400 Ipoh, Perak, Malaysia.

2.10.11. Swine influenza  
Dr S.L. Swenson  
National Veterinary Services Laboratories, P.O. Box 844, Ames, Iowa 50010, USA.

Dr P.L. Foley  
Center for Veterinary Biologics, 510 S. 17th St., Suite 104, Ames, IA 50010 USA

2.10.12. Toxoplasmosis  
Dr D. Buxton & Dr S.W. Maley  
Moredun Research Institute, Pentlands Science Park, Bush Loan, by Edinburgh EH26 0PZ, Scotland, UK.

2.10.13. Verocytotoxigenic Escherichia coli  
Dr F.A. Clifton-Hadley  
VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK.

2.10.14. Listeria monocytogenes  
Dr Jose Lopez  
Canadian Food Inspection Agency, National Centre for Foreign Animal Disease, 1015 Arlington Street, Winnipeg, Manitoba R3E 3M4, Canada.
SECTION 1.1
INTRODUCTORY CHAPTERS

CHAPTER I.1.1.
SAMPLING METHODS

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 I.1.6. Human safety in the veterinary microbiology laboratory). 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. Frequently, a combination of blood samples for serology and tissues from dead or culled animals for microbiological culture will be required. Recommendations for transport are described later in this chapter.
Chapter I.1.1. — Sampling methods

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 (2, 4, 8, 11, 12). 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. In birds, a wing vein (brachial vein) is usually selected. In small laboratory animals, the vena auricularis or vena retroorbitalis may be useful to obtain blood samples or it may be obtained by heart puncture. 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 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 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.

b) Faeces

At least 10 g of freshly voided faeces should be selected. Faeces for parasitology should fill the container and be sent refrigerated to prevent hatching of parasite eggs and should arrive at the laboratory within 24 hours. 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.
Chapter I.1.1. — Sampling methods

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 text books; a guide to necropsy procedures has been published (10). Post-mortem techniques are also included in some of the national guidelines (2, 4, 8). 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 scalpels, forceps and scissors, including scissors with rounded tip on one blade, for opening intestines. A plentiful supply of containers 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. 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. 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 examinations required. 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 insure 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.


Chapter I.1.1. – Sampling methods

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.

B. SAMPLE SIZE

When investigating a case of clinical disease, the samples collected should be representative of the condition being investigated and the lesions observed. When developing a programme of surveillance and monitoring for animal health, 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 (9). 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 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 (1, 3, 11) or by the use of a program (FreeCalc) available off the internet: http://www.ausvet.com.au/content.php?page=res_software#freecalc.

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 sample 59 animals to be 95% confident of finding at least one positive, assuming that both the sensitivity and specificity of the test were 100%. As most diagnostic tests do not have specificity and sensitivity of 100%, the number of samples collected must be adjusted to the sensitivity and specificity of the test that will be used (see also Chapter I.1.3. Principles of validation of diagnostic assays for infectious diseases).

\[
\ln(\alpha) = \frac{\ln(n)}{\ln(1-p)}
\]

In the above example \( \alpha = 0.05, 1-\alpha = 95\%, p = 0.05 \) and \( n = 59 \)

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

\[
\ln(\alpha) = \frac{\ln(n)}{\ln(1-p\cdot Se)}
\]

In the above example with \( \alpha = 0.05, p = 0.05 \), specificity (\( Sp \)) = 1 and \( Se = 0.95 \), a minimum of \( n = 62 \) animals instead of 59 would need to be sampled to have a probability of at least 0.95 of finding a positive animal. The 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 possible that a positive test result is a false positive.
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. Pencil has a tendency to rub off containers and labels attached to plastic will fall off when stored at –70°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. 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 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:

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 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) Any medication given to the animals, and when given.

i) Any vaccination given, and when given.

j) Other observations about the disease and husbandry

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 and must be obtained in advance for any biological material. This licence should be placed in an envelope on the outside of the parcel.

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 International Air Transport Association (IATA), Dangerous Goods Regulations (DGR) have explicit requirements for packaging and shipment of diagnostic specimens, by all commercial means of air transport (5, 6). In many countries, similar requirements are applicable to ground shipments and the postal service. These requirements for air transport are covered in detail in the IATA publications, 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 Terrestrial Manual was published and it should only be used as a guide for shipping. It is anticipated that there will be major changes to the DGR effective from 1 January 2005. If there are significant changes in the DGR, a revised version of this chapter will be posted on the OIE web site: www.oie.int. Shippers must also always check the latest version of the IATA 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 (2, 4, 8).

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 (5, 6) lists the following exemption from the Dangerous Goods Regulations:

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.

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 (5, 6):

Biological products are derived from living organisms. These are manufactured and distributed in accordance with the requirements of appropriate national governmental 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 and diagnostic products.
The DGR state that infectious substances (including diagnostic specimens likely to contain animal or human pathogens) are designated as UN 2814, UN 2900 or UN 3373.

Samples sent for diagnostic purposes should be designated as UN 2814 or UN 2900 if they include material derived from humans or animals with a disease that can be readily transmitted and for which effective treatment and preventative measures are not usually available. Infectious substances meeting this definition that affect humans, including zoonotic agents, are designated UN 2814; those affecting animals only are designated UN 2900.

Infectious substances shipped for diagnostic purposes that do not meet the criteria for assignment to UN 2814 or UN 2900 are assigned to UN 3373 and designated as ‘Diagnostic Specimens’.

The IATA DGR contain 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 (5, 6).

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

<table>
<thead>
<tr>
<th>Pathogen Name</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus anthracis</em> (cultures only)</td>
<td>Japanese Encephalitis virus (cultures only)</td>
</tr>
<tr>
<td><em>Brucella abortus</em> (cultures only)</td>
<td>Junin virus</td>
</tr>
<tr>
<td><em>Brucella melitensis</em> (cultures only)</td>
<td>Kyasanur Forest disease virus</td>
</tr>
<tr>
<td><em>Brucella suis</em> (cultures only)</td>
<td>Lassa virus</td>
</tr>
<tr>
<td><em>Burkholderia mallei – Pseudomonas mallei – Glanders (cultures only)</em></td>
<td>Machupo virus</td>
</tr>
<tr>
<td><em>Burkholderia pseudomallei – Pseudomonas pseudomallei (cultures only)</em></td>
<td>Marburg virus</td>
</tr>
<tr>
<td><em>Chlamydia psittaci – avian strains (cultures only)</em></td>
<td><em>Mycobacterium tuberculosis (cultures only)</em></td>
</tr>
<tr>
<td><em>Clostridium botulinum</em> (cultures only)</td>
<td>Monkeypox virus</td>
</tr>
<tr>
<td><em>Coccidioides immitis</em> (cultures only)</td>
<td>Nipah virus</td>
</tr>
<tr>
<td><em>Coxiella burnetii</em> (cultures only)</td>
<td>Omsk hemorrhagic fever virus</td>
</tr>
<tr>
<td>Crimean-Congo hemorrhagic fever virus</td>
<td>Poliovirus (cultures only)</td>
</tr>
<tr>
<td>Dengue virus (cultures only)</td>
<td>Rabies virus</td>
</tr>
<tr>
<td>Eastern equine encephalitis virus (cultures only)</td>
<td>Rickettsia prowazekii (cultures only)</td>
</tr>
<tr>
<td><em>Escherichia coli</em>, verotoxigenic (cultures only)</td>
<td>Rickettsia rickettsii (cultures only)</td>
</tr>
<tr>
<td>Ebola virus (cultures only)</td>
<td>Rift Valley fever virus</td>
</tr>
<tr>
<td>Flexal virus</td>
<td>Russian spring-summer encephalitis virus (cultures only)</td>
</tr>
<tr>
<td><em>Francisella tularensis</em> (cultures only)</td>
<td>Sabia virus</td>
</tr>
<tr>
<td>Guanarito virus</td>
<td><em>Shigella dysenteriae</em> type 1 (cultures only)</td>
</tr>
<tr>
<td>Hantaan virus</td>
<td>Tick-borne encephalitis virus (cultures only)</td>
</tr>
<tr>
<td>Hantaviruses causing hantavirus pulmonary syndrome</td>
<td>Variola virus</td>
</tr>
<tr>
<td>Hendra virus</td>
<td>Venezuelan equine encephalitis virus</td>
</tr>
<tr>
<td>Hepatitis B virus (cultures only)</td>
<td>West Nile virus (cultures only)</td>
</tr>
<tr>
<td>Herpes B virus (cultures only)</td>
<td>Yellow fever virus (cultures only)</td>
</tr>
<tr>
<td>Human immunodeficiency virus (cultures only)</td>
<td><em>Yersinia pestis</em> (cultures only)</td>
</tr>
<tr>
<td>Highly pathogenic avian influenza virus (cultures only)</td>
<td></td>
</tr>
</tbody>
</table>

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1 The definition that has been proposed for the 2005 version of the DGR (5) is: “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 to humans or animals.”
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>Pathogen Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>African horse sickness virus</td>
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<tr>
<td>African swine fever virus</td>
</tr>
<tr>
<td>Avian paramyxovirus Type 1 – Newcastle disease virus</td>
</tr>
<tr>
<td>Bluetongue virus</td>
</tr>
<tr>
<td>Classical swine fever virus</td>
</tr>
<tr>
<td>Foot and mouth disease virus</td>
</tr>
<tr>
<td>Lumpy skin disease virus</td>
</tr>
<tr>
<td>Mycoplasma mycoides – Contagious bovine pleuropneumonia</td>
</tr>
<tr>
<td>Peste des petits ruminants virus</td>
</tr>
<tr>
<td>Rinderpest virus</td>
</tr>
<tr>
<td>Sheep-pox virus</td>
</tr>
<tr>
<td>Goatpox virus</td>
</tr>
<tr>
<td>Swine vesicular disease virus</td>
</tr>
<tr>
<td>Vesicular stomatitis virus</td>
</tr>
</tbody>
</table>

Any infectious agent that can cause disease in humans or animals that has been amplified in culture and 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 (laboratory stocks) are the result of a process by which pathogens are amplified or propagated in order to generate high concentrations, thereby increasing the risk of infection when exposure to them occurs. This definition refers to cultures prepared for the intentional generation of pathogens and does not include cultures intended for diagnostic and clinical purposes.*

The following flow chart summarises the classification of diagnostic samples.

The packaging of infectious substances and specimens from suspected serious animal diseases, UN 2814 or UN 2900, are outlined in packing instruction 602; a Shipper's 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 IATA regulations for further information on all UN 2814 or 2900 shipments.

The other group, UN 3373, covers ‘Diagnostic Specimens’. This category has a lower risk and packages
containing these specimens should be labelled as ‘Diagnostic Specimens’; 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 compete procedure, as
outlined in the most recent IATA Dangerous Good Regulations, must be followed (5, 6).

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 specimen should be put into the primary receptacle(s), which can be glass bottles, tubes or plastic
containers.

iii) Adequate adsorbent material must be packed around the primary receptacle(s) to absorb all the fluid in the
primary receptacle(s).

iv) If multiple primary receptacles are used they should be individually wrapped or separated so as to prevent
contact.

v) The primary receptacle(s), and adsorbent material, are put into the secondary receptacle.

vi) The primary receptacle and the secondary packaging used for liquids must be leak-proof. The primary
receptacle or secondary packaging must withstand, without leakage, an internal pressure differential of not
less than 95 kPa in the range from –40°C to 55°C.

vii) The primary receptacle(s) used for solids must not leak (be siftproof).

viii) The primary receptacle must not contain more than 500 ml of liquids or 500 g of solid specimens. The outer
packaging must not contain more than 4 litres for liquid specimens or 4 kg for solids.

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

x) 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 taped shut.

xi) 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.

xii) The primary and secondary receptacles must be put into a shipping container with adequate cushioning
material.

xiii) 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.)

xiv) The package must be labelled Diagnostic Specimens. The 'Nature and Quantity of Goods' box of the airway
bill must state 'DIAGNOSTIC SPECIMENS PACKED IN COMPLIANCE WITH IATA PACKING
INSTRUCTION 650'.

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 have 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 (7).

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 (7).

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


CHAPTER 1.1.2.

QUALITY MANAGEMENT IN VETERINARY TESTING LABORATORIES

SUMMARY

Valid laboratory results are essential for diagnosis, surveillance, and trade. Such results may be 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 system. These subjects comprise one complex area of critical importance in the conduct of testing and in the interpretation of test results. This subject may be called laboratory quality management, and includes managerial, operational, and technical elements. A quality management programme should enable the laboratory to demonstrate that it operates a viable quality system, is technically competent, and is able to generate technically valid results. Additionally, a laboratory should implement a quality management programme that is appropriate for its mandate, clients, needs, and goals, and that can be shown to be effective in meeting quality objectives. The need for the mutual recognition of test results for international trade and the acceptance of international standards such as the ISO/IEC\(^1\) International Standard 17025 (4) for laboratory accreditation also affect the need and requirements for laboratory quality management programmes. The OIE has published detailed standard on this subject (7). This chapter is not intended to reiterate the requirements of these ISO or OIE documents. Rather, it outlines the important issues and considerations a laboratory should address in the design and maintenance of its quality management programme.

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

In order to ensure that the quality management programme is appropriate and effective, the design must be carefully thought out. The major categories of consideration and the key issues and activities within each of these categories are outlined in the following seven 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 programme. These factors include:

i) The type of testing done;

ii) The use of the test results;

iii) The impact of a questionable or erroneous result;

iv) The tolerance level of risk and liability;

v) Client needs (e.g. sensitivity and specificity of the test method, costs, turnaround time);

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; and

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

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1 International Organization for Standardization/International Electrochemical Commission.
Chapter I.1.2. — Quality management in veterinary testing laboratories

2. Standards, guides, and references

It is recommended that the laboratory choose reputable and accepted standards and guides to assist in designing the quality management programme. The OIE standard on this subject is a useful guideline (7). For laboratories seeking accreditation, the use of ISO/IEC 17025 (4) or the OIE standard (7) 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 American Association for Laboratory Accreditation [A2LA], United States of America; the United Kingdom Accreditation Service [UKAS], United Kingdom; and the Standards Council of Canada [SCC], Canada). Technical and international organisations such as the AOAC International (formerly the Association of Official Analytical Chemists) and the ISO publish useful references, guides, and/or standards that supplement the general requirements of ISO/IEC 17025. ISO International Standard 9001 (5), a general standard for quality management systems and one of the many standards in the group commonly termed the 'ISO 9000 series', is not usable for accreditation, as conformity with its requirements does not necessarily ensure or imply technical competence (see Section 3. below). While a laboratory may implement a quality management system meeting the requirements of ISO 9001, registration or certification is used to indicate conformity with this standard. ISO/IEC 17025 contains a tabular comparison by clause numbers of itself, ISO 9001 (1994), and ISO 9002 (1994). Both of these latter documents have been replaced by the 2000 version of 9001 (5).

3. Accreditation

If the laboratory has determined that it needs formal recognition of its quality management programme, 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. The terms accreditation, “a procedure by which an authoritative body gives formal recognition that a body or person is competent to carry out specific tasks” (2), and laboratory accreditation, “the formal recognition of the competence of a laboratory to carry out specific tests or specific types of tests” (2), are specific to ILAC requirements and to the use of ISO/IEC 17025 or the OIE standard as the basis for the accreditation. The use of competence is significant: it means much 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 selected standard(s) and/or guidelines;

ii) Has adequate qualified personnel who understand the science behind the procedures;

iii) Has correct and adequate equipment;

iv) Has adequate facilities and environmental control;

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

vi) Can foresee technical needs and problems;

vii) Can cope with and prevent technical problems that may arise;

viii) Can accurately estimate and control the uncertainty in testing; and

ix) Can demonstrate proficiency to conduct the test methods used.

4. Selection of an accreditation body

In order for accreditation to facilitate the acceptance of the laboratory's test results for trade, the accreditation must be recognised by the international community. Therefore, the accreditation body should be 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 (3). One may obtain information on recognised accreditation bodies from the organisations that recognise them, such as the National Cooperation for Laboratory Accreditation (NACLA), 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 programme and/or of the laboratory's accreditation

The quality management programme should ideally cover all areas of activity affecting all testing that is done at the laboratory. However, for the purpose of accreditation, the laboratory should determine the scope of testing to
be included in the accreditation. Factors that might affect the laboratory’s choice of scope of accreditation include:

i) The availability and cost of necessary personnel, facilities and equipment;
ii) The cost of environmental monitoring against the possibility of cross contamination;
iii) The deadline for accreditation;
iv) The impact of the test results;
v) The number of tests done;
vi) Whether the testing done is routine or non-routine;
vii) Whether any part of testing is subcontracted out;
viii) The quality assurance necessary for materials, reagents and media;
ix) The availability of reference standards (e.g. standardised reagents, internal quality control samples, reference cultures);
x) The availability of proficiency testing;
x) The availability, from reputable sources, of standard and/or fully validated test methods;
xii) The evaluation and validation of test methods to be done; and
xiii) The technical complexity of the method(s).

Accreditation bodies also accredit the providers and operators of proficiency testing programmes, and may require the use of an accredited provider in order to issue the laboratory a certificate of accreditation.

6. Test methods

ISO/IEC 17025 requires the use of appropriate test methods and has requirements for selection, development, and validation. The OIE document (7) also provides requirements for selection and validation.

This Terrestrial Manual provides recommendations on the selection of test methods for trade and diagnostic purposes in the chapters on specific diseases. In addition, a list of 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 are suitable for the diagnosis of disease within a local setting, but that may have had limited validation. These tests are generally serological tests.

In the veterinary profession, other standard (methods published in international, regional, or national standards) or fully validated methods (methods having undergone a full collaborative study and that are published or issued by an authoritative technical body such as the AOAC International), while preferable to use, may not be available. Many veterinary laboratories develop or modify methods, and most of these laboratories have test programmes 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.

Clients and laboratory staff must have a clear understanding of what performance can be expected from a test. 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 I.1.3. The following items discuss test method issues that are of most interest to those involved in the quality management of the laboratory.

a) Selection of the test method

Valid results begin with the selection of an appropriate test method for the diagnostic issues at hand. Considerations for the selection of a test method include:

i) International acceptance;
ii) Scientific acceptance;
iii) Method not outdated;
iv) Performance characteristics (e.g. analytical and diagnostic sensitivity and specificity, repeatability, reproducibility, isolation rate, lower limit of detection, precision, trueness, and uncertainty);

v) Behaviour in species and population of interest;

vi) Resources and time available for development, adaptation, and/or evaluation;

vii) Performance time and turnaround time;

viii) Type of sample (e.g. serum, tissue) and its expected quality or state on arrival at the laboratory;

ix) Analyte (e.g. antibody, antigen);

x) Resources and technology of the laboratory;

xi) Nature of the intended use (e.g. export, import, surveillance, screening, confirmatory, individual animal use, herd use);

xii) Client expectations;

xiii) Safety factors;

xiv) Number of tests to be done;

xv) Cost of test, per sample;

xvi) Existence of reference standards, including reference materials; and

xvi) Availability of 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. Whether the method was developed in-house or imported from an outside source, generally some additional optimisation is necessary. Optimisation is a series of experiments and subsequent data analysis. Optimisation establishes critical specifications and performance standards for the test process and for use in monitoring the correct performance of the test. Optimisation should ensure that a method is brought under statistical control. Optimisation should also determine:

i) Critical specifications for equipment and instruments;

ii) Critical specifications for reagents (e.g. chemicals, biologicals);

iii) Critical specifications for reference standards, reference materials, and internal controls;

iv) Robustness (if applicable);

v) Critical control points and acceptable ranges, attributes or behaviour at critical control points, using statistically acceptable procedures;

vi) The quality control activities necessary to monitor critical control points;

vii) The type, number, range, frequency, and/or arrangement of test run controls needed;

viii) The requirements for control behaviour for the non-subjective acceptance or rejection of test results;

ix) The elements of a fixed, documented test method for use by laboratory staff; and

x) The level of technical competence required of those who carry out and/or interpret the test.

c) Validation of the test method

Validation further evaluates the test for its fitness for a given use. Validation establishes performance characteristics for the test method, 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. Depending on logistical and risk factors, validation may involve any number of activities and amount of data, with subsequent data analysis using appropriate statistics. Test validation is covered in Chapter I.1.3. Principles of validation of diagnostic assays for infectious diseases, and Chapter I.1.4. Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases.

Validation activities might include:

i) Field and/or epidemiological studies;

ii) Comparison with other methods, preferably standard methods;

iii) Comparison with reference standards (if available);
iv) Collaborative studies with other laboratories using the same documented method, and including the exchange of samples, preferably of undisclosed composition or titre. It is preferable that these be issued by a qualified conducting laboratory that organises the study and evaluates the results provided by the participants;

v) Reproduction of data from an accepted standard method, or from a reputable publication;

vi) Experimental infection studies; and

vii) Analysis of internal quality control data.

ISO/IEC 17025 recognises that “Validation is always a balance between costs, risks, and technical possibilities.” It also recognises that there are many cases in which quantities such as accuracy and precision can only be given in a simplified way.

d) Uncertainty

Laboratories should be able to estimate the uncertainty of the test methods as performed in the laboratory. This includes methods used by the laboratory to calibrate equipment (4). ISO/IEC 17025 requirements concerning this topic are stated in sections 5.1.2, 5.4.4k, 5.4.6.2, 5.4.6.3 and 5.10.3.1c of that document.

The determination of measurement uncertainty (MU) is not new to some areas of measurement sciences. However, the application of the principles of MU to laboratories for the life sciences is new. Most of the work to date regarding MU is for areas of testing other than the life sciences, and much of the work has been theoretical. However, as accreditation becomes more important, applications are being developed for the other areas. It is important to note that MU does not imply doubt about the validity of a test result or other measurement, nor is it equivalent to error, as it may be applied to all test results derived from a particular procedure. It may be viewed as a quantitative expression of reliability, and is commonly expressed as a number after a +/− sign (i.e. the true value lies within the stated range, as MU is expressed as a range).

Standard deviation and confidence interval are examples of the expression of MU. An example of the use of standard deviation to express uncertainty is the allowed limits on the test run controls for an enzyme-linked immunosorbent assay, commonly expressed as +/− n SD.

Although the determination and expression of MU has not been standardised for veterinary testing laboratories (or medical, food, or environmental), some sound guidance exists.

MU must be estimated in the laboratory for each method included in the scope of accreditation. This can be estimated by a series of tests on control samples. MU can also be estimated using published characteristics (6), but the laboratory must first demonstrate acceptable performance with the method. Government agencies may also set goals for MU values for official methods (e.g. Health Canada). Reputable technical organisations, including accreditation bodies (e.g. AOAC International, ISO, NATA, A2LA, SCC, UKAS, Eurachem, and the Co-Operation on International Traceability in Analytical Chemistry [CITAC]) teach courses and/or provide guidance on MU for laboratories seeking accreditation. Codex Alimentarius, which specifies standards for food testing, has taken the approach that it is not necessary for a laboratory to take a further estimate of MU if the laboratory complies with Codex principles regarding quality; i.e. that the laboratory is accredited to ISO/IEC 17025, and therefore uses validated methods (e.g. knows applicable parameters such as sensitivity and specificity, as well as the confidence interval around such parameters), participates in proficiency testing programmes and collaborative studies, and uses appropriate internal quality control procedures.

The requirement for “use of appropriate internal quality control procedures” 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. Components can be estimated with experiments in the laboratory (Type A estimates), or from other sources (reference materials, calibration certificates, etc.) (Type B estimates). A traditional control sample procedure, run many times by all analysts and over all shifts, usually covers all the major sources of uncertainty except perhaps sample preparation. The variation of the control samples can be used as an estimate of those combined sources of uncertainty.

ISO/IEC 17025 requires the laboratory to identify all major sources of uncertainty, and to obtain reliable estimates of MU. Laboratories may wish to establish acceptable specifications, criteria, and/or ranges at critical control points for each component. Where appropriate, laboratories can implement appropriate quality control at the critical points associated with each source, or seek to reduce the size of a component. Sources of uncertainty include sampling, storage conditions, sample effects, extraction and recovery, reagent quality, reference material purity, volumetric manipulations, environmental conditions, contamination, equipment effects, analyst or operator bias, and other unknown or random effects. The laboratory would also be expected to account for any known systematic error. (See also Section 6.b. steps i–vii.) Systematic errors (bias) must either be corrected by changes in the method, adjusted mathematically, or have the bias noted in the report statement. If an adjustment is made to the procedure, there may or may
not be a need to reassess uncertainty. If there is an adjustment made to correct for bias, then a new source of uncertainty is introduced (the uncertainty of the correction). This must be added to the MU estimate.

There are three principal ways to estimate MU:

1. The components approach (or ‘bottom-up’ approach), where all the sources of uncertainty are identified, reasonable estimates are made for each component, a mathematical model is developed that links the components, and the variations are combined using rules for the propagation of error (1).

2. The control sample approach (or ‘top-down’ approach), where measurements on a stable control material are used to estimate the combined variation of many components. Variation from additional sources needs to be added.

3. The method characteristics approach, where performance data from a valid collaborative study are used as combined uncertainties (other sources may need to be added). Laboratories must meet defined criteria for bias and repeatability for the MU estimates to be valid. These should be larger than would be obtained by competent laboratories using their own control samples or components model.

e) Implementation and use of the test method

Analysts should be able to demonstrate proficiency in using the test method prior to producing reported results, and on an ongoing basis.

The laboratory should ensure, using appropriate and documented project management, records keeping, data management, and archiving procedures, that it can recreate at need all events relating to test selection, development, optimisation, standardisation, validation, implementation, and use. This includes quality control and quality assurance activities.

7. Strategic planning

Continuous improvement is essential. It is recommended that the laboratory be knowledgeable of and stay current with the standards and methods used to demonstrate laboratory competence and to establish and maintain technical validity. The methods by which this may be accomplished include:

i) Attendance at conferences;
ii) Participation in local and international organisations;
iii) Participation in writing national and international standards (e.g. participation on ILAC and ISO committees);
iv) Consulting publications;
v) 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; and
ix) Wherever possible, accreditation by a third party that is itself recognised as competent to issue the accreditation.

REFERENCES


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CHAPTER 1.1.3.

PRINCIPLES OF VALIDATION OF DIAGNOSTIC ASSAYS FOR INFECTIOUS DISEASES

INTRODUCTION

Validation is the evaluation of a process to determine its fitness for a particular use. A validated assay yields test results that identify the presence of a particular analyte (e.g. an antibody) and allows predictions to be made about the status of the test subjects. 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 [such as 99.99%] with associated lower diagnostic specificity [DSP] for a screening assay, or conversely, setting DSP high with associated lower DSe for a confirmatory assay).

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 – host/organism interactions affecting the analyte composition and concentration in the serum sample; (b) the assay system – physical, chemical, biological and technician-related factors affecting the capacity of the assay to detect a specific analyte in the sample; and (c) the test result – 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.

Factors that affect the concentration and composition of analyte in the serum sample are 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). Nonhost factors, such as contamination or deterioration of the sample, may also affect the analyte in the sample.

The principles of validation discussed in this chapter will focus primarily on methods to detect antibody in sera. However, these same principles could be applied to validation of tests for other analytes in sera or tissues. Chapter I.1.4. Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases extends the principles outlined here to a direct method of infectious agent detection, the molecular diagnostic assays.

Factors that interfere with the analytical accuracy of the assay system include instrumentation, technician error, reagent choice (both chemical and biological) and calibration, accuracy and acceptance limits of controls, reaction vessels, water quality, pH and ionicity of buffers and diluents, incubation temperatures and durations, and error introduced by detection of closely related analytes, such as antibody to cross-reactive organisms, rheumatoid factor, or heterophile antibody.

Measures that influence the capacity of the test result to predict accurately the infection or analyte status of the host are DSe, DSP, and prevalence of the disease in the population targeted by the assay. DSe and DSP are derived from test results on samples obtained from selected reference

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1 In this chapter, the terms ‘positive’ and ‘negative’ have been reserved for test results and never refer to infection or antibody/antigen status of the host. Whenever reference is made to ‘infection’ or ‘analyte’, any method of exposure to an infectious agent that could be detected directly (e.g. antigen) or indirectly (e.g. antibody) by an assay, should be inferred.
animals. The methods used to select the reference animals are critical to the accuracy of the estimates (5). 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 accuracy of test-result interpretation. For example, experienced diagnosticians are aware that an assay, validated by using samples from northern European cattle, may not give valid results for the distinctive populations of cattle in Africa.

The capacity of a positive or negative test result to predict accurately the infection status of the animal or population of animals is the most important consideration of assay validation. This capacity is not only dependent on a highly precise and accurate assay and carefully derived estimates of DSe and DSp, but is heavily influenced by prevalence of the infection in the targeted population or the likelihood that an animal is infected based on clinical criteria. Without a current estimate of the disease prevalence in that population or likelihood of infection in an individual animal, the interpretation of a positive or negative test result may be compromised.

Many factors obviously must be addressed before an assay can be considered to be ‘validated’ (5, 13). However, there is no consensus whether the concept of assay validation is a time-limited process during which only those factors intrinsic to the assay are optimised and standardised, or whether the concept includes an ongoing validation of assay performance for as long as the assay is used. Accordingly, the term ‘validated assay’ elicits various interpretations among laboratory diagnosticians and veterinary clinicians. Therefore, a working definition of assay validation is offered as a context for the guidelines outlined below. Ideally, all diagnostic assays would be fully validated for one or more purposes, but in practice there are sometimes limitations to the completeness of validation.

A. DEFINITION OF ASSAY VALIDATION

A validated assay consistently provides test results that identify animals as positive or negative for an analyte or process (e.g. antibody, antigen, or induration at skin test site) and, by inference, accurately predicts the infection status of animals with a predetermined degree of statistical certainty. This chapter will focus on the principles underlying development and maintenance of a validated assay. Guidelines for the initial stages in assay development are included because they constitute part of the validation process. How this early process is carried out heavily influences the capacity of the eventual test result to provide diagnostic accuracy.

It must be emphasised that an assay, when applied to target populations, will minimise misclassifications of animals as false positive or false negative only to the extent that validity is assured for all stages of the assay validation process. This assumes that a well designed and documented test method and proper standardised reagents, in combination with well trained technicians, will give a stable assay within the laboratory. It also assumes a thorough use of the most rigorous experimental design and epidemiological and statistical tools. These are required to reduce bias, random error, and false assumptions about the reference population of animals upon which the assay performance estimates are made (5). Furthermore, it assumes that the assay is fitted to the purpose for which it is intended (e.g. a confirmatory assay will likely yield many false-positive results if used as a screening assay). Finally, it assumes that when placed in practice, the assay is conducted within the context of a rigorous quality assurance programme.

The working definition of assay validation used in this paper generalises a process that takes on many different forms depending on the intended use of the assay being developed. No matter what the form of the assay, without scientific rigour in the validation process, the assay will not perform to expectations. However, there are many traditional techniques in widespread use that have not been subjected to the full validation process.

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2 In this definition, the DSe and DSp are performance characteristics of the assay for a given target population. They determine – together with the disease prevalence in the population – the probability that a given test result reflects the true status of the animal. An assay can be recognised as validated if reliable estimates of DSe and DSp for a given target population are available. This does not imply any minimum threshold values for these parameters. In practical applications, low values of DSe and DSp or diagnostic problems due to low disease prevalence are compensated by the sampling design or by combining multiple diagnostic assays into parallel or serial testing regimens. The selection of assays, the sampling process, the combination of multiple assays into a testing regimen and the interpretation rule for the results define the diagnostic process.
B. STAGES OF ASSAY VALIDATION

Development and validation of an assay is an incremental process consisting of at least five stages: 1) Determination of the feasibility of the method for a particular use; 2) Choice, optimisation, and standardisation of reagents, techniques and methods; 3) Determination of the assay’s performance characteristics; 4) Continuous monitoring of assay performance; and 5) Maintenance and enhancement of validation criteria during routine use of the assay (Figure 1). Although some scientists may question the relevance of the fourth and fifth stages as validation criteria, they are included here because an assay can be considered to be valid only to the extent that test results are valid, i.e. whether they fall within statistically defined limits and provide accurate inferences. In practice, there are many traditional techniques in widespread use that have not been through the formal validation process given above. Efforts should be made to formally validate the assay’s performance characteristics, either through use of historical data, or in a prospective study. This will lend credence to the belief that the assay is accomplishing what it purports to do. In such cases the process may appropriately be entered at stages 4 and 5, thus building up a data set of validation data for assays that lack the necessary information for stages 1–3.

It is of utmost importance not to stop after the first two stages of assay validation – that does not constitute a validated assay for diagnostic use. Although reagent and protocol refinement are important, the selection of the reference populations is probably the most critical factor. It is no surprise when reviewing the literature to find a wide range of estimates for diagnostic sensitivity (DSe) and diagnostic specificity (DSp) for the same basic assay. Although part of the variation may be attributed to the reagents chosen, it is likely that the variation in estimates of DSe and DSp is due to biased selection of sera on which the test was ‘validated’. This stage in assay validation needs more attention than it has been given previously. This is particularly true in the current atmosphere of international trade agreements and all their implications with regard to movement of animals and animal products.

An indirect enzyme-linked immunosorbent assay (ELISA) for detection of antibody will be used in this chapter to illustrate the principles of assay validation. It is a test format that can be difficult to validate because of signal
amplification of both specific and nonspecific components (2). This methodology serves to highlight the problems that need to be addressed in any assay validation process. The same basic principles are used in validation of other complex or simple assay formats. Chapter I.1.4. Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases describes the principles for validating gene-amplification techniques.

Because of space limitations, this introductory chapter provides only basic guidelines for the principles concerned with assay validation. It is derived from a more detailed treatment of the subject (8).

**STAGE 1. FEASIBILITY STUDIES**

In the ELISA example, feasibility studies are the first stage in validating a new assay. They are carried out in order to determine if the selected reagents and protocol have the capacity to distinguish between a range of antibody concentrations to an infectious agent while providing minimal background activity. Feasibility studies also give initial estimates of repeatability, and of analytical sensitivity and specificity.

1. **Control samples**

It is useful to select four or five samples (serum in our example) that range from high to low levels of antibodies against the infectious agent in question. In addition, a sample containing no antibody is required. These samples will be used to optimise the assay reagents and protocol during feasibility studies, and later as control samples. The samples ideally should represent known infected and uninfected animals from the population that eventually will become the target of the validated assay. The samples should have given expected results in one or more serological assay(s) other than the one being validated. The samples are preferably derived from individual animals, but they may represent pools of samples from several animals. A good practice is to prepare a large volume (e.g. 10 ml) of each sample and divide it into 0.1 ml aliquots for storage at or below –20°C. One aliquot of each sample is thawed, used for experiments, and ideally then discarded. If it is impractical to discard the aliquot, it may be held at –4°C between experiments for up to about 2 weeks; however, there is a possibility of sample deterioration under these circumstances. Then, another aliquot is thawed for further experimentation. This method provides the same source of serum with the same number of freeze–thaw cycles for all experiments (repeated freezing and thawing of serum can denature antibodies so should be avoided). Also, variation is reduced when the experimenter uses the same source of serum for all experiments rather than switching among various sera between experiments. This approach has the added advantage of generating a data trail for the repeatedly run samples. After the initial stages of assay validation are completed, one or more of the samples can become the serum control(s) that are the basis for data expression and repeatability assessments both within and between runs of the assay. They may also serve as standards if their activity has been predetermined; such standards provide assurance that runs of the assay are producing accurate data (13).

It is highly desirable to include OIE International Standard Sera or other international standard sera if they are available. This may lead to harmonisation between the assay under development and a standard test method in which international standard sera are normally used (12).

2. **Selection of method to achieve normalised results**

Normalisation adjusts raw test results of all samples relative to values of controls included in each run of the assay (not to be confused with transformation of data to achieve a ‘normal’ [Gaussian] distribution). The method of normalisation and expression of data should be determined, preferably no later than at the end of the feasibility studies. Comparisons of results from day to day and between laboratories are most accurate when normalised data are used. For example, in ELISA systems, raw optical density (absorbance) values are absolute measurements that are influenced by ambient temperatures, test parameters, and photometric instrumentation. To account for this variability, results are expressed as a function of the reactivity of one or more serum control samples that are included in each run of the assay. Data normalisation is accomplished in the indirect ELISA by expressing absorbance values in one of several ways (13). A simple and useful method is to express all absorbance values as a percentage of a single high-positive serum control that is included on each plate. (This control must yield a result that is in the linear range of measurement.) This method is adequate for most applications. More rigour can be brought to the normalisation procedure by calculating results from a standard curve generated by several serum controls. It requires a more sophisticated algorithm, such as linear regression or log-logit analysis. This approach is more precise because it does not rely on only one high-positive control sample for data normalisation, but rather uses several serum controls, adjusted to expected values, to plot a standard curve from which the sample value is extrapolated. This method also allows for exclusion of a control value that may fall outside expected confidence limits.

For assays that are end-pointed by sample titration, such as serum (viral) neutralisation, each run of the assay is accepted or rejected based on whether control values fall within predetermined limits. Because sample values usually are not adjusted to a control value, the data are not normalised by the strict definition of the term.
Whatever method is used for normalisation of the data, it is essential to include additional controls for any reagent that may introduce variability and thus undermine attempts to achieve a validated assay. The normalised values for those controls need to fall within predetermined limits (e.g. within an appropriate multiple of the standard deviation of the mean of many runs of each control). The chosen limits should reflect a reasonable and tolerable assay run rejection rate and an acceptable risk that some test samples may be misclassified.

**STAGE 2. ASSAY DEVELOPMENT AND STANDARDISATION**

When the feasibility of the method has been established, the next step is to proceed with development of the assay and standardisation of the selected reagents and protocols.

1. **Selection of optimal reagent concentrations and protocol parameters**

Optimal concentrations/dilutions of the antigen adsorbed to the plate, serum, enzyme–antibody conjugate, and substrate solution are determined through ‘checkerboard’ titrations of each reagent against all other reagents, following confirmation of the best choice of reaction vessels (usually evaluation of two or three types of microtitre plates, each with its different binding characteristics, to minimise background activity while achieving the maximum spread in activity between negative and high-positive samples). Additional experiments determine the optimal temporal, chemical, and physical variables in the protocol, including incubation temperatures and durations; the type, pH, and molarity of diluent, washing and blocking buffers; and equipment used in each step of the assay (for instance pipettes and washers that give the best reproducibility).

Optimisation of the reagents and protocol includes an assessment of accuracy by inclusion, in each run of the assay, of one or more serum standards of a known level of activity for the analyte in question. An optimised assay that repeatedly achieves the same results for a serum standard and the serum controls may be designated as a standardised assay.

2. **Repeatability – preliminary estimates**

Preliminary evidence of repeatability (agreement between replicates within and between runs of the assay) is necessary to warrant further development of the assay. This is accomplished by evaluating results from replicates of all samples in each plate (intraplate variation), and interplate variation using the same samples run in different plates within a run and between runs of the assay. For ELISA, raw absorbance values are usually used at this stage of validation because it is uncertain whether the results of the high-positive control serum, which could be used for calculating normalised values, are reproducible in early runs of the assay format. Also, expected values for the controls have not yet been established. Three-to-four replicates of each sample run in at least five plates on five separate occasions are sufficient to provide preliminary estimates of repeatability. Coefficients of variation (CV: standard deviation of replicates/mean of replicates), generally less than 20% for raw absorbance values for most samples (low-titrated samples may have larger CVs), indicates adequate repeatability at this stage of assay development. However, if evidence of excessive variation (>30%) is apparent for most samples within and/or between runs of the assay, more preliminary studies should be done to determine whether stabilisation of the assay is possible, or whether the test format should be abandoned. This is important because an assay that is inherently variable has a high probability of not withstanding the rigours of day-to-day testing on samples from the targeted population of animals.

3. **Determination of analytical sensitivity and specificity**

The analytical sensitivity of the assay is the smallest detectable amount of the analyte in question, and analytical specificity is the degree to which the assay does not cross-react with other analytes. These assay characteristics are distinguished from diagnostic sensitivity and specificity as defined below. Analytical sensitivity can be assessed by end-point dilution analysis, which indicates the dilution of serum in which antibody is no longer detectable. Analytical specificity is assessed by use of a panel of sera derived from animals that have experienced related infections that may stimulate cross-reactive antibodies. If, for instance, the assay does not detect antibody in limiting dilutions of serum with the same efficiency as other assays, or cross-reactivity is common when sera from animals with closely related infections are tested, the reagents need to be recalibrated or replaced, or the assay should be abandoned. However, there is a place for a test with low specificity, providing the sensitivity is high, for use as a ‘screening test’ in situations where a large number of samples have to be screened and where there is a ‘confirmatory test’ that has high specificity.

**STAGE 3. DETERMINING ASSAY PERFORMANCE CHARACTERISTICS**

If the feasibility and initial development and standardisation studies indicate that the assay has potential for field application, the next step is to establish the assay’s performance characteristics.
1. Diagnostic sensitivity and specificity

a) Principles and definitions

Estimates of DSe and DSp are the primary parameters established during validation of an assay. They are the basis for calculation of other parameters from which inferences are made about test results. Therefore, it is imperative that estimates of DSe and DSp are as accurate as possible. Ideally, they are derived from testing a series 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. Diagnostic sensitivity is the proportion of known infected reference animals that test positive in the assay; infected animals that test negative are considered to have false-negative results. Diagnostic specificity is the proportion of uninfected reference animals that test negative in the assay; uninfected reference animals that test positive are considered to have false-positive results.

The number and source of reference samples used to derive DSe and DSp are of paramount importance if the assay is ever to be properly validated for use in the population of animals targeted by the assay. It is possible to calculate the number of reference samples, from animals of known infection/exposure status, required for determinations of DSe and DSp that will have statistically defined limits. Formulae and tables for determining the number of samples required are provided elsewhere (5, 8). However, the number of reference samples derived from these formulae/tables is likely to be inadequate for a fully validated assay because of the very large number of variables in target populations that must be accounted for in the reference sera. These limitations and a discussion of the selection criteria for standard sera are detailed elsewhere (5, 8). Because of the many variables that must be accounted for, it is desirable to include at least 300 reference samples from known-infected animals, and about 1000 samples from known-uninfected animals to determine initial estimates of DSe and DSp, respectively. However, such a large number of samples from reference animals may be difficult or even impossible to achieve. Starting with fewer animals, followed by updating the DSe and DSp estimates as more samples from reference animals become available may be the only practical way to get initial estimates of DSe and DSp (See Stage 5 below).

b) Standards of comparison for the new assay

In serology, the ‘standard of comparison’ is the results of a method or combination of methods with which the new assay is compared. Although the term ‘gold standard’ is commonly used to describe any standard of comparison, it should be limited to methods that unequivocally classify animals as infected or uninfected. Some isolation methods themselves have problems of repeatability and sensitivity. Gold standard methods include unequivocal isolation of the agent or pathognomonic histopathological criteria. Because a true gold standard is impossible to achieve, relative standards of comparison are often necessary; these include results from other serological assays and from experimentally infected or vaccinated animals. Calculations of DSe and DSp are most reliable when the gold standard of comparison is available. When only relative standards of comparison are available, estimates of DSe and DSp for the new assay may be compromised because the error in the estimates of DSe and DSp for the relative standard is carried over into those estimates for the new assay. If no standard of comparison is available, DSe and DSp can be estimated (3, 7).

c) Precision, repeatability, reproducibility, and accuracy

Repeatability and reproducibility are estimates of precision in the assay. Precision is a measure of dispersion of results for a repeatedly tested sample; a precise assay has only a small amount of dispersion. Repeatability in a diagnostic assay has two elements: the amount of agreement between replicates (usually two or three) of each sample within a run of the assay, and the amount of between-run agreement for the same samples tested in different laboratories. Accuracy is the amount of agreement between a test value and the expected value for an analyte in a standard sample of known activity (e.g. titre or concentration). An assay system may be precise, but not accurate, if the test results do not agree with the expected value for the standard.

Reliable estimates of repeatability and accuracy, both within and between runs of the assay, can be obtained by use of normalised results from the many runs of the new assay that were required to assess the sera of reference animals (less reliable estimates were obtained from preliminary data using raw absorbance values). At least 10, and preferably 20, runs of the assay will give reasonable initial estimates of these parameters. Methods for evaluating these parameters have been described in detail (8).

Accuracy can be assessed by inclusion of one or more standards (samples of known titre, concentration, etc.) in each run of the assay. The standards may be control sera provided that the amount of analyte (e.g. titre, concentration) in each one has been previously determined by comparison with primary or secondary reference standards (13), and the control sera are not used in the data normalisation process.

Reproducibility of the assay is determined in several laboratories using the identical assay (protocol, reagents, and controls) on a group of at least 10 samples, preferably duplicated to a total of 20 samples.
These samples need to represent the full range of expected analyte concentrations in samples from the target population. The extent to which the collective results for each sample deviate from expected values is a measure of assay reproducibility. The degree of concordance of between-laboratory data is one more basis for determining whether the assay’s performance characteristics are adequate to constitute a validated assay.

2. Selection of the cut-off (positive/negative threshold)

To achieve estimates of DSe and DSp of the new assay, the test results first must be reduced to positive or negative categories. This is accomplished by insertion of a cut-off point (threshold or decision limit) on the continuous scale of test results. Although many methods have been described for this purpose, three examples will illustrate different approaches, together with their advantages and disadvantages. The first is a cut-off based on the frequency distributions (8) of test results from uninfected and infected reference animals. This cut-off can be established by visual inspection of the frequency distributions, by receiver-operator characteristics (ROC) analysis (6, 14), or by selection that favours either DSe or DSp, depending on the intended use for a given assay (11). A second approach is establishing a cut-off based only on uninfected reference animals, for instance the 99th percentile in a frequency distribution of assay values for uninfected reference animals; this provides an estimate of DSp but not DSe. The third method provides an ‘intrinsic cut-off’ based on test results from sera drawn randomly from within the target population with no prior knowledge of the animals’ infection status (4). Although no estimates of DSe and DSp are obtained by this method, they can be determined as confirmatory data are accumulated.

If considerable overlap occurs in the distributions of test values from known infected and uninfected animals, it is difficult to select a 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 would then be classified as suspicious or equivocal, and would require testing by a confirmatory assay or retesting for detection of seroconversion.

The purpose for which the assay is intended may dictate where the cut-off point is set. For example, a screening assay designed for high DSe versus a confirmatory assay designed for high DSp will require different cut-offs in the same assay system.

3. Calculation of diagnostic sensitivity and specificity

The selection of a cut-off allows classification of test results into positive or negative categories. Calculations of DSe and DSp are aided by associating the positive/negative categorical data with the known infection status for each animal using a two-way (2 x 2) table (Table 1). After the cut-off is established, results of tests on standard sera can be classified as true positive (TP) or true negative (TN) if they are in agreement with those of the gold standard (or other standard of comparison). Alternatively, they are classified as false positive (FP) or false negative (FN) if they disagree with the standard. Diagnostic sensitivity is calculated as TP/(TP + FN) whereas diagnostic specificity is TN/(TN + FP); the results of both calculations are usually expressed as percentages (Table 1).

Table 1. Calculations of DSe and DSp aided by a 2 x 2 table that associates infection status with test results from 2000 reference animals

<table>
<thead>
<tr>
<th>Reference animals of known infection status</th>
<th>Infected (n = 600)</th>
<th>Uninfected (n = 1400)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Result</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Test Result</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Reference animals of known infection status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected (n = 600)</td>
<td>570</td>
<td>30</td>
</tr>
<tr>
<td>Uninfected (n = 1400)</td>
<td>46</td>
<td>1354</td>
</tr>
<tr>
<td>Diagnostic sensitivity</td>
<td>TP = 570</td>
<td>FN = 30</td>
</tr>
<tr>
<td>Diagnostic specificity</td>
<td>TN = 1354</td>
<td>FP = 1400</td>
</tr>
<tr>
<td>TP = 570</td>
<td>TP + FN = 600</td>
<td></td>
</tr>
<tr>
<td>95.0%</td>
<td>96.7%</td>
<td></td>
</tr>
</tbody>
</table>
4. Harmonisation of assays

When an international standard method (12) is available for detection of an analyte, it is possible to compare the performance of that method with the one under development. This process requires use of the same serum controls and/or standards in both assays. If OIE Standard Sera or other international standard sera are available, preferably at least three (negative, low positive, and high positive), they should be included in the assay-comparison study. This could lead to a new assay that is indexed to an international standard method and international standard sera (12). Harmonisation of the two assays may then be realised.

STAGE 4. MONITORING VALIDITY OF ASSAY PERFORMANCE

1. Interpretation of test results – factors affecting assay validity

An assay's test results are useful only if the inferences made from them are accurate. A common error is to assume that an assay with 99% DSe and 99% DSp will generate one false-positive and one false-negative result for approximately every 100 tests on animals from the target population. Such an assay may be precise and accurate, but produce test results that do not accurately predict infection status. For example, if the prevalence of disease in a population targeted by the assay is only 1 per 1000 animals, and the false-positive test rate is 1 per 100 animals (99% DSp), for every 1000 tests on that population, ten will be false positive and one will be true positive. Hence, only approximately 9% of positive test results will accurately predict the infection status of the animal; the positive test results will misclassify the animal 91% of the time. This illustrates that the capacity of a positive or negative test result to predict infection status is dependent on the prevalence of the infection in the target population (9). Of course, the prevalence will probably have been determined by use of a serological test with its own inherent misclassification of results.

An estimate of prevalence in the target population is necessary for calculation of the predictive values of positive (PV+) or negative (PV–) test results. When test values are reported without providing estimates of the assay’s DSp and DSe, it is not possible to make informed predictions of infection status from test results (8). It is, therefore, highly desirable to provide an interpretation statement with test results accompanied by a small table indicating PV+ and PV– for a range of expected prevalences of infection in the target population. Without provision of such information, test results from the assay may have failed to accurately classify the infection status of animals, and thus do not reflect a fully validated assay.

STAGE 5. MAINTENANCE AND ENHANCEMENT OF VALIDATION CRITERIA

A validated assay needs constant monitoring and maintenance to retain that designation. Once the assay is put into routine use, internal quality control is accomplished by consistently monitoring the assay for assessment of repeatability and accuracy (1).

Reproducibility between laboratories should be assessed at least twice each year. It is highly desirable to become part of a consortium of laboratories that are interested in evaluating their output. In the near future, good laboratory practice, including implementation of a total quality assurance programme, will become essential for laboratories seeking to meet national and international certification requirements (see Chapter I.1.2.).

Proficiency testing is a form of external quality control for an assay. It is usually administered by a reference laboratory that distributes panels of samples, receives the results from the laboratories, analyses the data, and reports the results back to the laboratories. If results from an assay at a given laboratory remain within acceptable limits and show evidence of accuracy and reproducibility, the laboratory may be certified by government agencies or reference laboratories as an official laboratory for that assay (10). Panels of sera for proficiency testing should contain a full representation of an analyte’s concentration in animals of the target population. If the panels only have high-positive and low-positive sera (with none near the assay’s cut-off), the exercise will only give evidence of reproducibility at the extremes of analyte concentration, and will not clarify whether routine test results on the target population properly classify infection status of animals.

Because of the extraordinary set of variables that impact on the performance of serodiagnostic assays, it is highly desirable to expand the number of standard sera from animals of known infection status because of the principle that error in the estimates of DSe and DSp is reduced with increasing sample size. Furthermore, when the assay is to be transferred to a completely different geographical region, it is essential to re-validate the assay for this new intended use by subjecting it to sera from populations of animals that reside under local conditions. The same is true for establishing DSe and DSp for subpopulations (e.g. age groups, vaccinated/non-vaccinated, etc.).
When a serum control sample is nearing depletion, it is essential to prepare and repeatedly test a replacement before the serum control is depleted. The prospective control sample is included in 10–20 runs of the assay before depletion of the original control to establish its proportional relationship to the nearly depleted control. If the depleted sample was a positive control in ELISAs where the normalised value is expressed as a per cent of that positive control, the proportional difference in ELISA activity between the original and replacement sera must be factored into the normalisation algorithm to retain the same cut-off, and thus the same DSe and DSp in the assay. When other reagents, such as antigen for capture of antibody, must be replaced, they should be produced using the same criteria as for the original reagents, and tested in at least five runs of the assay using a panel of sera that has been designed for this purpose. Reagent lots (serials) need to be evaluated for consistency so variability is not introduced into the assay as new lots are required. Whenever possible, it is important to change only one reagent at a time to avoid the compound problem of evaluating more than one variable at a time.

**REFERENCES**


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CHAPTER I.1.4.

VALIDATION AND QUALITY CONTROL OF POLYMERASE CHAIN REACTION METHODS USED FOR THE DIAGNOSIS OF INFECTIOUS DISEASES

INTRODUCTION

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 nonstructural proteins, enzymes, etc., are detected. The indirect methods demonstrate the antibodies induced by the infections.

The most common direct detection methods are isolation or in-vitro cultivation, electron microscopy, immunofluorescence, immunohistochemistry, antigen enzyme-linked immunosorbent assay (ELISA), nucleic-acid hybridisation (NAH) and nucleic acid amplification, such as the polymerase chain reaction (PCR). As NAH and PCR assays have nucleic acid molecules as targets, they are also termed methods of molecular diagnosis.

The most common indirect methods of infectious agent detection are serological assays, such as virus neutralisation, ELISA and haemagglutination inhibition tests. In general, diagnostic laboratories simultaneously apply both the direct and the indirect methods, in order to provide more certainty in a diagnosis.

To date, OIE principles of validation have been developed for indirect detection methods, i.e. for antibody ELISA (see Chapter I.1.3.). The purpose of this chapter is to extend the rules to a direct method of infectious agent detection, i.e. to adapt the principles of validation to the PCR assays.

The experiences of the last decade indicate that the PCR techniques will eventually supersede many of the classical direct methods of infectious agent detection. It is clear that the PCR is replacing virus isolation or bacteria cultivation for the detection of agents that are difficult or impossible to culture. There are several reasons for this trend, including that virus isolation requires: i) the presence of replicating viruses; ii) expensive cell culture and maintenance facilities; iii) as long as several weeks to complete the diagnosis; and iv) special expertise, which is missing or diminishing today in many laboratories. Although PCR assays were initially expensive and cumbersome to use, they have now become relatively inexpensive, safe and user-friendly tools in diagnostic laboratories (2, 7). The sensitivity and specificity of PCR is generally greater than isolation or capture ELISA procedures.

A. PCR METHODS USED IN ROUTINE MOLECULAR DIAGNOSTICS

1. The principles of the PCR

Polymerase chain reaction (PCR) implies that there is an enzyme-based amplification reaction in the assay. The term ‘chain reaction’ refers to several cycles of copying a specified stretch of DNA or target nucleic acids (nucleotides), in this case from the genome of an infectious agent. The region to be amplified is defined by two (or more) short nucleotide sequences, termed primer sites, that flank the target sequence. Primers, short oligonucleotides that are complementary to the primer sites, bind to the DNA strand to be copied. Using a polymerase, which is not denatured during heat cycling, it is possible to copy the target sequence by joining free nucleotides to the primers. By repeating the heat-cycling regime 20–40 times, the amount of copied target DNA gained is enough for further operations, such as detection, cloning or sequencing. The diagnostic sensitivity of the PCR is very high because several million copies of the selected target are produced. The specificity may also be very high, as determined by the specific nucleotide sequences of the selected target, as well as primer
design. The primers can be designed to detect very specific nucleotide sequences in the genomes of the selected target infectious agents, or can be designed to be complementary to very common sequences occurring in nature. These nonspecific primers, termed universal primers, are able to bind to a wider range of DNA and can be used to detect members within a family or genus of infectious agent.

a) DNA amplification
If the genome of the infectious agent is DNA, the amplification is performed directly, with or without previous purification of the target DNA. In many cases, use of DNA extracted and purified from the material to be tested (e.g. blood) will result in increased analytical and diagnostic sensitivity.

b) RNA amplification (reverse-transcription PCR)
The genomes of many infectious agents contain ribonucleic acid (RNA) that cannot be amplified directly by the PCR. For PCR amplification, a single-stranded DNA target is necessary, and this is not available in the case of RNA viruses. This problem can be solved by the addition of a step before the PCR is begun. Using reverse transcriptase it is possible to transcribe the RNA into complementary DNA (cDNA), which is double-stranded DNA and hence can be used in a PCR assay (the procedure is termed reverse transcriptase PCR: RT-PCR). Traditionally, the reverse transcription reaction was performed in a separate reaction vessel and the cDNA produced is then transferred to a new tube for the PCR. However, heat-stable DNA polymerases with reverse transcriptase activity and specific buffers in which RT and DNA polymerases are active are now readily available. Both allow an RT-PCR reaction to take place in the same tube and in direct sequence without any further handling with less chance of carry-over contamination. In most cases, it will be necessary to extract and purify RNA prior to reverse transcription; however, in some cases, it is sufficient to boil the sample before RT-PCR (e.g. faecal samples in PBS).

c) PCR amplicon detection
The PCR product, or amplicon, can be detected using a variety of procedures. The most common include nonspecific detection of the PCR product based on amplicon size using electrophoresis in agarose gel and staining of the DNA with a nonspecific dye, such as ethidium bromide, or specific recognition of the amplified target sequence using Southern blot transfer of the DNA followed by hybridisation with oligonucleotide probes complementary to the target sequence. Hybridisation probes can be enzyme, chemiluminescent, or radionucleotide-labelled to allow visual detection of the specific target sequence.

Some examples of PCR methods currently used are given below.

2. Conventional PCR
‘Conventional PCR’ (or simply PCR) uses one pair of oligonucleotide primers to amplify a small part of the genome of the infectious agent. Analytical sensitivity is typically high with a minimum number of 100 to 1000 copies of the target DNA detectable. Analytical specificity can be high, dependent on target selection, primer design, and assay optimisation. Both analytical sensitivity and specificity can be further improved by applying nested PCR (see point 3 below). Detection methods, such as Southern blotting followed by hybridisation probes, can further improve sensitivity and specificity, but are time-consuming, require laboratory handling of amplified DNA, and interpretation of results can be technically subjective. Based on complexity and expense, these detection methods are not generally considered suitable procedures for common practice in diagnostic laboratories today.

3. Nested PCR
Nested PCR assays use two sets of amplification cycles with four primers, termed external and internal primers. In general, nested PCR assays provide higher analytical sensitivity and specificity compared with conventional PCR assays; however, there is a substantial increased risk of cross contamination. The lower limit of detection with the nested PCR is typically <10 genomic copies of the target DNA, and analytical specificity is also enhanced because in the nested PCR, four oligonucleotide primers have to bind specifically to the selected targets in order to yield a positive reaction (2).

4. Real-time PCR
Real-time PCR differs from standard PCR in that the amplified PCR products are detected directly during the amplification cycle using hybridisation probes, which enhance assay specificity. Various real-time methods, such as TaqMan, Scorpions, FRET, or Molecular Beacons assays, have become popular tools for detection of infectious agents. Real-time PCR has been used for the detection of bacteria, viruses or parasites from a range of animal species (2, 6, 8). These new assays have several advantages over the ‘classical’ conventional or nested PCR methods. Only one primer pair is used, providing sensitivity often close or equal to traditional nested
PCR but with a much lower risk of contamination. Fluorescence, indicating the presence of the amplified product, is measured through the lid or side of the reaction vessel thus there is no need for post-PCR handling of the amplified DNA. These procedures are considerably less time-consuming compared with traditional post-amplification PCR product detection in agarose gels followed by ethidium bromide staining and again, the risk of contamination is reduced. The use of a 96-well microtitre plate format, without the need for nested PCR, allows the procedure to be automated and suitable for large-scale testing (5). Diagnosis can be further automated by using robots for DNA/RNA extractions and pipetting. Compared with classical amplification methods, a further advantage of the real-time PCR is that it is possible to perform quantitative assays (6).

5. Multiplex PCR

PCR reactions using multiple primers directed at different targets in a single assay are referred to as multiplex PCR assays. In multiplex PCR, various infectious agents can be detected and differentiated in a single reaction vessel at the same time. The different PCR targets amplified in a standard PCR assay are identified based on PCR product size. The use of ‘classical’ nested PCR methods for the construction of a multiplex assay is complicated by the need for targets of different sizes, as well as primers that may ‘compete’ with each other in the same reaction mix, both of which can negatively impact PCR efficiency. In contrast, the concept of real-time PCR (single primer pairs) provides excellent possibilities for the construction of highly sensitive multiplex systems (2, 4) based on more uniform target size, uniform amplification conditions, and differential detection of targets using specific hybridisation probes labelled with different fluorophores.

B. VALIDATION OF MOLECULAR DIAGNOSTIC ASSAYS

When performing diagnostic analyses of clinical material it is important to produce data of good quality. For this, some key criteria have to be fulfilled. The establishment of quality assurance (QA) and quality control (QC) systems is required, i.e. a set of quality protocols, including the use of 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. Assay validation is another essential factor for assuring that test results reflect the true status of the samples (3).

To predict the diagnostic performance of a diagnostic assay, it is necessary to use a validation methodology to document the expected analytic performance of the assay in question. Validation is the evaluation of a diagnostic assay for the purpose of determining how fit the assay is for a particular use. The general principles of assay validation can be found in Chapter I.1.3. Principles of Validation of Diagnostic Assays for Infectious Disease. This chapter extends these validation principles to molecular diagnostic assays. For explanations of terms and definitions please consult Chapter I.1.3.

C. MEASURES OF VALIDITY

Performance characteristics (or assay parameters) give information about how a method functions under specified conditions. Some typical performance characteristics are given in Chapter I.1.3. and some others, important to PCR methods, are given here.

D. STAGES OF ASSAY VALIDATION

In Chapter I.1.3., the five stages of assay validation are described in detail. In this chapter, these stages are presented briefly with special emphasis on molecular diagnostic assays.

STAGE 1. FEASIBILITY STUDIES

A feasibility study is a preliminary step in validating a new assay. The goal is to determine whether or not a new assay can detect a range of target concentrations without confounding background activity. At least ten samples (for example, infectious agents produced in the laboratory in cell or bacterial culture) are chosen, that are at detection levels consistent with that expected to be encountered in clinical specimens ranging from low to high levels of the infectious agent. It is also necessary to include at least ten samples containing no target. Usually it is difficult to separate this stage from stage 2, as preliminary optimisation is necessary before further studies can be conducted. Assays that look promising are subjected to further development in stage 2. Note that it is sometimes possible to substantially improve an assay by proper optimisation schemes and thus exclusion of non-optimal assays, at least when suitable target sequences are difficult to find, should be done with caution.
Target selection and primer design are critical, and account should be taken of the nature of the infectious agent, its genome structure and the diversity of genetic sequences among different strains or isolates, and the intended use of the assay (e.g. surveillance testing, strain typing, etc.).

The result obtained by PCR may be influenced by the performance of the laboratory equipment, and in particular the thermocycler, which should therefore be monitored routinely as part of a QA programme. Regular temperature calibration is crucial and, for real-time PCR instruments, the optical systems must be calibrated regularly according to the laboratory’s QA protocols. Assays developed and validated using key equipment, such as robotic extraction or a thermocycler produced by a specific manufacturer should be revalidated or have equivalency data generated prior to use with alternate equipment platforms.

STAGE 2. ASSAY DEVELOPMENT AND STANDARDISATION

1. Selection of optimal reagent concentrations, protocol parameters and equipment

Sample collection, preparation and transport (see Chapter I.1.1.) and nucleic acid extraction methods (see Chapter I.1.8.) are all critical parameters in test performance and should be optimised for disease diagnosis. Suitable methods vary depending on sample and organism type. In general, blood serum, body tissues and swab samples are suitable samples for easy extraction of target nucleic acids, while faeces and semen samples are more difficult to handle. Extraction of RNA targets differs from extraction of DNA targets, and RNA is more prone to degradation. Both commercial (robotic, spin columns, 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 before further validation of the assay is performed. If the method of extraction is changed, equivalency data should be generated or the entire validation procedure should be repeated.

All equipment used during the process must be properly maintained. Apparatus (heating blocks, refrigerators, freezers, thermocyclers, pipettes, etc.) that require calibration must be calibrated according to the laboratory’s quality assurance protocols.

When developing ‘classical’ or real-time PCR assays, all parameters, protocols and reagents need to be optimised. A standardised assay is a method that consistently gives the same result for a given sample when repeated several times and when performed by different analysts in different laboratories.

2. Repeatability – preliminary estimates

Agreement between replicates within and between runs of the assay should be accessed at this stage. This gives important information about the assay before further validation is carried out. If excessive variability is encountered, it should be corrected before continuing the validation process.

3. Determination of critical control parameters

During the optimisation of the PCR assay, it is also possible to estimate the capacity of the method to remain unaffected by small changes in the main parameters. Documentation of intentional variations during performance of the assay is necessary to characterise critical parameters in the assay. Examples of such parameters include: incubation times and temperatures, concentrations of buffers, primers, MgCl₂, etc., pH, amounts of other components added (e.g. dNTP, bovine serum albumin, etc.). The characterisation of critical control parameters is crucial for identifying critical points that must be properly controlled in the assay.

4. Analytical sensitivity and specificity

Analytical sensitivity (or limit of detection) is defined as the smallest amount of an agent detected by the assay, and may be represented as number of genome copies, infectious dose, colony-forming units, plaque forming units, etc. of the agent that can be detected and distinguished from a zero result. To determine analytical sensitivity, an end-point dilution is used until the assay can no longer detect the target in question in more than 5% of the replicates (2 standard deviations). Cloned fragments of the PCR products in question can be used as standard samples, either as DNA or for RNA targets, the RNA being transcribed in vitro into DNA.

Analytical specificity is defined as the ability of an assay to distinguish the target agent from other infectious agents. This ability is determined by analysing genetically related pathogens and clinical material obtained from animals with diseases that may mimic that for which the assay is being designed.
5. **Range**

Analytical techniques should be optimised in the linear phase of the response curve. The range of an assay is defined as the interval between the upper and lower concentration of an infectious agent in a sample in which the agent can be reliably and reproducibly detected.

**STAGE 3. DETERMINING ASSAY PERFORMANCE CHARACTERISTICS**

1. **Diagnostic sensitivity and specificity**

Diagnostic sensitivity (D-SN; proportion of known infected reference animals that are tested positive in the assay) and specificity (D-SP; proportion of known uninfected reference animals that are tested negative in the assay) are the most important parameters obtained during the validation of an assay. They form the basis for calculating other parameters and hence they are critical to the whole validation process. The number of reference samples required to determine estimates and allowable error of both D-SN and D-SP can be calculated. To do this, a reasonable prediction of both D-SN and D-SP must be used. Generally, confidence in the estimate is set at 95%. However, no formula can account for the numerous host/organism factors that can affect the outcome of the test. The number of samples to determine estimates of D-SN and D-SP is outlined in Chapter I.1.3. In practice, this is unlikely to be achievable with molecular methods, especially with regard to positive samples in the case of a disease that is not endemic or widespread. It is usually only at the final stage, when the assay is in practical use, perhaps in parallel with the gold standard, that a sufficient number of animals is tested. As the use of the test progresses, the additional data collected will help to reduce the error in the estimations. The status of known infected and uninfected animals should be established using comparisons with other assays. The use of spiked samples in PCR is not appropriate as these might not be representative of naturally infected samples and thus the whole validation process could potentially be jeopardised.

2. **Repeatability and reproducibility**

Repeatability and reproducibility are both important parameters in assay precision. Repeatability is measured as both the amount of agreement between replicates within the same run or between replicates tested in different runs. Reproducibility is determined in several laboratories using the identical assay (protocol, reagents and controls).

Currently, OIE stage 3, as outlined in Chapter I.1.3, is rarely performed to its full extent in veterinary diagnostic laboratories carrying out PCR assays. Traditionally, many laboratories have used tests developed in-house, probably for practical reasons. Where there are published standardised and validated methods, these should be followed. Inter-laboratory validation processes have to be carried out even if they are costly and labour intensive. This work will lead to standardised assays, allowing harmonised diagnostic activity in various countries.

**STAGE 4. MONITORING VALIDITY OF ASSAY PERFORMANCE**

The estimation of the prevalence of an infectious agent in the population is necessary for calculating the predictive value of positive (PV+) or negative (PV–) test results. This applies equally to molecular test methods as it does to other methods such as the enzyme-linked immunosorbent assay.

Reference Laboratories are encouraged to determine values for D-SN and D-SP as accurately as possible, as these are extremely important for judging the real performance of an assay when used in the field. It is also important to estimate the predictive values (PV+ or PV–) in the local situation. During initial validation and use of a new assay, investigation of false negative and false positive results is encouraged. For example, false positive reactions in molecular-based assays can be evaluated by sequence analysis of the amplified DNA to assist in correcting errors due to nonspecific target or primer binding, lack of assay stringency, etc.

**STAGE 5. MAINTENANCE AND ENHANCEMENT OF VALIDATION CRITERIA**

When the assay is used as a routine test it is important to maintain the internal QC. The assay needs to be consistently monitored for repeatability and accuracy. Reproducibility between laboratories (ring tests) is recommended by the OIE to be estimated at least twice a year (9).

If the assay is to be applied in another geographical region and/or population, it might be necessary to revalidate or document equivalency under the new conditions. Revalidation may also be necessary if the test is applied to a
different sample matrix, e.g. validated on blood and used on other tissue, or validated for cattle tissue and used on other species. This is especially true for PCR assays as it is very common for point mutations to 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 by doing so the established performance criteria are no longer valid. 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 sites, to ensure that they remain stable so that the validation of the assay cannot be questioned. Validation and robustness may be needed to be repeated when the test is transferred from the developing laboratory to the field as the conditions may be less than optimal and the staff less knowledgeable.

1. Precautions and controls

Considering the uncertainty about the safety and reliability of the PCR in routine diagnosis, special precautions should be applied in any laboratory using PCR for detecting infectious agents, in order to avoid false-positive or false-negative results. These, together with internal controls (mimics) assure the safe evaluation of the results.

a) Precautions to avoid false-positive results

False-positive results (negative samples showing a positive reaction), may arise from either laboratory-related issues, such as cross-contamination, or assay-related factors, such as inefficient optimisation or assay performance. Product carry-over from positive samples or, more commonly, from cross-contamination by PCR products from earlier experiments is a possible source of error, and various practices and tools have been applied to prevent false-positive PCR results. Samples and reagents should be handled in separate laminar air-flow hoods, which are regularly decontaminated using UV light and bleach. Constructing and using special tube-holders and openers also help prevent false-positive PCR. In addition, safe laboratory practices should be applied, i.e. to perform the basic steps (DNA extraction, mix and primer preparation, sample preparation, agarose gel electrophoresis of amplification products, etc.) in separated laboratory areas or rooms. Different sets of pipettes should be used for each of the steps. The use of positive displacement and filtered tips is advisable. It is also advisable to have different persons perform the different steps, who are restricted to the respective laboratory areas. Precautions should be taken to prevent the introduction of amplified material from potentially contaminated laboratories into ‘clean’ laboratory areas by movement restrictions on samples, papers, equipment, persons or any other potential method of contamination. Movement in the opposite direction should only occur after decontamination, showering and clothes changing. Furthermore, it is preferable to dilute samples with an expected high, but unknown, amount of agent or target nucleic acid prior to introduce in ‘clean’ laboratory areas.

It is also very important to include negative controls, i.e. samples that are as similar to the test samples as possible but without having the target. In laboratories experiencing problems with cross-contamination, at least one negative control per five diagnostic samples has been recommended. Both positive and negative control samples should routinely be interspersed with diagnostic samples to assess PCR assay performance.

b) Internal controls (mimics) to avoid false-negative results

False-negative results (samples containing the agent of interest but tested as negative) occur mostly due to inhibitory effects and/or pipetting errors. Therefore, internal controls are used as indicators of PCR assay efficiency. PCR internal controls may include foreign DNA added to the sample or ubiquitous DNA naturally occurring in the sample. Foreign DNA added to the sample, may include DNA or RNA mimics. DNA mimics, manufactured oligonucleotides, have the same primer-binding sequences as the PCR target, but flank a heterologous DNA fragment of a different size. The identical primer-binding nucleotide sequences allow co-amplification of the target and the mimic in the same tube with minimal competition. The size differences provide easy discrimination by Southern blot analysis. Armored RNA®, an identical concept to DNA mimics, uses a control RNA fragment packaged in bacteriophage coat proteins to protect or stabilise the RNA for control or standardisation of RT-PCR assays.

With real-time PCR assays, it is also possible to use internal controls, a naturally occurring housekeeping gene, a selected fragment of the host animal’s genome such as beta-actin, GAPDH, or ribosomal RNA. By multiplexing such an intrinsic control with a specifically coloured reporter fluorophore, it is possible to check the sample quality and confirm PCR efficiency, as the target agent and intrinsic DNA are simultaneously detected.

Internal controls increase the reliability of diagnostic PCR. Caution must be used when designing and validating internal controls. 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. Internal controls are used in concentrations slightly lower than the detection limit of the diagnostic PCR to ensure the test’s performance. It should also be remembered that internal controls have a disadvantage similar to spiked samples and not representative of target nucleic acid and can lead to false-negative results.
2. Preparation of standards

Reference laboratories should provide standard samples representative of a given infectious agent. Such samples can be cultivated infectious agents or clinical specimens, etc., which are distributed in such a manner that the infectious agent is well preserved. Thus, the samples are distributed frozen, in organic solvents (e.g. Trizol) or other suitable ways. The samples can also be sent as nucleic acids (frozen, freeze-dried or in ethanol). For specific details, see the individual disease chapters. Reference laboratories should also provide the appropriate mimics.

REFERENCES


* * *

NB: There is an OIE Reference Laboratory for the Application of polymerase chain reaction methods for diagnosis of viral diseases in veterinary medicine (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 1.1.5.
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 so as to reduce internal contamination to a minimum.

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 line cell 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.

3. Each batch of vaccine shall pass a test for sterility that is similar to published methods (1–3, 5).

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 mycoplasmas. Suitable test methods have been published (1–3, 5).

6. Tests for freedom from certain specific bacteria may be required, e.g. for Salmonella pullorum, Mycobacterium tuberculosis and M. paratuberculosis, Brucella spp. and Leptospira spp.

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


2. A limited number of contaminating, non-pathogenic bacteria and fungi may be permitted (see Section J.2.5. below).

D. INACTIVATED VIRAL VACCINES

1. Paragraphs B.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.1. and B.4.

E. LIVING BACTERIAL VACCINES

1. Paragraph 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 vaccine passes a test for freedom from mycoplasmas. Suitable test methods have been published (ref. 5, and for avian mycoplasmas ref. 2).

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.

2. Paragraph B.2. or E.2. applies, as appropriate, if a virus or a bacterium is used in serum production.

3. Each batch of serum shall pass a test for sterility that conforms to that found in ref. 5. Suitable test methods have been published (1–3).

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 (ref. 5, and for avian mycoplasmas ref. 2).

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 (4).

J. PROTOCOL EXAMPLES

1. General procedures

Materials used in the production of biological products should be sterilised and/or tested to ensure the absence of 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 thiglycollate 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.
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>Bacillus subtilis ATCC # 6633</td>
<td>30–35</td>
<td>Aerobic</td>
</tr>
<tr>
<td>FTM</td>
<td>Candida krusei ATCC # 6258</td>
<td>20–25</td>
<td>Aerobic</td>
</tr>
<tr>
<td>SCDM</td>
<td>Bacillus subtilis ATCC # 6633</td>
<td>30–35</td>
<td>Aerobic</td>
</tr>
<tr>
<td>SCDM</td>
<td>Candida kursei ATCC # 6258</td>
<td>20–25</td>
<td>Aerobic</td>
</tr>
<tr>
<td>FTMB</td>
<td>Clostridium sporogenes ATCC # 11437</td>
<td>30–35</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>FTMB</td>
<td>Staphylococcus aureus 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. The amount of sterility test inoculum is dependent on the quantity of biological in each container. If the quantity is less

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\(^1\) American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, USA.
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 no 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 test). A sterile pipette or syringe and needle is used to aseptically transfer the quantity of biological 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 controls 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, such as Frey's mycoplasma medium, that will support the growth of small numbers of test organisms, including Acholeplasma laidlawii (ATCC # 23206), Mycoplasma arginini
identification. If samples of each batch should be tested; 5 ml or one-half of the container contents, whichever is the lesser, of cells are passaged two or three times at 7-day intervals, and tested for cytopathology, haemadsorption and flasks with 3.75 ml of test material in 25 ml of media or 15% of the test material, whichever is the lesser. The

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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, 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 pestiviruses.

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. 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 fluorescent antibody staining.

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. 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 tetraionate 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, 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 pestiviruses.

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 antisera. The serum must be shown to be free from antibodies against any of the contaminants for which the test is intended. At least two cell types are always required, so a minimum of 2 ml of MSV and 2 ml of antisera are required. The antisera is allowed to neutralise the MSV at room temperature for 1 hour. Of the MSV/antisera mixture, 2 ml is then inoculated on to 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 antisera 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 slides and rinsing them in deionised water 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 usually 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 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.
• **Acknowledgement**

Parts of this chapter were taken from or based on the chapter on Tests for sterility and freedom from contamination of biological materials in previous editions of the *Terrestrial Manual*.

**REFERENCES**


**FURTHER READING**

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


* * *
CHAPTER I.1.6.

HUMAN SAFETY IN THE VETERINARY MICROBIOLOGY LABORATORY

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. This requires careful consideration of the risks involved in a particular procedure, followed by appropriate measures to minimise the risk of human disease. 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; the other is to prevent disease in animals. 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 disease. 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 disease 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 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. Work with it can then be assigned to an appropriate containment category. To assess the risk to humans from a particular pathogen it is necessary to know whether infection with that organism can cause death, disease or inconvenience to the people working with it, and whether it could then spread to cause disease in the general human population. (There are additional considerations relating to the containment of animal pathogens and the prevention of the spread of infection to animals, which is a separate, though related, subject. Information on this may be found in the OIE Terrestrial Animal Health Code, Chapter 1.4.6., which for convenience is reproduced at the end of this chapter as Appendix 1.) To assess this risk it is necessary to know the epidemiological background of the organism and also such attributes of the organism as infectivity for humans, stability in the environment, ability to infect by different routes, and susceptibility to specific treatments or prophylaxis \(1, 2, 5, 6\). 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. Some of the considerations to take into account when evaluating risk are:

1. Known occurrence of human 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 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 Mycobacteria are inoculated into animals. To evaluate the impact of animal inoculation, a risk assessment should be conducted and the following factors should be considered:

   a) Host species versus inoculated species.
   b) Strain/treatment and concentration of the inoculum.
   c) Route of inoculation.
   d) Animal housing.
   e) Types of sampling during the experiment.

B. GROUPING OF MICROORGANISMS

The considerations outlined above have been used by several National Authorities to designate microorganisms into four hazard groups (2, 4) representing increasing hazards 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 disease;

Group 2 – Organisms that may cause human disease but are unlikely to be spread in the community and for which effective prophylaxis and treatment are available;

Group 3 – Organisms that can cause severe human disease and may spread in the community but for which there is usually effective prophylaxis and treatment;

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

Additional considerations apply to animal disease agents that are controlled by veterinary authorities, where it is necessary to prevent spread to domestic animals or wildlife. These aspects are dealt with in the OIE Terrestrial Animal Health Code; Chapter 1.4.6 of the Terrestrial Code deals with containment groups and is attached to this chapter as an Appendix. These OIE Standards are similar to those published by the European Union for laboratory containment of agents affecting animals.

Infectious organisms that might be encountered in laboratory work have been assigned to Hazard Groups 1–4 by authorities in several countries (2, 4). Some examples of the dangerous pathogens that may be found in a veterinary laboratory are listed in Table I. Bovine spongiform encephalopathy (BSE) has been placed in Hazard Group 3 by the European Union. 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 Hazard 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>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viruses:</strong></td>
<td><strong>Viruses:</strong></td>
</tr>
<tr>
<td>Influenza viruses types A, B, C; Newcastle disease virus; Orf (parapox virus)</td>
<td>Rabies virus; Equine encephalomyelitis virus (Eastern, Western and Venezuelan); Japanese B encephalitis virus; Louping ill virus</td>
</tr>
<tr>
<td><strong>Bacteria:</strong></td>
<td><strong>Bacteria:</strong></td>
</tr>
<tr>
<td>Alcaligenes spp.; Arizona spp.; Campylobacter spp.; Chlamydia 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>
<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

A. 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. 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 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 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 must 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 must be established for frequent cleaning and disinfection during and at the end of the work period;

2. Personnel access to the laboratory should be restricted;

3. Protective clothing, including gloves, masks, eye shields, and oro-nasal respirators, as appropriate, must 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 biohazard cabinets are used, care must be taken to balance ventilation systems.);

5. Food and/or drink must not be stored or consumed in laboratories;
6. Smoking and/or application of cosmetics must not take place in the laboratory;

7. Pipetting must not be done by mouth;

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

9. Emergency response plans should be developed to deal with biohazard 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 materials must be stored safely before disinfection. Materials for disposal must be transported without spillage in strong containers. Waste material should be autoclaved, incinerated or otherwise made safe before disposal. Reusable material must be decontaminated by appropriate means;

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

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

**Containment level for Group 2 pathogens** (see Appendix 1), in addition to the points given above, a microbiological safety cabinet should be used but can be operated in the open front mode (Class I cabinet). A Class II cabinet may also 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** (see Appendix 1), it is advisable that the laboratory be in an isolated location; access should be limited to qualified 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) (15) and the United States Department of Agriculture (USDA) (14).

In addition to the previous requirements, the laboratory must 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. A ventilation system is required that removes air from the laboratory through a high efficiency particulate air (HEPA) filter. HEPA filters must 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. Safety cabinets of Class I, II or III must be provided (16). 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 Hazard 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 (3).

**Containment level for Group 4 pathogens** (see Appendix 1), the most stringent precautions are required, including access to the building through a system of air locks, and the building being maintained under negative air pressure. Inlet air to the laboratory must be filtered through a single HEPA filter and extracted air through double HEPA filters. All work with infective agents must be conducted in a Class III cabinet or in a Class II cabinet in conjunction with the use of positive-pressure personnel suits. All sewage from the laboratory, laboratory effluent and autoclave drain effluent must be treated by appropriate means to ensure that all infectious material is destroyed before entering the public sewerage system. Staff must 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 is generally equal to the USDA’s biosafety level 3 Ag guidelines (14). 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 must pass a pressure decay test to confirm that it does not surpass the prescribed maximum leak rate.
B. 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 and mucous membrane exposure. 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 should be worn, 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 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.A. above. They are of three types:

Class I: An open-fronted cabinet designed specifically to provide operator 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 protection as well as product 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 (8, 10, 16).

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 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.

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 must be special facilities for the 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 must be fitted with safety devices to prevent accidental opening of doors when under pressure, and be regularly serviced and tested. Heat-protective gloves must 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 masks and boots should also be worn when working with liquid nitrogen.

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 must be regularly serviced and tested. All use of radioactive material must be meticulously recorded. All staff must be provided with a radiation monitoring device and have annual health checks. Local and national regulations must be followed (10).
A wide range of chemicals is in use 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 (i.e. in sufficient quantities, even a ‘harmless’ substance can become toxic). Vapours are especially hazardous, and some chemicals can be absorbed by penetration of intact skin. Procedures sufficient to protect pregnant laboratory workers should be followed at all times. A list of hazardous chemicals must 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 must be correctly stored in appropriate containers and at the correct temperature. Those that are very flammable must 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 (11, 12).

G. LABORATORY ANIMAL FACILITIES

Work with pathogens in laboratory animals poses special hazards (see Appendix 1). 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. Any such incidents should be recorded and wounds appropriately treated. There must be provision for incineration or rendering of carcasses 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 attendants. This is a large subject that can only be referred to briefly here (4, 7). An excellent book on health and safety in laboratory animal facilities has been published recently (17).

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 must be appropriate to the work and properly maintained. It must be kept ready to hand for immediate emergency use. Bandages, dressings and simple medicines should be available. Some staff should receive training in safety and first aid from recognised authorities and should possess a valid certificate as evidence of 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 accidents.

There should be written procedures for dealing with emergency failure of air-handling 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. Managers should be 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 their staff.

There should be an emergency procedure for calling medical assistance if required, and for hospitalisation when needed. Fire alarms must be fitted, and tested regularly. Each unit should designate a fire officer who conducts periodic fire drills to make staff aware of what to do and where to assemble in the event of fire. The fire officer is responsible for checking that everyone has left the building. Procedures for other 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 for transport, to ensure that there is no breakage of containers or leakage of contents that could put at risk postal workers, couriers or staff at the receiving laboratory. Applicable local, national and international regulations for the transportation of dangerous goods and importation of animal pathogens must be followed. These are summarised in Chapter I.1.1. Sampling methods.

J. CONCLUSION

High standards of laboratory safety that will ensure healthy working conditions for laboratory staff 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 has been completed and documented. There is a large published literature on all aspects of the subject, and further reading is recommended (6, 9, 13, 14, 15).

REFERENCES


APPENDIX I.1.6.1.

INTERNATIONAL TRANSFER AND LABORATORY CONTAINMENT OF ANIMAL PATHOGENS¹

A. OBJECT

To prevent the introduction and spread of animal diseases caused by pathogens.

B. INTRODUCTION

1. The consequences of the introduction into a country of an infectious disease or an animal pathogen or new strain of animal pathogen from which it is currently free, are potentially very serious. This is because animal health, human health, the agricultural economy and trade may all be adversely affected to a greater or a lesser degree. Countries will already have in place a range of measures, such as requirements for pre-import testing and quarantine, to prevent such introductions through the importation of live animals or their products.

2. However, there is also the risk that disease may occur as a result of the accidental release of animal pathogens from laboratories that are using them for various purposes such as research, diagnosis or the manufacture of vaccines. Such pathogens may already occur in the country or they may have been imported deliberately or inadvertently. It is therefore necessary to have in place measures to prevent their accidental release. These measures may be applied (a) at national borders by prohibiting or controlling the importation of specified pathogens or their carriers (see G. Importation of animal pathogens) or (b) within national boundaries by specifying the conditions under which laboratories must handle them. In practice, a combination of external and internal controls is likely to be applied depending on the risk to animal health posed by the pathogen in question.

C. PURPOSE

1. To provide guidance on the laboratory containment of animal pathogens according to the risk they pose to animal health and the agricultural economy of a country, particularly when the disease they cause is not enzootic.

2. To provide guidance on the import conditions applicable to animal pathogens.

3. Where animal pathogens also pose a risk to human health, guidance on their laboratory containment should be sought from the Terrestrial Manual and other relevant published documents.

D. CLASSIFICATION OF ANIMAL PATHOGENS

1. Animal pathogens should be categorised on the risk they pose to animal health, should they be introduced into a country or accidentally released from a laboratory. In categorising pathogens into four groups according to containment requirements, the following factors should be taken into account: the organism's pathogenicity, the biohazard it presents, its ability to spread, the economic aspects and the availability of prophylactic and therapeutic treatments.

2. 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,

¹ This Appendix is taken from Chapter 1.4.6. of the OIE Terrestrial Animal Health Code, 2003.
the pathogen poses a lower risk to animal health than in countries where such vectors or intermediate hosts occur naturally or could survive.

3. 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.
      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) The need to confine diseased or infected nondiseased animals is minimal.
      vi) The disease is of limited economic and/or clinical significance.

   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.
      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.

   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.
      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) The statutory confinement of diseased, infected and in-contact animals is necessary.
      vi) The statutory control of animal movements over a wide area is necessary.
      vii) The disease is of extremely severe economic and/or clinical significance.
      viii) No satisfactory prophylactic and/or therapeutic treatments are available.

### E. CONTAINMENT LEVELS

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.
2. The level of physical containment and biosecurity procedures and practices should be related to 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 Table 1.

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.

Table 1. Guidance on the laboratory requirements for the different containment groups

<table>
<thead>
<tr>
<th>Requirements of the laboratory</th>
<th>Containment Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>A) Laboratory siting and structure</td>
<td></td>
</tr>
<tr>
<td>1. Not next to known fire hazard</td>
<td>Yes</td>
</tr>
<tr>
<td>2. Workplace separated from other activities</td>
<td>Yes</td>
</tr>
<tr>
<td>3. Personnel access limited</td>
<td>Yes</td>
</tr>
<tr>
<td>4. Protected against entry/exit of rodents and insects</td>
<td>Yes</td>
</tr>
<tr>
<td>5. Liquid effluent must be sterilised</td>
<td>Yes and monitored</td>
</tr>
<tr>
<td>6. Isolated by airlock. Continuous internal airflow</td>
<td>Yes</td>
</tr>
<tr>
<td>7. Input and extract air to be filtered using HEPA or equivalent</td>
<td>Single on extract</td>
</tr>
<tr>
<td>8. Mechanical air supply system with fail-safe system</td>
<td>Yes</td>
</tr>
<tr>
<td>9. Laboratory sealable to permit fumigation</td>
<td>Yes</td>
</tr>
<tr>
<td>10. Incinerator for disposal of carcasses and waste</td>
<td>Available</td>
</tr>
<tr>
<td>B) Laboratory facilities</td>
<td></td>
</tr>
<tr>
<td>11. Class 1/2/3 exhaust protective cabinet available</td>
<td>Yes</td>
</tr>
<tr>
<td>12. Direct access to autoclave</td>
<td>Yes with double doors</td>
</tr>
<tr>
<td>13. Specified pathogens stored in laboratory</td>
<td>Yes</td>
</tr>
<tr>
<td>14. Double-ended dunk tank required</td>
<td>Preferable</td>
</tr>
<tr>
<td>15. Protective clothing not worn outside laboratory</td>
<td>Yes</td>
</tr>
<tr>
<td>16. Showering required before exiting laboratory</td>
<td>Yes</td>
</tr>
<tr>
<td>17. Safety Officer responsible for containment</td>
<td>Yes</td>
</tr>
<tr>
<td>18. Staff receive special training in the requirements needed</td>
<td>Yes</td>
</tr>
<tr>
<td>C) Laboratory discipline</td>
<td></td>
</tr>
<tr>
<td>19. Warning notices for containment area</td>
<td>Yes</td>
</tr>
<tr>
<td>20. Laboratory must be lockable</td>
<td>Yes</td>
</tr>
<tr>
<td>21. Authorised entry of personnel</td>
<td>Yes</td>
</tr>
</tbody>
</table>
### Table 1. Guidance on the laboratory requirements for the different containment groups (cont.)

<table>
<thead>
<tr>
<th>Requirements of the laboratory</th>
<th>Containment Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>22. On entering all clothing removed and clean clothes put on</td>
<td>Yes</td>
</tr>
<tr>
<td>23. On exiting all laboratory clothes removed, individual must wash and transfer to clean side</td>
<td>Yes</td>
</tr>
<tr>
<td>24. Individual must shower prior to transfer to clean side</td>
<td></td>
</tr>
<tr>
<td>25. All accidents reported</td>
<td>Yes</td>
</tr>
</tbody>
</table>

D) Handling of specimens

<table>
<thead>
<tr>
<th></th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>26. Packaging requirements to be advised prior to submission</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>27. Incoming packages opened by trained staff</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>28. Movement of pathogens from an approved laboratory to another requires a licence</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>29. Standard Operating Procedures covering all areas must be available</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

### F. POSSESSION AND HANDLING OF ANIMAL PATHOGENS

1. A laboratory should be allowed to possess and handle animal pathogens in Group 3 or 4 only if it can satisfy the relevant authority that it can provide containment facilities appropriate to the group. However, depending on the particular circumstances of an individual country, the authority might decide that the possession and handling of certain pathogens in Group 2 should also be controlled. The authority should first inspect the facilities to ensure they are adequate and then issue a licence specifying all relevant conditions. There should also be a requirement for appropriate records to be kept and for the authority to be notified if it is suspected that a material being handled contains a pathogen not covered by the licence. The authority should visit the laboratory periodically to ensure licence conditions are being complied with. It is important that authority staff carrying out the visit should not have any contact with species susceptible to the pathogens being handled at the laboratory for a specified period after visiting the laboratory. The length of this period will depend on the pathogen.

2. Licences should specify:
   
   a) how the pathogen is to be transported and the disposal of the packaging;
   
b) the name of the person responsible for the work;
   
c) whether the pathogen may be used in vivo (and if so whether in laboratory animals or other animals) and/or only in vitro;
   
d) how the pathogen and any experimental animals should be disposed of when the work is completed;
   
e) limitations on contact by laboratory staff with species susceptible to the pathogens being used;
   
f) conditions for the transfer of pathogens to other laboratories;
   
g) specific conditions relating to the appropriate containment level and biosecurity procedures and practices.

### G. IMPORTATION OF ANIMAL PATHOGENS

1. The importation of any animal pathogen, pathological material or organisms carrying the pathogen should be permitted only under an import licence issued by the relevant authority. The import licence should
contain conditions appropriate to the risk posed by the pathogen and, in relation to air transport, the appropriate standards of the International Air Transport Association concerning the packaging and transport of hazardous substances. The import licence for Group 2, 3 or 4 should only be granted to a laboratory that is licensed to handle the particular pathogen as in F. Possession and handling of animal pathogens.

2. When considering applications to import pathological material from other countries, the authorities should have regard to the nature of the material, the animal from which it is derived, the susceptibility of that animal to various diseases and the animal health situation of the country of origin. It may be advisable to require that material is pretreated before import to minimise the risk of inadvertent introduction of a pathogen.

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CHAPTER 1.1.7.
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 also prevents infection and replication 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 concern about the animal welfare implications of veterinary vaccine production and use. 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, 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), 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 90/677/EEC. 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, immunostimulation, 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 other 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, cell cultures, or avian embryos to select an isolate 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. This 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. These 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, are also used.

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 should, 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 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 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 a blueprint (building plan) and 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 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; 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; prion) 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 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.

**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. All records relevant to a batch of product should be retained for at least 2 years after the expiry date on the label. 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.
Chapter 1.1.7. — Principles of veterinary vaccine production

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

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. The master seed should consist of a single uniform batch of seed that has been mixed and filled into containers as one batch. 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. 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. Purity of MCSs should be established by testing to ensure freedom from extraneous bacteria, fungi, mycoplasma, and viruses.

- Primary cells

These 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 agents and other extraneous viruses. Some control authorities only allow the use of primary cells in exceptional cases.

- Embryonating eggs

These 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 sterilisation 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 minimise the risk of TSE (prion) contamination in 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 also 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.
EFFICACY TESTS

The efficacy of veterinary vaccines should be demonstrated by statistically valid vaccination-challenge studies in the host animal, using 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 onset of protection when claims for onset are made in 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. The antigen level per dose in the vaccine tested for efficacy must be at or below this minimum 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 establish 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.

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 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.

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, usually using the natural route of infection for that organism. 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 five passages (more for poultry products), the isolate must be fully characterised, using the same procedures used to characterise the master seed. 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 nontarget animals, and to persist in the environment must be evaluated to provide information for assessing the risk of the vaccine to the environment.

In some cases this may be done in conjunction with the increase in virulence tests.
Chapter I.1.7. — Principles of veterinary vaccine production

CONSISTENCY OF PRODUCTION

Prior to marketing approval of any new product, each establishment should produce in its facilities three consecutive production batches of completed product to evaluate the consistency of production. These batches 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. The size of each of the three batches should be at least one-third the size of the average batch that will be produced once the product is in production.

The manufacturer should test each of these batches 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 EU Directive 92/18/EEC, in the European Pharmacopoeia, or as described in this Terrestrial Manual may be used. Satisfactory test results should be demonstrated for all three batches prior to approving the production of the product in the facilities and its release for marketing. Each subsequent batch should be tested in the same manner with satisfactory results prior to release for marketing.

POTENCY TESTS

Potency tests, required for each batch 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. The potency of live vaccines is generally measured by means of bacterial counts or virus titration.

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.

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. For estimating stability to determine a provisional expiry date, a new product may be subjected to accelerated stability tests, 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 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 nonviable products, each batch presented for licensing is tested at release and at, or after, the requested expiry date. Some authorities also require an intermediate test. 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.

SAFETY TESTS

The intrinsic safety of vaccines should be demonstrated early in the development stage. Live vaccines should be evaluated using increase in virulence tests and by assessing risk to the environment as discussed above. Vaccines derived through biotechnology should be evaluated as discussed in the classification of biotechnology-derived vaccines and release of live rDNA vaccines discussed below. Safety studies for all products should include the safety of a single dose, of an overdose and of repeated single doses.
Safety tests for release of a batch are described in CFR Title 9 part 113, in the European Pharmacopoeia, in this Terrestrial Manual and elsewhere. Standard procedures are given for mouse, guinea-pig, cat, dog, horse, pig, and sheep safety tests. Products may require more than one type of safety test. The required safety test for a poultry product is described in the specific Standard Requirement or the Outline of Production for that product. 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 daily observation of the vaccinates during the prechallenge period of the potency tests. Further evidence concerning the safety of products is derived from field safety trials (discussed below), but these tests are not required for each batch.

**PURITY TESTS**

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 of final product prior to release.

Purity test procedures have been published, for example in CFR Title 9 part 113, in EU Directive 92/18/EEC, 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, pathogens detected by a 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 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 bovine spongiform encephalopathy 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 such as: verification that sources of all ingredients of animal origin in production facilities are from countries free of bovine spongiform encephalopathy.

**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 92/18/EEC, in the European Pharmacopoeia, or in this Terrestrial Manual.

**SAMPLING**

Samples should be selected from each batch of product. The selector should pick representative final containers from each batch 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 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);
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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;
7. The batch 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. When an antibiotic is 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 large 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. 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 I.1.2. 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. Periodic inspections also encourage continual improvements in production procedures and facilities that are consistent with advances in technology.
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TESTING PRIOR TO RELEASE FOR DISTRIBUTION

Prior to release, the manufacturer must test each batch 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 check testing final product, samples of each batch 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 should not be released. In such cases, subsequent batches of the product should be given priority for check testing by competent authorities.

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 they have 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 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 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 effects. 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 in 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 much 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 for plasmid DNA vaccines may be found on the Internet at the following addresses:

http://www.fda.gov/cber/points.htm; http://www.cba.unige.it/VL/bio-info.html

RELEASE OF LIVE rDNA PRODUCTS

The release of live rDNA and plasmid DNA vaccines (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 Directives 90/219/EEC and 90/220/EEC.

FURTHER READING

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


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


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CHAPTER 1.1.8.

BIOTECHNOLOGY IN THE DIAGNOSIS
OF INFECTIOUS DISEASES
AND VACCINE DEVELOPMENT

INTRODUCTION

Molecular biological methods have become increasingly applicable to the diagnosis of infectious
diseases and vaccine development. To become widely used the methods need to be easy, safe,
sensitive, reproducible and eventually automated to facilitate the evaluation of large numbers of
samples.

The purpose of this chapter is to provide general background information for the nonspecialist.
Two issues of the OIE Scientific and Technical Review are concerned with biotechnology and the
diagnosis of animal diseases, and may be consulted for a more detailed review (99, 100). The
following is an outline of the topics briefly reviewed in this chapter.

A. Detection of nucleic acids
1. Diagnosis by restriction fragment length polymorphisms and related DNA-based approaches
2. Polymerase chain reaction (PCR) and real-time PCR
3. Diagnosis by DNA probes and DNA microarray technology

B. Detection of protein
1. Immunohistochemistry
2. Immunoblotting
3. Antigen-capture enzyme-linked immunosorbent assay (ELISA)
4. Proteomics

C. Antibody detection
1. Competitive ELISA (C-ELISA)
2. Production of antigens by recombinant DNA technology

D. Vaccines
1. Gene deletion vaccines – bacteria
2. Marker vaccines and companion diagnostic tests
3. Virus vectored vaccines
4. DNA vaccines
5. Other developments in vaccine technology

A. DETECTION OF NUCLEIC ACIDS

1. Diagnosis by restriction fragment length polymorphisms and related DNA-based
approaches

Serological tests that are commonly used to identify microorganisms may be insufficiently discriminatory to
distinguish between isolates of closely related pathogens, whether they be viruses, bacteria, fungi or parasites. A
DNA-based procedure will offer the better discrimination that is often required and an appropriate starting point
may be analyses for restriction fragment length polymorphisms (RFLP).

The RFLP approach is based on the fact that the genomes of even closely related pathogens are defined by
variation in sequence. Thus, instances where the linear order of adjacent nucleotides comprising the recognition
sequence of a specific restriction enzyme in one genome may be absent in the genome of a closely related strain or isolate.

In practice the RFLP procedure consists of isolating the target pathogen, extracting DNA or RNA (with subsequent reverse transcription to DNA) and then digesting the nucleic acid with one of a panel of restriction enzymes. The individual fragments within the digested DNA are then separated within a gel by electrophoresis and visualised by staining with ethidium bromide. Ideally each strain will reveal a unique pattern, or fingerprint. Many different restriction enzymes may be considered at the outset of a new piece of work, so that analyses of many molecular fingerprints from digestions with several individual restriction enzymes may be undertaken and combination of the best set of results will allow a comprehensive differentiation between strains or isolates. A good example of the application of this technique is the differentiation between rabies virus biotypes from dog or vampire bats origin in Latin America (81).

Of greater utility to the study of pathogens is a modification to the basic RFLP technique whereby the polymerase chain reaction (PCR) is incorporated as a preliminary step. The PCR method (described in more detail in Section 2 below) is used to amplify a specific region of the genome (known by the investigator to be variable in sequence between pathogens), which then serves as the template DNA for the RFLP technique. This new combination (PCR-RFLP) offers a much greater sensitivity for the identification of pathogens and is especially useful when the pathogen occurs in small numbers or is difficult to culture, two features that characterise the intestinal protozoan parasite Cryptosporidium spp. Both RFLP and, more especially, PCR-RFLP are immensely useful for the genotyping of strains of Cryptosporidium as they can identify sources of human infection and provide a commentary on their epidemiology and occurrence (13, 115, 142). The involvement of specific strains or types in a disease outbreak can be thus defined and the epidemiological tracing of isolates within a country or between countries should be possible.

There are many other examples in which the RFLP/PCR-RFLP techniques are proving useful for discriminating between genotypes; for example, the fungus Candida (32, 33), the porcine reproductive and respiratory syndrome virus (154) and the bacterium Helicobacter pylori (51).

The human pathogen Candida krusei provides a good illustration of the general application of a range of molecular techniques. Dassanayake et al. (33) investigated the genetic diversity of eleven oral isolates of C. krusei and identified five different genotypes by pulsed field gel electrophoresis (PFGE), nine genotypes by RFLP using the enzyme HinfI, while DNA fingerprinting by the randomly amplified polymorphic DNA approach (RAPD-PCR) revealed three, eight or eleven genotypes depending on the primers used.

The incorporation of PFGE facilitates the separation of large (up to megabase size) fragments of DNA and can be a useful adjunct to the basic RFLP analysis. Jager et al. (61) used a combination of the rare-cutting restriction enzyme NotI and PFGE to characterise 80 isolates of Coxiella burnetii derived from animals and humans in Europe, USA, Africa and Asia. They distinguished 20 different restriction patterns and phylogenetic analysis of the different RFLP patterns revealed evolutionary relationships among groups that corresponded to the geographical origin of the isolates. No correlation between restriction group and the virulence of an isolate was detected in this study, but similar approaches on some other pathogens have made such a connection. Grigg and Boothroyd (50), for example, identified three restriction sites within the 35-fold-repetitive B1 locus that were capable of discriminating type I (mouse-virulent) from type II or III (mouse-avirulent) strains of Toxoplasma gondii.

RFLPs have clear value for use in epidemiological studies but more critical interpretation of RFLP data involves the construction of databases to determine whether the RFLP profiles are linked to factors such as virulence, host range and clinical significance. In practice, it is usual not to rely on one restriction site but to use sites from several locations within the genome to classify the isolate. A continuing issue for veterinary diagnostics is the correct assessment of any molecular differences found between isolates of a pathogen as the loss or acquisition of restriction endonuclease site(s) may not be associated with differences in the ability of the pathogen to cause disease, i.e. an RFLP difference may not be functionally significant, except as a distinguishing feature.

The technique of RAPD-PCR exploits the ability of short primer sequences to reproducibly amplify random fragments of DNA from a template of genomic DNA in a PCR with a low annealing temperature. The amplified fragments are again separated by electrophoresis and visualised by staining with ethidium bromide. Thus genomes from closely related pathogens yield DNA fingerprints that are more similar to each other than the fingerprints from the genomes of unrelated pathogens. Keil & Fenwick (68), for example, evaluated the degree of genetic diversity in 26 strains of the respiratory tract pathogen of canines, Bordetella bronchiseptica, and with one 10-nucleotide primer were able to identify four distinct fingerprint patterns. The technique has also proved useful for studies on Cryptosporidium (e.g. 91, 135).

Polymorphic RAPD markers that define individual strains, etc. may be sequenced and thence used as a sequence-confirmed amplified region (SCAR). Thus conversion of an anonymous polymorphic marker to a SCAR means that a single PCR may be done to more simply identify a specific genome. Lewin et al. (74) used the approach to identify 19 unique multilocus genotypes among 29 strains of the protozoan, Leishmania donovani.
The techniques by which DNA from a pathogen may be detected and characterised continue to improve and evolve. The ultimate discriminatory procedure is that of genome sequencing, but this is practicable at present only for significant pathogens with small genomes, such as viruses. For example, the outbreak of severe acute respiratory syndrome (SARS) and the sequencing of the 29,751-base genome of the associated coronavirus (85) usefully revealed that the virus was only moderately related to other known coronaviruses, including two human coronaviruses and did not closely resemble any of the three previously known groups of coronaviruses. This degree of interrogation at the level of nucleic acid will not be available to studies of the majority of pathogens for many years hence. Thus techniques such as RFLP, PCR-RFLP, RAPD-PCR and SCAR analyses will continue to play a central role in the identification of, and discrimination between, isolates of most pathogens.

2. Polymerase chain reaction (PCR) and real-time PCR

The PCR exploits natural DNA replication mechanisms and results in the in-vitro production of large quantities of a desired sequence of DNA from a complex mixture of heterogeneous sequences (44, 129). PCR can amplify a selected region of 50 to several thousand base pairs into billions of copies. A detailed discussion on the methodology and applications of PCR is given in Mullis et al. (94).

The amplification of DNA by the PCR is accomplished via a cyclic succession of incubation steps at different temperatures. The target DNA is first heat-denatured to separate the two complementary strands to provide a single-stranded template. Specific primers (short synthetic molecules of DNA complementary to both strands and flanking the target sequences) are then annealed to the single-stranded template at low temperature and extended with DNA polymerase at an intermediate temperature. Once the polymerase has synthesised a new strand of DNA, the product is separated from the template by heating to a higher temperature. These steps, referred to as cycles, are repeated 20–40 times, resulting in amplification of target DNA sequences. The key to the geometric amplification of target DNA sequences by the PCR is the selection of paired primers that, when extended, will create additional reciprocal primer-annealing sites for primer extension in subsequent cycles. The amplification of DNA by the PCR is accomplished via a cyclic succession of incubation steps at different temperatures. The target DNA is first heat-denatured to separate the two complementary strands to provide a single-stranded template. Specific primers (short synthetic molecules of DNA complementary to both strands and flanking the target sequences) are then annealed to the single-stranded template at low temperature and extended with DNA polymerase at an intermediate temperature. Once the polymerase has synthesised a new strand of DNA, the product is separated from the template by heating to a higher temperature. These steps, referred to as cycles, are repeated 20–40 times, resulting in amplification of target DNA sequences. The key to the geometric amplification of target DNA sequences by the PCR is the selection of paired primers that, when extended, will create additional reciprocal primer-annealing sites for primer extension in subsequent cycles. To detect RNA (e.g. RNA viruses), a cDNA copy of the RNA must first be made using reverse transcriptase (RT). The cDNA then acts as the template for amplification by the PCR. This technique is referred to as RT-PCR.

Any PCR product generated has, by definition, a characteristic size. Its identity is generally confirmed using DNA probes (see below) or restriction digests, which can be used to provide RFLPs (see above). More commonly, the advent of automated cycle sequencing techniques, identification is via direct sequencing of the PCR product. For example, sequencing has been used in the virulence typing of avian influenza A virus, in which virulence-associated structural motifs at the haemagglutinin gene cleavage site are reliable indicators of high pathogenicity in chickens (58). The sensitivity of a PCR may be enhanced by the use of a second set of primers to amplify a sub-fragment from the PCR product of the first reaction. This technique is commonly referred to as ‘nested PCR’ and has been used to detect low levels of Anaplasma marginale in persistently infected cattle (37). However, the use of nested PCR can increase the rate of false-positive results.

PCR is a highly sensitive procedure for detecting infectious agents in host tissues and vectors, even when only a small number of host cells are infected. PCR can target and amplify a gene sequence that has become integrated into the DNA of infected host cells. It can also target and amplify unintegrated viral gene sequences. It is clear that PCR has a role in the testing of vaccines to detect contamination. However, it does not differentiate between viable and nonviable organisms or incomplete pieces of genomic DNA, and this may complicate interpretation of results and affect the applicability of PCR in this role.

PCR may prove to be very useful in the diagnosis of chronic-persistent infections, such as those caused by retroviruses (bovine leukaemia virus, caprine arthritis/encephalitis virus, etc.). These diseases present serious problems in terms of diagnosis and prevention since infected animals are a constant potential source for transmission.

When PCR is used for diagnosis, a great deal of care is required to avoid contamination of the samples because the exquisite sensitivity of the technique can easily lead to false-positive results. Multicentre studies have shown that positive samples are detected consistently between laboratories, but that false positives are frequently obtained with known negative samples, indicating the continuing presence of contamination problems (133). Systems have been developed to deal with this problem, for example the dUTP-UNG system (d-uracil triphosphate and uracil-N-glycosylase). This system uses an enzymatic reaction to specifically degrade PCR products from previous PCR amplification (in which dUTP has been incorporated) without degrading native nucleic acid templates (23). This, of course, does not exclude contamination of the sample with extraneous virus. A new generation of robotic workstations is now available where PCR reactions may be set up with only a single tube open at any one time. This greatly reduces the risk of contamination. It is also important to control for potential ‘negative’ results caused by the presence of interfering substances in the PCR reaction mixture or patient’s sample by the inclusion of a template known to produce a PCR product (23). Use of these precautions allows the PCR to become a realistic option for the diagnostician.
Classical PCR methods for diagnosis of pathogens, both bacterial and viral, are now being complemented and in some cases replaced with real-time PCR assays. Real-time PCR monitors the accumulation of PCR product during the amplification reaction, thus enabling identification of the cycles during which near-logarithmic PCR product generation occurs. In other words, the assay can be used to reliably quantify the DNA or RNA content in a given sample. In contrast to conventional PCR, real-time PCR requires less manipulation, is more rapid than conventional PCR techniques, has a closed-tube format therefore decreasing risk of cross-contamination, is highly sensitive and specific, thus retaining qualitative efficiency, and provides quantitative information. In many cases, the real-time PCR assays have proved to be more sensitive than existing reference methods (43, 56, 82, 86, 166). The recent development of portable real-time PCR machines and assays (55, 124) raises the exciting prospect of these techniques being used for rapid (less than 2 hours) diagnosis of disease outbreaks in the field.

Validation of PCR techniques is covered in Chapter I.1.4. Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases.

### 3. Diagnosis by DNA probes and DNA microarray technology

Conventional DNA probing and microarray analysis are two sides of the same coin. Fundamental to both processes is the binding (hybridisation) of DNA, derived from a sample suspected of containing a pathogen (the ‘unknown’), with highly characterised DNA derived in advance from a pathogen of interest (the ‘known’ DNA).

In conventional DNA probing the unknown DNA (or RNA), the target, is immobilised on a solid surface e.g. a filter. The known DNA, made into a probe by labelling or tagging it in some way, is in the liquid phase and is applied to the target. In microarray diagnosis it is the known DNA (large oligonucleotides or complementary DNA) that is the target, immobilised on a glass slide, and the unknown DNA, in the liquid phase, that is labelled to make a probe.

In conventional DNA probing the target can be nucleic acids extracted from clinical material or cultured cells and either (a) added to filters (a dot or slot blot) or (b), less conveniently in a diagnostic context, transferred to a filter after gel electrophoresis. The amount of pathogen in a clinical sample might be too low for detection. Consequently one might amplify the nucleic acid by PCR or reverse transcription PCR (RT-PCR), the PCR product being applied to a filter. In order to visualise a probe bound to its target, the probe can be labelled with a radioactive nuclide or, more commonly and safely, ‘tagged’ non-radioactively. For example, biotin or psoralen–biotin may be incorporated into the probe, bound probe being detected by addition of streptavidin linked to an enzyme for subsequent generation of colour or light (chemiluminescence).

A microarray is so-called because it can comprise 20,000 or more different known DNAs, each DNA being spotted onto glass slides, to form the array. Each spot is only around 10 μm in diameter. DNAs complementary to parts of selected genes of pathogens can be used to make the arrays (14). However, if large numbers of pathogens are to be investigated then it would be logistically easier to use large oligonucleotides. The microarray that was used to identify the SARS virus as being a coronavirus had oligonucleotides comprising 70 nucleotides (70-mer) (165). In microarray probing it is the sample from which a probe is made. Essentially nucleic acid is extracted from a sample and (RT-) PCR performed using random oligonucleotide primers. In this way part of all the nucleic acids in the sample – both of host and pathogen origin – are amplified. These PCR products, representative of every nucleic acid in the sample, are labelled with a fluorescent dye and applied to the microarray. Under optimised conditions only the DNA derived from the pathogen will bind to the DNA on the glass slide. If one is interested in detecting only a particular pathogen or group of related pathogens then pathogen-specific oligonucleotides can be used to amplify these within the sample for probe production.

Microarrays for detecting pathogens can be designed for several levels of differentiation. In the case of oligonucleotide target DNAs one might initially design oligonucleotides to be able to detect and differentiate pathogens at the genus level. One would choose a number, perhaps 10 or so, of oligonucleotides with a high degree of sequence conservation (consensus oligonucleotides) within a given genus, such that a probe made from a field sample containing a member of that genus would be likely to hybridise to at least some of the oligonucleotides, whilst not hybridising (or hybridising to a lesser degree) to those corresponding to related genera, e.g. to differentiate *Aptelloivirus* (foot and mouth disease, FMDV) isolates from *Enterovirus* strains in the *Picornaviridae* family. One could then select other sets of oligonucleotides, placed on the same array slide, able to characterise a pathogen more specifically, e.g. to differentiate the seven types of FMDV, and potentially to even further refinement at subtype level.

In conventional DNA probing the detection of a pathogen is limited by the number of probes used, whereas in microarray analysis one is limited only by the number of target DNAs on the array. If a microarray has 1000 different oligonucleotides, then to achieve the same resolving power by conventional probing would require 1000 probes, 1000 separate probing reactions. The great advantage of microarray analysis in searching for pathogens is thus that hundreds of pathogens can be looked for simultaneously when probing a single microarray slide. Clearly microarray analysis has great potential when one is investigating diseases of unknown aetiology, diseases where more than one pathogen might be present, and when subtyping is required. When one
has a particular pathogen in mind, then the use of a microarray would be less justifiable, since the production and hybridisation of slides is relatively expensive. Instead, for these more simple cases, one might use pathogen/subtype specific PCRs, followed by sequencing or restriction fragment analysis for confirmation.

If previous experience of biotechnology is indicative of the future, then one would expect microarray equipment and reagents to become less expensive, leading to greater application of this technology in animal disease diagnosis. It will make the search for hitherto undiscovered viruses very much easier, allowing a much broader picture of the prevalence of infection in animal species.

**B. DETECTION OF PROTEIN**

1. **Immunohistochemistry**

As an adjunct to the isolation of causative organisms from tissue, immunohistochemistry is rapidly becoming a standard tool in diagnostic laboratories for the identification of antigens associated with viral, bacterial and protozoal microorganisms (121). The detection of antigens in fixed tissues offers a number of advantages over other diagnostic techniques. These advantages are: (a) convenience of sample submission; (b) safe handling of potential human pathogens; (c) retrospective studies of stored specimens; (d) rapidity; and (e) the detection of nonviable organisms (52). Immunohistochemistry is also used for the detection of abnormal prion protein (PrPSc) in brain tissue to confirm scrapie, bovine spongiform encephalopathy and other transmissible spongiform encephalopathies, and has proved to be more sensitive than the standard histopathological examination for diagnosis of these diseases (151). Demonstration of PrPSc in lymphoid tissue biopsies, e.g. nictitating membrane, can also be used for the preclinical diagnosis of scrapie (102). As the number of monoclonal antibodies (MAbs) to defined antigens increases, the use of immunohistochemistry for the identification of organisms and other specific markers for autoimmunity and neoplasia will increase. The limiting step in the process of immunohistochemistry is identifying a MAb/antigen combination that will bind in formalin-fixed tissues. This may be overcome by using frozen sections or employing antigen retrieval techniques (e.g. proteolytic enzyme digestion, microwaving) before immunostaining.

2. **Immunoblotting**

Immunoblotting combines the high resolution of gel electrophoresis with the specificity of immunochernical detection and offers a means of identifying immunodominant epitopes recognised by antibodies from infected animals. The immunoblotting procedure can be divided into six steps:

a) Preparation of the antigen
b) Resolution of the antigen by gel electrophoresis
c) Transfer of the separated polypeptides to a membrane support (nitro-cellulose membrane)
d) Blocking nonspecific binding sites on the membrane
e) Addition of detecting antibody
f) Detection of bound antibody

The choice of detecting antibody is critical. Polyclonal sera are composed of a range of antibodies reflecting the full repertoire of the immune response to a particular complex antigen. They will therefore detect a number of distinct polypeptides giving a characteristic ‘profile’ of reactivity. MAbs bind to only one epitope, therefore they are useful in identifying highly specific polypeptides. After incubation with the detecting antibody, any antibodies bound to specific protein bands are visualised using enzyme-labelled conjugated anti-species antisera and a suitable substrate/chromogen.

Immunoblotting is performed chiefly in diagnostic laboratories to identify and/or characterise infectious agents based on antigen specificity or to use known antigens to look for a specific serological response. False-positive and false-negative results in other diagnostic assays can often be resolved by immunoblotting (90). Immunoblotting is also often used to determine the specificity of individual MAbs. Individual purified polypeptides (or recombinant expressed proteins) may also be transferred to nitrocellulose membranes by immunoblotting to examine the reactivity of test sera to individual proteins. This characteristic profile of reactivity may be used to help distinguish between animals that have been vaccinated or infected (7). The major factor affecting the success of an immunoblotting technique is the nature of the epitopes recognised by the antibodies. Most high resolution gel techniques involve some form of denaturation of the antigen. This destroys conformational determinants and allows only the detection of linear or non-conformational epitopes. Most polyclonal antisera contain antibodies to both linear and conformational epitopes, but MAbs often are directed at conformational epitopes.
3. Antigen-capture enzyme-linked immunosorbent assay (ELISA)

The antigen-capture enzyme-linked immunosorbent assay (ELISA) facilitates detection of antigen from pathogens directly from an animal prior to or during clinical disease. The ELISA commonly follows a sandwich assay format using capture and detecting antibodies (either specific MAbs or polyclonal antibodies). Antigen from the test sample is first captured by a specific MAb or polyclonal antibody bound to a solid-phase support and its presence is detected through use of a second MAb or polyclonal antibody, which may either be radio- or more generally, enzyme-labelled (conjugated). If the detecting antibody is not conjugated then an anti-species conjugate (reactive to the detector antibody) is used. The capture antibody selects the target antigen from other competing protein in sample suspensions and ensures that it is semi-concentrated to increase the chances of its detection. The desired characteristics of the capture MAb are strong binding to the pathogen, recognition of a conserved epitope highly specific for the target agent, and the ability to attach to an ELISA plate without loss of reactivity. In addition, a second MAb recognising an epitope other than that recognised by the capture MAb that is bound to the ELISA plate is often used as part of the indicator system. However, it may be difficult to identify MAbs of comprehensive intra-typic reactivity and polyclonal antisera may be preferred to increase the likelihood of reaction against all antigenic variants. Examples of antigen-capture ELISAs are the system for detection of *Anaplasma marginale* in the blood of preclinical cattle (152), the use of antigen-capture ELISA on cattle blood samples for the detection of bovine viral diarrhoea virus (88, 89, 131), and the rapid detection of rinderpest and peste des petits ruminants virus antigens in clinical samples (76). Respiratory syncytial antigen in nasal secretions was captured using ELISA with MAbs directed against epitopes of the viral capsid (98). Related antigen-capture methods using immunomagnetic beads are now important and well accepted methods for detecting certain bacterial infections, including *Listeria*, *Salmonella* and *Escherichia coli*.

Immunocapture PCR is a promising method for detection of antigen that combines the principles of ELISA diagnostics with the amplification power of PCR and has been reported for the detection of plant and human viruses (62, 97). The principles involve antigen capture by antibody coated to a solid phase and subsequent DNA (or RNA) extraction, reverse transcription in the case of RNA) and PCR amplification. Several studies have shown this procedure to increase the sensitivity of ELISA techniques significantly, by as much as tenfold in the case of bovine herpesvirus 1 (BHV-1) (95).

Validation of tests to detect antibody is addressed in Chapter I.1.3. Principals of validation of diagnostic assays for infectious disease.

4. Proteomics

The proteome is the total complement of proteins expressed within a cell, a tissue or an organism and proteomics is the study of proteins, including their expression level, post-translational modification and interaction with other proteins, on a large scale. Since not all proteins are expressed at all times, but are dependent on physiological and environmental factors, proteomics can provide an excellent global view of disease processes at the protein level. Because the application of proteomics to novel drug discovery promises huge economic returns, companies all over the world have rapidly poured resources into this new research field (21).

Many methods used in proteomics, including two-dimensional gel electrophoresis (2DGE) and mass spectrometry (MS) were established years ago. However recent advances in MS techniques, together with whole genome sequencing and the development of powerful bioinformatics and robotics platforms, have revolutionised protein identification. The general principle of proteomics is that proteins are separated, usually by 2DGE on polyacrylamide gels, then protein spots are excised, digested with trypsin, and the resultant peptides analysed by MS. The masses of these peptides are then compared to the predicted masses of peptides derived by computational analyses of genome databases, resulting in gene identification. MS can also be used to deduce the amino acid sequence of peptides and to characterise post-translational modifications such as glycosylation or phosphorylation. 2DGE shows some drawbacks, particularly for the separation of hydrophobic proteins, and other separation techniques based on liquid chromatography are now finding favour for some applications. Nevertheless, 2DGE is the method of choice for creating quantitative maps of protein expression and many thousands of proteins can be analysed in a short space of time.

Alterations in the proteome of body tissues or of fluids such as serum, urine or cerebro-spinal fluid can be measured directly so changes that occur in a disease state can be accurately pinpointed. As well as identifying molecules that may be targets for novel therapies, this approach is a very powerful tool for early-stage diagnosis of disease. The best-established clinical applications of proteomics are so far in the identification of markers for the early diagnosis of cancers, such as bladder cancers in urine (123). However, considerable research efforts are also ongoing on other areas such as heart disease (64), Alzheimer’s disease (25) and insulin-dependent diabetes (1).

The use of proteomics for the diagnosis of infectious disease is in its infancy but may prove to be of considerable importance. For example, definitive diagnosis of chronic hepatitis B virus (HBV) infection still relies on liver biopsy, but proteomic analysis of serum samples shows that the expression of at least seven serum proteins is...
changed significantly in chronic HBV patients (54). Similarly, the ante-mortem differential diagnosis of Creutzfeldt-Jakob disease (CJD) may be aided by proteomics since preliminary data show that seven proteins in cerebro-spinal fluid (CSF) are differentially expressed between patients with variant or sporadic CJD (26).

An extremely useful application of proteomics to the diagnosis of infectious disease is in the identification of novel diagnostic antigens by screening serum from infected and uninfected individuals against immunoblotted, 2DGE mapped proteomes of infectious agents. Using this type of approach with human sera, nine new potential immunodiagnostic antigens were identified in Helicobacter pylori (49), over 80 antigens in Borrelia burgdorferi that could potentially differentiate between patients with early or late symptoms of lyme disease (64) and seven antigens of Toxoplasma gondii that could potentially differentiate between acute and latent toxoplasmosis (64).

Within the veterinary field, proteomics-based research projects are now underway and these will undoubtedly yield novel diagnostic tools for the future. Proteome maps are being derived for a range of veterinary pathogens including bacteria such as Brucella melitensis (93) and Streptococcus agalactiae (60), protozoa such as Toxoplasma gondii (27), Eimeria tenella (20) and Trypanosoma brucei (126) and nematodes such as Haemonchus contortus (171).

C. ANTIBODY DETECTION

1. Competitive enzyme-linked immunosorbent assay (C-ELISA)

The competitive ELISA (C-ELISA) has largely replaced the indirect ELISA for large-scale screening and serosurveillance. The C-ELISA offers significant advantages over the indirect assay since samples from many species may be tested without the need for species-specific enzyme-labelled conjugates for each species under test. Many antigens are extremely difficult or time consuming to purify. If used in an indirect assay, they would result in high background values due to nonspecific binding. However, relatively crude antigens may be used in the C-ELISA provided the ‘detecting antibody’ has the desired specificity. The principle of a competitive assay for the detection of antibodies is competition between the test serum and the detecting antiserum. Specific binding of the detecting antibody is detected using an appropriate anti-species conjugate. A reduction in the expected colour obtained is due to binding of antibodies in the test serum, which prevent binding of the detecting antibody.

The detecting antibody may be polyclonal or monoclonal depending on the required specificity. MAbs directed against highly conserved epitopes will give broadly reactive assays whereas those directed against highly specific epitopes will result in a highly specific test. One of the early reports on the use of the C-ELISA was its use in detecting anti-bluetongue virus antibody (2). This used an MAb against a highly conserved epitope on bluetongue virus (BTV) P7 and allowed detection of antibodies to all 24 serotypes of BTV. The epitope was not shared in any of the other closely related Orbivirus serogroups, therefore the test was also BTV-specific. The specificity of the assay can therefore be tailored depending on the specificity of the detecting antibody.

The C-ELISA format has been successfully used in the screening of large numbers of pig sera for classical swine fever antibodies (168), the detection of antibody to malignant catarhal fever virus in apparently infected sheep, deer and bison (75) and antibodies to Babesia equi and B. caballi in persistently infected horses (67, 70). More recently, a solid-phase C-ELISA was used for the large-scale serological surveillance during the UK FMD outbreak in 2001 (104). This facilitated the testing of some 3 million sera over a period of less than one year.

2. Production of antigens by recombinant DNA technology

Advances in molecular biology and genetics in the 1970s initiated the development of recombinant DNA technology. Since then the impact of this technology is such that it plays a vital role in scientific research as well as in the diagnosis and treatment of disease. Recombinant DNA technology simply refers to the transfer of a gene from one organism into another – literally, the recombination of DNA from different sources. The objectives of recombinant DNA technology include identifying genes, isolating genes, modifying genes, and re-expressing genes in other hosts or organisms. These steps permit scientists and clinicians to identify new genes and the proteins they encode, to correct endogenous genetic defects, and to manufacture large quantities of specific gene products such as hormones, vaccines, and other biological agents of interest. Of particular importance is the degree of specificity in diagnostic tests attainable by the use of recombinant protein. One example is the use of ESAT-6, (early secretory target antigen 6) present in virulent Mycobacterium bovis and M. tuberculosis but not in avirulent BCG or most environmental mycobacteria, for the diagnosis of tuberculosis in cattle and humans (22, 153). This has the potential for providing a degree of specificity in diagnosis not achievable with purified protein derivative (PPD), the bacterial extract currently used.

Natural proteins are perhaps the ideal antigens, providing sequence-specific and surface structural epitopes. Many current diagnostic tests require test antigens that need to be continuously produced from cell culture or harvested from an infected animal. These antigen preparations are expensive and often have a short shelf-life, with each new batch of antigen requiring standardisation. Natural proteins are rarely available in a completely
pure form, and antibodies often develop against contaminating polypeptides that can lead to false-positive results. Recombinant DNA technology produces antigens that offer many advantages over antigens isolated from other biological sources. These advantages include a high purity, high specific activity and since the protein is synthesised in genetically modified laboratory-grown cells, each preparation of the protein product is identical to the previous preparation, ensuring batch-to-batch consistency. When recombinant antigens are used in combination with the C-ELISA format, purification of the recombinant antigen from the lysate may not be necessary as the specificity of the C-ELISA resides mainly in the MAb used. An example of the procedure is the cloning of the envelope genes of caprine arthritis/encephalitis lentivirus in a vaccinia expression vector (77).

An outline of the procedure for the production of an antigen by recombinant DNA technology is as follows. The identification of an antigen of potential diagnostic or scientific significance is achieved through the study of the antibody response of the host to the proteins of the organism in question. Immunodominant antigens, defined proteins of the organism against which the host responds with the highest potential diagnostic titre, are of particular interest as they are major stimulants of cellular and humoral immunity against the disease of interest. Antigen discovery studies are widely used to identify biologically relevant, immunodominant antigens for use in generating MAbs as well as in vaccine development. Once a protein of interest has been identified, the gene encoding the protein is generated using messenger RNA (mRNA) from the organism as a template for making cDNA. This method of cloning the gene encoding the protein of interest requires a prior knowledge about the gene sequence, either directly from the organism of interest or through the use of gene sequences from closely related species. An alternative method, when gene sequence data is not available, is the generation of recombinant libraries from the genomic DNA of the organism or from cDNA synthesised from mRNA. Fragments of the recombinant libraries can be cloned into an expression system, which may be prokaryotic or eukaryotic, and the gene library screened for expression of the protein.

There is a wide choice of expression systems. Protein may be expressed in bacteria, usually *E. coli* (118), yeast (24), insect cells using baculovirus (143), or in eukaryotic cells by infection with appropriate viral vectors (138) or by permanent transfection. Differences in glycosylation when prepared in bacterial, insect or mammalian cell cultures can modify protein structure and its reactivity with antibody. Antigen may need extracting from the cell or be secreted. Purification is often, but not always, necessary. An upcoming trend in the production of antigens for use in assays is in the development of synthetic peptide antigens. This allows antigens to be tested as diagnostic reagents from knowledge of the gene sequence, without expression of the whole protein being necessary, thus curtailing the process. An example is the production of peptide antigens to two immunodominant antigens, reported to be promising candidates as diagnostic reagents for the detection of *M. bovis* infection in cattle (164).

### D. VACCINES

1. **Gene deletion vaccines – bacteria**

Live attenuated bacterial vaccines confer better protection against challenge than killed vaccines (59). The reasons for this improved protection are not yet clear, but one could be that live vaccines are able to express antigens *in vivo* necessary for protection that killed vaccines preparations do not contain. Another reason could be that live vaccines are able to stimulate antigen presenting cells (APC) in a manner in which killed vaccine preparations are unable to. Most likely it is a combination of both, novel antigen expression and interaction with APC. Generation of live attenuated bacterial vaccines relied mainly on the generation of mutants by prolonged culture in vitro, changes in temperature growth or chemical modification, which resulted in undefined attenuations. In some cases, for unknown reasons, these mutants reverted to wild-type phenotype and therefore could not be used as vaccines (141). In 1981 Hosieth & Stocker (57), using transposon technology, developed *Salmonella typhimurium* strains with defined genetic mutations auxotrophic for aromatic amino acids (*Aro* that were unable to survive in the immunocompetent host. These strains were able to confer protection against virulent challenge in the murine model of salmonellosis and in several domestic species, although for unknown reasons, not all the mutants were able to confer protection in the domestic species (136, 137). In 1992, Jones et al. (63) developed a live attenuated salmonella mutant using precise genomic excision of two genes involved in the aromatic amino acid pathway, which resulted in an even lower probability of the strain reverting to wild-type phenotype. This mutant proved to be a vaccine with relatively mild clinical secondary effects and able to confer protection in cattle against virulent challenge at the age in which the host is more susceptible. This vaccine has also been used as a delivery vector for guest antigens, which brings closer to reality the ideal single dose multivaccine (162). Developments in molecular biology and a greater understanding of the host pathogen interaction will permit the rational design of safer and more efficient vaccines with markers that will allow the distinction between vaccinated and infected hosts. Although most of the developments described in here focus on salmonella, similar technologies are being applied to other bacterial pathogens.

2. **Marker vaccines and companion diagnostic tests**

In animal health, one can either vaccinate animals in order to prevent a disease or try to eliminate the infection through strict application of sanitary measures such as slaughtering of infected and in-contact animals. For
certain diseases for which no vaccine exists (e.g. African swine fever) and particularly for zoonotic infections (e.g. Nipah virus infection of pigs), the systematic slaughtering of infected animals is the only available solution. Diagnosis of infection is of paramount importance whatever the measures taken to fight the disease. Diagnosis can be direct, through the detection of the infectious agent using immunological or molecular technologies, or indirect, based upon the detection of specific antibodies against the suspected infectious agent. The latter methods have a major drawback in that one must wait until antibodies are synthesised by the animal after infection and generally they do not allow distinction between a humoral immune response resulting from an infection or a vaccination.

This problem can be overcome by adopting new approaches to vaccine development (101) using molecular technologies that allow the production of marker vaccines associated with companion diagnostic tests. There are currently two types, either based on the detection of a serological response against a protein whose gene has been deleted in the vaccine strain (either used as a replicating vaccine or as an inactivated vaccine derived from such a deleted virus vaccine), or on the detection of the serological response to virus nonstructural proteins (purified inactivated vaccines). In the case of the deletion vaccines the gene coding for a non-essential protein, the marker characteristic, is always linked with the detection test while in the case of subunit vaccines (e.g. protein E2 of classical swine fever virus expressed in baculovirus) the choice of the marker test assay may be linked to several other of the virus proteins. For harmonisation purposes, an agreed protein should be chosen for the test (e.g. protein gE of pseudorabies virus). In the first type of marker vaccines, the marker must always be negative since a positive marker, for instance provided through the insertion of a gene coding for a foreign protein, is not suitable; such a vaccine will only show if the animal has been vaccinated but will not indicate if the animal was also infected with the wild virus. Marker vaccine used with the intention of distinguishing a serological response resulting from either vaccination or infection must always be associated with a companion diagnostic test that can be used during a prophylactic campaign with the aim of eliminating the infectious agent. Previous veterinary vaccines were mainly designed to prevent clinical signs in animals following an infection without taking too much account of the epidemiological impact of vaccination on the excretion of wild virus following infection and on its dissemination/circulation. If marker vaccines are used with the aim of eliminating a virus they must have a clear impact on the epidemiology of the infection.

There can be problems with this approach, for example if wild virus multiplication is inhibited to the point that it does not induce the synthesis of specific antibodies in all animals. Therefore, most of the available marker vaccines can only be used for herd certification and not for individual animal certification.

a) Marker vaccines with one gene deletion: the examples of pseudorabies and infectious bovine rhinotracheitis

Pseudorabies in pigs and infectious bovine rhinotracheitis are two infections caused by herpesviruses that become latent in an animal, even when it has already been vaccinated (108, 112, 113). The first marker vaccine became available to prevent pseudorabies infection in pigs (156) due to the development of an attenuated strain of pseudorabies virus by Bartha in Hungary (5) that had a spontaneous deletion in the gE glycoprotein. Analogous vaccines were later developed for infectious bovine rhinotracheitis.

As mentioned above, the herpesvirus responsible for infectious bovine rhinotracheitis becomes latent after infection, whether or not the animal has been vaccinated. It does not matter if the vaccine is an inactivated or an attenuated one, either way the animal becomes a latent carrier after infection with a wild virus. Moreover, all the attenuated vaccine strains establish latency after vaccination, including gE deleted strains. It should be borne in mind that attenuated vaccines produced with identical strains, deleted or not, are generally more efficacious than their inactivated counterparts (15, 65, 66).

In an area where vaccination is prohibited, all animals serologically positive with regard to infectious bovine rhinotracheitis virus must be considered as potentially infected and latent carriers of a wild virus. Similarly, in an area where animals are vaccinated with a conventional (non-deleted) vaccine, either attenuated or inactivated, it is impossible to distinguish between vaccinated and infected cattle and so if an elimination programme is in place, all the seropositive animals must also be eliminated from the herd.

A solution may come from the use of a marked/deleted vaccine. The deleted protein in the vaccine strain must have the following characteristics:

1) Be a structural protein, in order to be able to produce inactivated vaccines;
2) Be non-essential in order to be able to produce the vaccine;
3) Not be an essential protective immunogen in order to still have an efficacious vaccine;
4) Induce a significant and long lasting humoral immune response when present in order to be used (when deleted) as a marker;
5) Be present in all the wild virus strain;
6) Induce a humoral immune response by wild virus in already vaccinated animals.
If such a marker vaccine is used, whenever an animal is seropositive towards the deleted protein, it must be seen as infected and eliminated. The gD protein of herpesviruses, being a major protective immunogen, cannot be deleted but contrarily may be used to develop subunit vaccines. The main problem encountered with the use of marker vaccines against infectious bovine rhinotracheitis is their inability to completely prevent wild virus circulation when used within the framework of an elimination programme.

No available vaccine is able to induce sterile immunity. As a consequence the vaccination schedule must be more stringent than a conventional one designed merely to protect against clinical signs in the herd. Vaccination must be repeated according to a strict schedule to reduce the possibility of wild virus excretion and must, in addition, be associated with strict sanitary measures (78). Within the framework of a coordinated virus elimination campaign, vaccination must prevent the excretion of wild virus by naive animals and prevent re-excretion by latently infected ones.

The efficacy of a repeated vaccination using an inactivated gE negative vaccine administered intramuscularly has been investigated under field conditions in the Netherlands. This study showed a significantly reduced incidence of seroconversion against wild virus in the vaccinated group compared with the placebo injected control animals. In addition, wild virus circulation, while not completely restricted, was nevertheless significantly reduced (15) and in some circumstances even prevented (157).

b) Vaccination against classical swine fever with subunit vaccines

An elimination programme for classical swine fever has been set up within the European Union. Vaccination using conventional vaccines is now prohibited and a slaughter policy is in place. This policy is challenged by the existence of a strong antigenic relationship with other pestiviruses, such as the virus responsible for bovine viral diarrhoea (BVD/MD), that impede serological diagnosis, the insidious circulation of hypovirulent strains (10) and, last but not least, the presence of a wild reservoir in wild boar (Sus scrofa) in continental Europe (3).

The classical, conventional, vaccines had a well proven efficacy (120) and even prevented the emergence of asymptomatic carriers when they were of sufficient potency (11, 73). Live attenuated vaccines were more efficacious than their inactivated counterparts in this respect (31) and they contributed greatly to the elimination of the disease. Their one disadvantage was the creation of a population of serologically positive animals, which is not acceptable if a slaughter policy is in place. The solution for countries that prohibit vaccination but which are still facing recurrent episodes of classical swine fever may come from the use of subunit vaccines that act as ‘marker vaccines’.

Subunit vaccines have recently been developed by expressing the E2 protein, a major immunogen of classical swine fever virus, either in a baculovirus system (71, 158) or in vaccinia or pseudorabies viruses (E1) (128, 160). The baculovirus expressed E2 protein vaccine allows distinction between infected or vaccinated animals when used with reliable companion diagnostic tests to detect the presence of specific antibodies directed against other major immunogens of classical swine fever virus not present in the subunit vaccine, such as NS2 protein, a conserved virus protein. Unfortunately, inactivated vaccines are not sufficiently efficacious from an epidemiological standpoint (40) when compared to the former conventional vaccines (38, 155). Moreover, the companion diagnostic tests currently available are not fully reliable and therefore limit the use of these subunit vaccines in the field. This is worrying since it would be difficult to completely eliminate classical swine fever from Europe without vaccination (161).

c) Vaccination against foot and mouth disease using highly purified vaccines

Elimination of foot and mouth disease in continental Europe was achieved by mass vaccination of cattle and pigs (83), notwithstanding the difficulties encountered with all such vaccination campaigns (35). Preventive vaccination has been prohibited in the European Union since 1991. This prohibition ended a 30-year period of vaccination and consequently completely naïve cattle herds now exist in Europe (145). This situation is particularly detrimental when the disease is accidentally reintroduced (42). The contingency plan that has evolved to deal with unexpected outbreaks is mainly based on information and training of the concerned partners in the European Union. In order to overcome the risks associated with the complete susceptibility of European livestock, concentrated, highly purified virus antigen vaccine banks have been established (80, 130) and there is the possibility of using these as marker vaccines in case of an emergency outbreak (34).

This is possible since, when highly purified vaccines are used, whenever an animal is found that is seropositive to the nonstructural proteins (NSP) coded by the virus using an ELISA diagnostic test (36), it must have been infected by a wild virus. The NSP are only produced when virus multiplication occurs and are not present in the extracellular virions used to produce purified inactivated vaccines. The NSP are synthesised at the same level as the structural proteins during infection and so produce a good humoral immune response. In order to completely remove containing NSP, the vaccines must be submitted to a special purification procedure in order to ensure that they only contain structural proteins before
formulation. Unfortunately, the companion diagnostic tests currently available only permit certification of freedom from foot and mouth disease at a herd level and not the individual animal level.

d) Equine influenza as a special case

A similar approach as that used for foot and mouth disease has been applied, in a different context, to equine influenza (107). When carrying out studies on the duration of protective immunity with equine influenza inactivated vaccines, it is useful to have a diagnostic tool that allows the exclusion of antibodies due to intercurrent infection of the experimental animals by a wild influenza virus. A diagnostic test has been developed (9) based on the serological response to a nonstructural protein coded by the virus.

3. Virus-vectored vaccines

Many virus species, including adenoviruses, herpesviruses and poxviruses, have been used as delivery systems (vectors) for foreign antigens. The virus can be used simply as a vector, for example the vaccinia-rabies recombinant vaccine, or as both a vector and a vaccine against the infection by the wild vector itself. An example of a virus acting both as a vector and a self vaccine is the recombinant capripox virus expressing a peste des petits ruminants virus antigen (8). A vector virus may undergo full multiplication cycle leading to the production of progeny virus or abortive multiplication cycle without the production of progeny virus, such as in the case of the avipoxvirus vector in mammalian species.

The most commonly used vectors are poxviruses and this chapter will therefore focus on the use of poxviruses as vaccine vectors (114).

A number of features make poxvirus recombinants suitable as vaccines:

i) the stability of freeze-dried vaccine (28), its low cost, ease of manufacture and administration;

ii) the vaccine can be administrated by several routes (46) and in the case of vaccinia virus it has even been shown that the virus can be administrated per os (this feature has been used for vaccinating wildlife) (109);

iii) the ability to induce both antibody and cytotoxic T cell responses against the foreign antigen with long lasting immunity after a single inoculation (139, 140, 170);

iv) the packing flexibility of the genome, which allows large amounts of the genome to be lost or deleted and foreign DNA to be inserted in its place (at least 25 kb), thus enabling multivalent vaccines to be created (116, 117, 139, 140);

v) the use of recombinant poxviruses as vaccines allows discrimination between naturally infected versus vaccinated animal since the recombinant vaccine displays a defined subset of the antigens of the pathogens concerned.

Within each genus of the Poxviridae family the members are antigenically related (92). This antigenic relationship has raised an important question concerning the use of poxvirus-derived vectors as live vaccines, as pre-existing immunity against the vector could reduce the success of a subsequent vaccination performed with a homologous poxvirus vector (29, 30, 72). To circumvent this problem, the use of different combinations of vectors and/or routes of immunisation has been implemented (45, 122).

a) Vaccinia virus as a vector

The first recombinant vaccinia to be used in the field is the recombinant vaccinia-rabies vaccine (VRG) used for oral vaccination of foxes against rabies. This was developed using the Copenhagen strain and tested in many potential target species under laboratory conditions (12, 18, 69, 110, 150) before it was eventually used under field conditions in 1987 (111) and proved to be safe and efficacious (17). It has been used on a large-scale in several European countries that were, as a consequence, freed from rabies (16) as well as in North America.

The safety of vaccinia virus can be enhanced by multiple gene deletions. This has been demonstrated by the engineering of the NYVAC strain of vaccinia virus (147). For this the Copenhagen strain of vaccinia virus was chosen as the vaccine substrate and based on the entire DNA sequence (48), on extensive knowledge of virulence-related genes and on genes determining host range replication competency, unwanted genetic information was deleted from the viral genome in a very precise manner. The resulting virus, named NYVAC, has 18 open reading frames deleted compared with the parental strain. NYVAC is highly attenuated as demonstrated in many animal studies. Intracranial inoculation of newborn and young adult mice demonstrated a very favourable dose range compared with either the parental or other vaccinia strains. Most significantly there is no dissemination of the virus in immunocompromised hosts. NYVAC has dramatically reduced the ability to replicate in a variety of human tissue culture cells and is unable to
produce infectious particles in humans. Several animal and human trials have demonstrated the safety of the NYVAC strain-derived vectors (19, 105, 106, 146, 167).

b) Avipoxvirus vectors

When considering the development of avipox-derived vectors for the production of vaccines for birds, the use of attenuated strains is recommended in order to reduce the safety risk and the potential consequences arising from environmental spread to other avian species. Attenuated derivatives of fowlpox virus, like TROVAC, and canarypox virus, like ALVAC, have been extensively tested and their safety demonstrated in a variety of species, including immunocompromised animals and human volunteers. These viruses can be used under laboratory safety conditions level 1, the lowest category for recombinant organisms (106).

Despite the fact that their multiplication is restricted to avian species, attenuated strains of avipoxviruses have been demonstrated to be efficacious and extremely safe vectors for mammals. Inoculation of avipox based recombinants in mammalian cells results in expression of the foreign gene and inoculation into mammalian species induces protective immunity without producing progeny viruses (148, 149). This observation demonstrates that they have a significant safety advantage for human and animal use. Since immunisation can be achieved in the absence of productive replication it eliminates the potential for dissemination of the vector within the vaccinates and, therefore, the spread of the vector to non-vaccinated contacts or to the general environment. Moreover, the use of this vector in species that are not a reservoir of avipoxviruses renders the likelihood of recombination in vivo nil. Additionally, these vectors can be used for vaccination of individuals with pre-existing immunity to vaccinia virus.

In the past decade, a great number of recombinant viruses have been produced using the attenuated canarypox ALVAC strain as the parental strain. An impressive number of trials, both in humans and animals, have demonstrated the safety and protective efficacy of vaccines using this vector.

4. DNA vaccines

DNA vaccination is the direct introduction into host cells of a bacterial plasmid DNA that expresses an antigenic protein under the control of a eukaryotic cell promoter (125). As a consequence, the foreign antigen is expressed within the host cell and can stimulate the induction of both humoral and cell-mediated immune responses. This approach to vaccination has been effective against a wide-range of viruses, bacteria and parasites and not only has many of the benefits of live vaccines but also has several advantages over more conventional approaches to vaccination. For example, DNA vaccines encoding foreign genes are inexpensive and easy to produce; they obviate the need for complex carrier organisms; the risks associated with live vaccines are absent; and the impact of pre-existing immunity to the organism or vector on vaccine efficacy is circumvented. However, a disadvantage of DNA vaccination is that, as the plasmid persists for a long time, there is a potential for chromosomal integration with resulting cell transformation.

The immune response of DNA vaccines can be further improved by simultaneous inoculation of immunostimulators, such as CpG motif sequences (119), plasmids expressing cytokines (169), plasmids expressing co-stimulatory molecules (84), or even conventional adjuvants (159). Immunogenicity can also be improved by first priming with a plasmid DNA vaccine expressing an immunogenic protein followed by subsequent boosting with the protein or with a recombinant virus vector expressing the protein, the so-called ‘prime-boost’ approach (163).

Several DNA vaccines for veterinary use are currently being developed in cattle, pigs and poultry (96, 103, 159). Delivery of the DNA is either by intramuscular, intradermal or intranasal inoculation, particle-mediated intradermal delivery using a gene gun, in which the DNA is precipitated onto gold microspheres (79), or it can be accomplished using attenuated intracellular bacteria, such as *Shigella flexneri* or *Salmonella typhimurium* (41). Whilst this latter approach has the advantage of targeting the DNA to a large number of antigen-presenting cells, and could be delivered via the oral route, there are a number of safety issues that need to be addressed before this method of delivery is accepted.

Another DNA vaccine strategy is based on the use of a DNA vector consisting of recombinant Semliki Forest virus (SFV) cDNA under the control of a eukaryotic promoter and expressing a foreign gene (6). Unlike conventional DNA vectors, the promoter is not directly driving the expression of the foreign antigen, but directs the synthesis of a recombinant SFV replicon RNA transcript. Translation of this RNA molecule produces a SFV replicase complex that allows replication of the RNA in the cell cytoplasm and results in high-level production of the mRNA for the encoded foreign antigen. Since expression mediated by the SFV vector is transient and lytic, there is less risk from possible chromosomal integration.

5. Other developments in vaccine technology

Subunit vaccines, which contain purified protein or glycoprotein components of a pathogen that have been identified as carrying critical epitopes involved in inducing a protective immune response (4) have distinct safety advantages and recent improvements in their production using recombinant DNA technology may facilitate their
more widespread use (39). Synthetic peptide vaccines have also been engineered (87), however, thus far they have not been shown to be very effective in inducing protection against infectious diseases. There may be many reasons why synthetic peptides may not induce protective immunity. For example, even so-called linear peptides exhibit a degree of conformational flexibility so that they adopt a different structure from that of the parent molecule and therefore induce antibodies of low avidity for the pathogen in question. A potential disadvantage of using peptides that represent single antigenic sites to stimulate a protective antibody response is the possibility of selecting for antigenic mutations in the pathogen.

A number of strategies have been developed for inducing cytotoxic T cell (CTL) responses using peptides, such as coupling CTL epitopes to toxins that are able to invade eukaryotic cells or constructing virus-like particles carrying foreign CTL epitopes (132, 134). However, the utility of this approach in outbred populations is limited by the polymorphism of the major histocompatibility complex molecules. Other virus-like particle vaccines that involve self-assembling proteins that can be used to carry foreign antigens have been made from particles produced from the TYA gene of the yeast retrotransposon Ty (47). A vaccine composed of empty virus-like particles produced by expressing the four main structural proteins of bluetongue virus in baculovirus has been shown to protect against challenge with bluetongue virus (127).

Another interesting approach is the development of 'edible vaccines'. Plants can be engineered to express a number of foreign proteins and can express multiple transgenes at one time (144). The oral delivery of subunit vaccines expressed in plants would be particularly suited to protect against intestinal pathogens. A disadvantage would be that antigens delivered orally would be susceptible to proteolytic degradation. Moreover, oral delivery of antigens tends to induce tolerance rather than active immunity. However, tolerance can be circumvented by expression of a fusion protein composed of the antigen with the B subunit of the heat labile toxin (53).

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Chapter I.1.8. — Biotechnology in the diagnosis of infectious diseases and vaccine development


* * *
SUMMARY

The official control of veterinary biologicals is vested in various national and regional organisations that differ in their approach to ensuring the quality, safety and efficacy of the products. International harmonisation of regulations concerning biological products did not begin until well after those concerning chemically defined products. The first biological products for veterinary use were not manufactured and distributed until the end of the nineteenth century. They were often produced under unsophisticated conditions, and distributed or sold without any control other than those of their manufacturers. Later, each manufacturer developed its own standards. In Europe, these were subject to State controls as early as 1895 for certain diagnostic products (e.g. mallein, tuberculin) or vaccines. Gradually the conditions for international harmonisation of standards evolved, beginning with the comparative testing of products being issued by different European laboratories. It was only in the second half of the twentieth century that national laws covering veterinary biologicals were imposed. These demanded that precisely defined techniques be followed before biological products for veterinary use could be licensed. This was followed by considerable efforts to harmonise these national regulations, first at the regional level (notably in Europe and the Americas) then at the global level, notably by the Office International des Épizooties (OIE) with the publication of the first edition of the OIE Manual of Standards for Diagnostic Tests and Vaccines in 1989.

World-wide harmonisation of standards for veterinary biologicals will be of help to Chief Veterinary Officers who must follow the instructions given in the OIE International Animal Health Code, as they apply to all biological products for use in international trade. It will also be of assistance to vaccine producers, who have expressed their wish for world-wide harmonisation of registration rules so as to simplify and facilitate marketing of their products. Evidently, it will also be of interest to farmers and to consumers, who would benefit from the fact that the safety and efficacy of the products that they use would have been assured to a uniformly high level.

The different sections of this chapter will review and compare regulations from the regions of the world that have made most progress in this field and will describe current attempts at harmonising these regulations on an international scale.

Note: In this chapter the term ‘veterinary biological’ will be taken to include vaccines for use in animals, antisera for use in animals, and in-vivo diagnostic preparations.

A. REGULATION OF VETERINARY BIOLOGICALS: PRESENT SITUATION

1. In Japan

1.1. Introduction

Medicinal products that are exclusively used for animals, including veterinary biologicals, are under the jurisdiction of the Ministry of Agriculture, Forestry and Fisheries, and securing their quality, efficacy and safety is stipulated in the Pharmaceutical Affairs Law (1). Since 1972, registration procedures have been developed with the aim of rationalising the examination procedure and facilitating the acquisition of approval. These procedures are stipulated in the Pharmaceutical Affairs Law and other related regulations. Consequently, a speedy and
simple examination procedure has been achieved with emphasis on the assurance of quality, safety and efficacy. The Food Safety Commission was established in the Cabinet Office, Government of Japan, in July 2003. In the case of approval examination, re-examination and re-evaluation, all veterinary vaccines, except products of dogs and cats, must comply with the Food Safety Basic Law of the Food Safety Commission.

1.2. Regulations governing the approval and quality assurance of veterinary biologicals

a) Application for approval and licence

Those who intend to manufacture or import veterinary biologicals must prepare an application using the designated format for each product, and obtain approval from the Minister of Agriculture, Forestry and Fisheries. The application should be submitted with designated appended documents, such as those on clinical studies. Of the latter, the safety studies and clinical studies using the target species should have been performed in compliance with GLP (Good Laboratory Practice) and GCP (Good Clinical Practice).

A licence to manufacture or import and sell veterinary biologicals is issued by the Minister of Agriculture, Forestry and Fisheries to each applicant and the licence must be renewed every 5 years. Conformity to GMP (Good Manufacturing Practice) is stipulated as one of the conditions for obtaining a licence to manufacture.

b) National assay

After receiving a licence, each batch of the veterinary biological must be examined by the National Veterinary Assay Laboratory according to the procedures of the Assay Standard for Veterinary Biological Products (3, 9). Each product for marketing must include an official identification stamp on the container or the package as a seal.

c) Re-examination and re-evaluation

Re-examination is performed on newly approved veterinary biologicals. Usually a field assessment of the drug is conducted over a period of 6 years following initial approval of the vaccine. During this investigation, efficacy and safety are re-examined.

Re-evaluation is performed on approved products available on the market by order of the Minister of Agriculture, Forestry and Fisheries. This may happen when it is suspected that a veterinary biological does not conform to the latest standards.

d) Minimum requirement of veterinary biological products

The examinations provide information about the consistency of the manufacturing process and the quality of the product: manufacturing methods, properties of strains used for manufacturing, methods of quality control, methods of storage and shelf life, according to the standards given in the ‘Minimum Requirement of Veterinary Biological Products’ (2). Any product that does not conform to these product standards cannot be manufactured, imported or marketed.

e) Cases of rejection of approval

When the quality of the veterinary biological that has been submitted for approval is found to be unsatisfactory, or its adverse effects are marked as compared with its indications, the product is judged to be of little value and approval is not given.

f) Cancellation of approvals

At the time of granting approval to manufacture or import, the quality, safety and efficacy of the product are carefully examined with reference to the latest available technology. However, if scientific knowledge acquired since the granting of approval indicates that there could be a health hazard associated with the product, re-examination and re-evaluation are performed and an order of ‘cancellation of approval’ may be made.

1.3. Procedure for approval to manufacture (or import)

The quality, safety and efficacy of the product as a veterinary vaccine are the most important criteria when manufacturing (or importing) is intended. A licence is not required for each manufacturing facility. One approval for each product is sufficient even if the manufacturer intends to manufacture (or import) in more that one facility. It is also possible to apply simultaneously for approval of the quality of the product and for a licence to manufacture.

When a manufacturer (or importer, distributor) manufactures (or imports) veterinary biologicals, an application for approval to manufacture (or import) the veterinary drug must be submitted on a designated form to an official in
charge of veterinary drugs at the Department of Livestock Industry of each Prefecture. If the documentation is satisfactory, the application for approval to manufacture (or import), together with appended documents, are sent to and reviewed by the Office of the Ministry of Agriculture, Forestry and Fisheries. At that time, a hearing may be conducted if necessary. The application is then discussed in the Central Pharmaceutical Affairs Council, and if no problems are found, notice of approval to manufacture (or import) the veterinary product is sent to the applicant.

2. In the European Union

2.1. Introduction

The pharmaceutical legislation of the European Union (EU), which has evolved over a 30-year period, covers both medicinal products for human and veterinary use. Harmonisation of requirements in the area of veterinary medicines began in 1981 with the adoption of Directives 81/851/EEC and 81/852/EEC, laying down common requirements for manufacturing and marketing authorisations, based on the evaluation of the quality, safety, and efficacy of the product. These Directives, and subsequent veterinary and human pharmaceutical legislation, were consolidated into Directive 2001/82/EC and 2001/83/EC for veterinary and human products, respectively. A series of detailed guidelines were first published in 1994 entitled 'Rules Governing Medicinal Products in the EU' (7). These have since been updated and describe in detail the legal basis for obtaining marketing authorisations, how dossiers should be compiled and how they should be assessed. These rules serve as extremely useful reference publications for any authority that is setting up a system for authorisation of veterinary biologicals. The rules were formally adopted and applied specifically to veterinary biologicals from 1993. Many additional measures were taken to further harmonise the procedures and the criteria for the evaluation of veterinary medicinal products, such as framework requirements and interpretive guidelines for their testing, principles and guidelines of GMP, and a Community procedure for the evaluation of high-technology products. However, granting of authorisations remained at the national level. As a consequence, although applications were evaluated on the basis of these harmonised criteria and procedures, and in some cases simultaneously by the authorities of the Member States, there were differences in the decisions reached by the Member States on individual products. This was why in 1990 the Commission proposed a new system for marketing authorisation for medicinal products, which was adopted by the Council of Ministers in 1993 and entered into force on 1 January 1995.

One of the first consequences was the creation of the European Medicines Evaluation Agency (EMEA) in London, United Kingdom (UK).

2.2. The role of the European Medicines Evaluation Agency

In 1995, a new European system for the authorisation of medicinal products came into force. After 10 years of cooperation between national registration authorities at the EU level and 4 years of negotiations, the Council of the EU adopted, in June 1993, three directives and one regulation, which together form the legal basis of the system (5).

The EMEA was established by Council Regulation 2309/93/EEC of 22 July 1993, (OJ No. L 214, 24.8.1993), and London, UK, was chosen as its location by decision made by the Heads of State and Government on 29 October 1993. This agency formulates opinions and, apart from the administrative staff and the management board, is composed of two scientific committees, the CPMP (Committee for Proprietary Medicinal Products) in charge of medicinal products for humans and the CVMP (Committee for Veterinary Medicinal Products) in charge of medicinal and other products for use in animals.

The CVMP is responsible for the evaluation of applications for marketing authorisation for products derived from biotechnology, for productivity enhancers, new chemical entities and other innovative new products. In addition, the CVMP makes recommendations regarding MRLs (maximum residue limits) for substances used in food-producing animals. To support its activities, the CVMP relies on a pool of 400 experts put at the disposal of the agency by the EU Member States. These experts may participate in any of the CVMP working parties. Among the working parties, the Immunologicals Working Party (CVMP/IWP) has a double mandate: to assist the CVMP, if necessary, by examining on behalf of the Scientific Advice Coordinator of the CVMP, in part or in whole any request for scientific advice made by a company during the development of a new vaccine, and to advise the CVMP on more general policy issues such as the elaboration and revision of guidelines on immunological products. The guidelines for the testing of veterinary medicinal products are contained within ‘The Rules Governing Medicinal Products in the EU’, last published by the European Union in 1999 (7). New guidelines, and revisions of old guidelines, are no longer produced in hardcopy form and may be accessed on the EMEA web site at www.emea.eu.int and/or the DG Enterprise web site at www.pharmacos.eudra.org.

2.3. Present European procedures for marketing authorisation

Since 1995, two new registration procedures for human and veterinary medicinal products have become available through the EU: the centralised and the decentralised procedures (12).
a) The centralised procedure

This applies to high technology products defined in the Annex to Council Regulation 2309/93. It is compulsory for some products (‘Part A’ – certain biotechnological products and novel growth promoters), and optional for others (‘Part B’ – other innovative products). The assessment is co-ordinated by one of the members of the CVMP, known as the ‘rapporteur’, under contract to the EMEA. Data requirements and criteria are the same as those for national procedures. The authorisation subsequently issued by the Commission is valid in all Community Member States. However, in the case of veterinary vaccines, one or more Member States may prohibit the use of a vaccine either because of the absence of the infection in their territory or because an eradication programme is being implemented there, as certified by Competent Authorities.

b) The decentralised procedure

Applications for authorisation of a product may still be obtained in a single Member State (the ‘Reference Member State’) by means of a national procedure. The same ‘Rules Governing Medicinal Products in the EU’ apply. Following approval in the Reference Member State, applications may be made, if desired, to other ‘Concerned’ Member States for identical authorisations to be granted on the basis of ‘mutual recognition’. If another Member State has grounds for considering that the product, as authorised, constitutes a ‘risk to animal or public health or to the environment’, it may refer the dossier to the EMEA for arbitration. The Commission then issues a binding decision based on the opinion of the CVMP.

2.4. Manufacturing authorisation and batch release control

In accordance with Directive 2001/82/EC, authorisation is also required for the manufacture of veterinary medicinal products, including immunologials. This directive provides for regular inspections and stipulates that manufacture must be supervised by a ‘qualified person’, who certifies that each batch is in conformity with the approved specifications for the product. For the implementation of these requirements, the Commission has adopted Directive 91/412/EEC relating to the principle and guidelines of GMP, and published a detailed guide on GMP developed by a group of pharmaceutical inspectors from the Member States.

Manufacturers are required to have the services of a qualified person at their disposal to certify that each batch of product has been manufactured and checked in accordance with the conditions for marketing authorisation. This is a basic requirement of the pharmaceutical legislation. In the case of batches imported from third countries, each batch has to undergo a full qualitative and a quantitative analysis of at least the active ingredients in the first Member State of import into the EU, under the supervision of a qualified person. Not until this control by the qualified person has been carried out can a batch circulate within the EU without further control. In the special case of immunological veterinary medicinal products, an additional step may be introduced. Directive 90/677/EEC allows those Member States that consider it necessary to ask for the submission of samples of each production batch of the bulk and/or finished product for examination by a control laboratory before that batch is placed on the market. This official batch release does not relinquish the requirement of batch control by the qualified person. Except in specially justified circumstances, batch release carried out by one national control laboratory must be recognised without repetition by the other Member States. To ensure the smooth operation of this provision, an administrative information exchange procedure has been agreed between the Competent Authorities. Although all Member States do not require official batch release for veterinary immunologials, it was felt by all that they had to be involved in this information exchange scheme.

Discussions are currently in progress (year 2002) to establish a harmonised system of batch release in all Member States of the EU. Following review of European medicines legislation (Review 2001), the European Commission has produced proposals to amend the legislation and these are being considered by the Council and European Parliament under the Co-decision Procedure.

2.5. The role of the European Pharmacopoeia

The past 30 years have seen profound changes in the organisation and regulation of medicinal products in European countries (4). Thirty years ago, each country had its own regulations, and among them the European countries had two-thirds of the world’s pharmacopoeias. The European Pharmacopoeia Convention has now been signed by 24 parties: 23 countries1, and just recently by the Commission of the European Communities; moreover 10 European and non-European countries2, and the World Health Organisation (WHO) have observer status. Close relations are maintained with the licensing authorities of the European Economic Area, where integration is developing through contact with the EMEA and the implementation of common directives and guidelines on medicines for human and veterinary use. In 1990, the European Pharmacopoeia co-founded, with

1 Austria, Belgium, Cyprus, Croatia, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, the Netherlands, Norway, Portugal, Slovenia, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and the Former Yugoslav Republic of Macedonia; Member States must apply the standards of the European Pharmacopoeia.
2 Albania, Australia, Bulgaria, Canada, Hungary, the Czech Republic, the People’s Republic of China, Lithuania, Slovakia, and Poland; Observer States do not have to apply the European Pharmacopoeia standards. Some of them apply the standards on a voluntary basis.
the Japanese Pharmacopoeia and the United States (US) Pharmacopoeia, the Pharmacopoeial Discussion Group (PDG); this group is working assiduously for harmonisation at the world level, and it participates in the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) programme. A parallel programme for veterinary medicinal products, the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), has recently turned its attention to harmonising the requirements for certain types of tests routinely performed on extraneous agents (see Section C.4. below). The European Pharmacopoeia defines the minimum acceptable standards for products to be authorised within the European Union because compliance with monographs is a mandatory requirement within Directive 2001/82/EC. This requires that products must comply with the relevant specific monograph where one exists or with the general monographs where one does not.

The European Pharmacopoeia also contains a department called the ‘European Department for the Quality of Medicines’ (EDQM). This creates, maintains and distributes the international standard reagents referred to in monographs of the European Pharmacopoeia. To date there are few standards for veterinary biologicals, but the EDQM is becoming increasingly active in this area and it is anticipated that several more will be available in the near future.

3. In the United States of America

3.1. Introduction

In the United States of America (USA), veterinary biologics or veterinary biological products are defined as all viruses, sera, toxins (excluding substances that are selectively toxic to microorganisms, e.g. antibiotics), or analogous products at any stage of production, shipment, distribution, or sale, that are intended for use in the treatment (prevention, diagnosis, management, or cure) of diseases of animals and that act primarily through the direct stimulation, supplementation, enhancement, or modulation of the immune system or immune response. The term biological products includes, but is not limited to, vaccines, bacterins, allergens, antibodies, antitoxins, toxoids, immunostimulants, certain cytokines, antigenic or immunising components of live organisms, and diagnostic components that are of natural or synthetic origin or that are derived from synthesising or altering various substances or components of substances such as microorganisms, genes or genetic sequences, carbohydrates, proteins, antigens, allergens, or antibodies.

3.2. Legal basis

The Virus/Serum/Toxin Act of 1913 (the ‘VST Act’), as amended, 21 U.S.C. Sections 151 to 159, provides the legal authority for the regulation of immunologicals and biologicals for animal use in the USA. The regulatory programme implementing the requirements of the VST Act is administered by the Center for Veterinary Biologics (CVB), Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA). Administrative regulations, duly promulgated and with effect of law, are published in Title 9. Code of Federal Regulations Parts 101 to 118 (6). In addition, APHIS has issued programme guidance in CVB Notices, Veterinary Services Memoranda, Veterinary Biologics General Licensing Considerations, and the Veterinary Biologics Program Manual.

The VST Act requires that products governed by the Act that enter channels of commerce be ‘not worthless, contaminated, dangerous or harmful’. The regulatory scheme implementing these standards is structured to require manufacturers of these products to apply for licences prior to marketing, and to place certain evidentiary responsibilities on those applicants, i.e. manufacturers are required to demonstrate through the submission of certain information, research data, and test results that their products are ‘pure, safe, potent and efficacious’. The APHIS programme for immunologicals and biologicals for animal use regulates the manufacture and release of products on to the market through a system of licensing, inspection, testing and post-marketing surveillance that ensures that the statutory and regulatory standards are met.

3.3. Licensing and initial inspection

Any person or firm seeking to manufacture in the USA an immunological or biological for animal use must obtain from APHIS both a licence to manufacture at a specified facility (Establishment Licence), and a licence for every particular product to be manufactured (Product Licence). These licence requirements apply whether the product is to be released on to the US market or is to be exported to markets abroad. Typically, an applicant will request a facility licence at the same time as the licence for the first product. Once the facility licence and one product licence have been obtained, a firm that seeks to manufacture and market new products needs only to apply for additional product licences. A person or firm located overseas that seeks to market its product in the USA must also apply for marketing authorisation. In the case of an imported product, however, the authorisation is termed a ‘permit’ rather than a ‘licence’.

To obtain a facility licence, the applicant must submit for approval the blueprint (that is, the architects plan of the buildings) and blueprint legends for the facility. APHIS reviews these blueprints and legends to ensure that the
facility will operate in a manner consistent with GMP. If the applicant subsequently makes any physical or operational changes to the facility, revised blueprints and legends must be submitted immediately.

To obtain a product licence, the applicant must establish the purity and identity of all master seeds and master cell stocks that will be used in the manufacture of the product, and must submit for approval a detailed outline of production. The outline of production includes not only the details of the method of product manufacture, but also a description of the procedures for collecting and submitting samples and for releasing batches. The applicant must also provide information regarding the professional and technical credentials of company personnel, and must identify a qualified individual (termed under US regulations as the ‘government liaison’) who acts as the official contact with CVB during the licensing process, and who is subsequently responsible for the submission of the firm’s test reports in conjunction with the release of the product on to the market. The applicant is required to submit test data that demonstrate that the product produced in accordance with the outline is pure, safe, potent and efficacious. To verify the accuracy and proficiency of the manufacturer’s tests, CVB selects samples on test within 14 days of the date on which the samples are received; ordinarily, samples are put on test sooner than the 14-day limit so that the testing of production by the firm and the CVB proficiency testing programme are effectively being conducted at the same time.

Finally, before the facility or product licences are issued, the applicant’s premises are subject to a comprehensive inspection by APHIS examiners. The inspection ensures that the facility is operating in a manner consistent with GMP by confirming that the establishment is configured in the manner set out in the approved blueprints and legends, that the production line is set up and operating in accordance with the approved outline of production, and that records are adequately kept and maintained for each step in production. The inspection also confirms that the applicant follows procedures consistent with GLPs, that the in-process and final product testing programme is conducted properly and appropriately documented, that the sampling is conducted properly, and that adequate procedures for determining and documenting the release of the product on to the market are in place.

3.4. Post-licensing inspection

Once a firm has been issued facility and product licences, APHIS will routinely conduct thorough follow-up inspections of the facility to ensure that the licensee continues to operate the establishment in accordance with the programme regulations and in the manner represented at the time of licensing. Post-licensing inspections are conducted unannounced, and ordinarily within 12–18 months after the last inspection. If the licensee proposes any significant changes to the facility or to the method of production of a licensed product, APHIS retains the right to conduct a special inspection prior to approving the changes.

3.5. Testing

Each licensee is responsible for thoroughly testing all of its production processes and each serial (or lot) of every product prior to release on to the market. The type and amount of testing required depends on the particular product, but is determined and approved by the regulatory authority prior to the issuing of the product licence. A qualified individual employed by the licensee (‘government liaison’) is responsible for selecting the samples to be tested, for monitoring the licensee’s testing programme, and for certifying the test results to the regulatory authority.

At the same time that the firm selects its samples for its own in-house testing, it also selects samples to be submitted to the CVB laboratories. Using a programme for random selection, CVB then selects a percentage of the samples submitted for confirmatory testing to verify the accuracy and proficiency of the manufacturer’s tests. The testing is conducted prior to marketing authorisation for each serial. By regulation, CVB is required to put its selected samples on test within 14 days of the date on which the samples are received; ordinarily, samples are put on test sooner than the 14-day limit so that the testing of production by the firm and the CVB proficiency testing programme are effectively being conducted at the same time.

When the firm receives the results of its own tests, the government liaison submits those results to the regulatory authority along with a batch release form, initiating the release procedure. If the batch has not been selected as part of the proficiency testing programme, or if it has been selected but the CVB tests confirm the company’s test results, the release form is counter-signed by the regulatory authority completing the release procedure. If either the company tests or the proficiency tests indicate that the batch may be unsatisfactory, the batch is not eligible for release.

If the licensee makes a proposal to modify its facility or its operation in any way that could affect the purity, safety, potency or efficacy of the product, the regulatory authority may require the licensee to provide data demonstrating the purity, safety, potency and efficacy of the product as well as to submit samples of the product to CVB’s laboratories for confirmatory testing.

3.6. Post-marketing surveillance

CVB operates a post-marketing surveillance programme to monitor the performance of products on the market. Under this programme, CVB typically learns of any problems relating to product quality through consumer reports.
or complaints, although the programme regulations also place an obligation on the licensee to inform CVB of any problem that comes to its attention regarding the purity, safety or potency of the product. CVB has the legal authority to intervene in the marketplace where there are serious concerns with respect to the purity, safety, potency or efficacy of the product.

B. COMPARISON OF EUROPEAN UNION AND UNITED STATES REGULATIONS

Veterinary biologicals must meet certain basic criteria, regardless of the Regulatory Agency overseeing their production. These criteria include:

- Safety: the product must be safe in the target species and, if live, in species exposed to shed organisms;
- Efficacy: the product should be effective according to claims indicated on the label;
- Quality: includes purity, potency and consistency;
- Purity: the product must be free from contaminating agents;
- Potency: each batch of product should be formulated, and tested, to ensure effectiveness and reproducibility of activity as demonstrated in the registration data.

Although, on a global basis, agencies and regulations differ, all strive to ensure that products offered to the end-consumer conform to these basic standards.

The EU uses a complete system that is a combination of GMP, including validated processes and specifications of materials, together with production methods that ensure the quality of the final product. In-process and batch controls (tests) constitute additional guarantees of the quality of IVMPs. The safety tests are conducted under GLP and the field efficacy tests under GCP. The USA defines acceptable manufacturing processes in the outline of production and detailed facility description (blueprints and blueprint legends), and relies on inspection and confirmatory testing to achieve the same goal. Although different, both systems are designed to allow only pure, safe, potent, and effective biologicals to be released to the consumer.

In addition to the data provided by the applicant, expert reports have to be included in the EU marketing authorisation application file (dossier). Each main section of the EU dossier, including analytical, safety and efficacy, must be reviewed by an independent expert. The assessment of the expert is included in the marketing authorisation file. No such system of third-party review exists under the USDA registration system.

There are many procedural differences between the EU and the USA. Harmonisation between the two systems should be established where possible, on the recognition of equivalence for tests and procedures that are performed to assess a vaccine and that ensure quality, safety and efficacy of the product. Mutual recognition agreements (MRAs) covering veterinary biologicals have been signed between the EU and Australia and between the EU and New Zealand. These MRAs are now at an operational stage. Progress on MRAs between the EU and the USA, regarding veterinary biologicals, is likely to take longer to achieve.

C. THE ROLE OF INTERNATIONAL ORGANISATIONS

Most nations have a range of official acts that regulate the sale and use of veterinary biologicals. Almost all of these acts stipulate ‘minimum requirements’ for quality, safety and efficacy of veterinary biologicals (mostly vaccines), to be tested at independent laboratories, usually under State supervision. These acts and tests may differ from one country to another, and they involve costs and restrictions for producers, users and testers.

Many of the vaccines described in this Terrestrial Manual are produced and/or used in countries that do not currently apply regimens of authorisation and testing as stringent as those described in this chapter. Nevertheless, it is useful to be aware of the regulations operating in different regions and, therefore, the testing and inspection that is likely to have been carried out there on a veterinary biological.

The idea of harmonising this testing to simplify and reduce costs on a regional, or even world scale, is not new, and much has been accomplished during the past 20 years. The purpose of this section is to review the current situation by describing the role of international organisations in the regulation of veterinary vaccines.

In this section the term ‘international organisation’ refers to those concerned with animal health on a world-wide scale (OIE, the Food and Agriculture Organization of the United Nations [FAO] and the WHO).

1. The role of the OIE (World Organisation for Animal Health)

The OIE was founded in Paris in 1924 as the world organisation for animal health, and comprises 166 Member Countries in the year 2004. The principal aims of the OIE are: to ensure transparency in the global animal
Chapter I.1.9. – The role of official bodies in the international regulation of veterinary biologicals

Within the OIE there are four Specialist Commissions: the Terrestrial Animal Health Standards Commission, which deals with the Terrestrial Animal Health Code, the Biological Standards Commission, the Scientific Commission for Animal Diseases and the Aquatic Animal Health Standards Commission (including diseases of molluscs and crustaceans). In addition, there are three Working Groups: the Working Group on Wildlife Diseases, the Working Group on Animal Production Food Safety and the Working Group on Animal Welfare.

Among the Specialist Commissions, the one most closely connected with standardisation is the Biological Standards Commission. This Commission establishes standards for diagnostic methods (including diagnostic preparations) and for vaccines. Its terms of reference reflect the Commission's obligation to participate in the standardisation of biological products, including vaccines used for prophylactic purposes. The Biological Standards Commission is responsible for the preparation of the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (11), and the organisation of Reference Laboratories for many of the diseases contained in OIE Lists A and B.

However, full standardisation of vaccine testing can be achieved only when the necessary standards have been devised. It is hoped to reach the goal of standardisation and wide availability of standards through the participation of OIE Reference Laboratories. The functions and responsibilities of experts at the over 150 OIE Reference Laboratories include the provision of a centre of excellence in a designated activity; standardisation of methods; preparation, storage and distribution of standard antisera, antigens and other reagents.

Among the 15 OIE Collaborating Centres, three may be involved at some stage in veterinary vaccine control and/or harmonisation: the Collaborating Centre for Veterinary Medicinal Products in Fougeres (France), the Collaborating Centre for ELISA (enzyme-linked immunosorbent assay) and Molecular Techniques in Animal Disease Diagnosis in Vienna (Austria), and the Collaborating Centre for the Diagnosis of Animal Diseases and Vaccine Evaluation in the Americas in Ames (USA).

In 1994, following discussions with the International Technical Consultation on Veterinary Drug Registration (ITCVDR), the OIE set up an Ad hoc Group on the Harmonisation of Veterinary Medicines, which was the first step towards the creation of the VICH (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products) (see Section C.4. below).

In May 2003, the OIE International Committee adopted a resolution entitled OIE Procedure for Validation and Certification of Diagnostic Assays (Test Methods) for Infectious Animal Diseases. This resolution requires the OIE Director General to make provisions to establish an OIE registry for assays with levels of validation specified. Fitness for purpose should be used as a criterion for validation.

2. The role of the Food and Agriculture Organization of the United Nations

FAO, established in 1945, is responsible for agricultural development and food production. The Animal Production and Health Division ("AGAH") within the Agriculture Department is concerned with livestock development, and it includes the Animal Health Service ("AGAH"), the main role of which is to assist Member Countries in the control of animal diseases, with the aim of improving livestock production as an integral component of general social, economic and agricultural development. FAO's involvement in testing veterinary biologicals is primarily through its technical assistance system to Member Countries to set up and even execute independent quality control of vaccines and other biologicals. One example is FAO's assistance to the AU (African Union) in setting up a system for continental testing of veterinary vaccines, especially against rinderpest and contagious bovine pleuropneumonia, by the Pan African Veterinary Vaccine Center (PANVAC). FAO also commissions, at the request of Member Countries, initiatives for either quality assurance of vaccines and other biologicals or expert consultations on this subject, or publication of manuals on the production and quality control of vaccines. Furthermore, two auxiliary services can be asked to intervene on matters concerning these products, namely Codex Alimentarius and the Division of Nuclear Techniques in Food and Agriculture. The latter is operated jointly by FAO and the International Atomic Energy Agency (IAEA) based in Vienna (Austria). It has an Animal Production and Health Section, which assists veterinary services and research institutes in developing countries to establish radio-immunoassay (RIA) and ELISA techniques. Linked to this activity is a quality assurance scheme under which laboratories in receipt of FAO/IAEA ELISA kits are required to routinely monitor internal quality controls and to periodically (once or twice a year) test a batch of unknown samples, and to forward the results to IAEA. The overall aim is to provide assurance to all concerned that the results being generated through the use of such internationally standardised and validated kits can be relied upon as correct.
3. The role of the World Health Organization

Currently WHO is not directly involved in preparing international reference preparations (i.e., antigens or antibodies) for purely veterinary use, but has developed and still retains in the National Institute for Biological Standards and Control, Potter’s Bar (UK) some materials related to purely veterinary diseases (e.g. Newcastle disease live vaccine, anti-swine fever serum). WHO wishes to retain a role in this area in instances where the veterinary reference preparations and guidance documents have a direct relevance to human health (8, 10, 15, 16). This involves zoonotic and potentially zoonotic agents and other infectious agents of animal origin that are potential contaminants of biological products, whether these are vaccines produced in cell cultures or organs for xenotransplantation. At the meeting of the Expert Committee on Biological Standardization in October 1998, a review of currently retained international standards and reference preparations for veterinary medicine was carried out and a list of candidates for discontinuation, replacement and revision was suggested (8). The Expert Committee however decided to defer taking action on preparation of veterinary reference materials pending an evaluation by WHO with its partners in the veterinary field of the need for these various biological products. In addition, the present day topicality of certain preparations, especially veterinary vaccines against known zoonoses (e.g. anthrax, brucellosis) adopted and/or revised in the 1960s and 1970s, also needs to be evaluated.

The format of the list of Requirements for Biological Substances published as an Appendix to each report of the Expert Committee on Biological Standardization was revised in 1998 and should facilitate the retrieval of information and achieve the aim of improved transparency.

4. The role of VICH

4.1. The terms of reference

The objectives of the VICH are to:

- Provide a forum for a constructive dialogue between regulatory authorities and the veterinary medicinal products industry on the real and perceived differences in the technical requirements for product registration in the EU, Japan and the USA, with the expectation that such a process may serve as a catalyst for wider international harmonisation;

- Identify areas where modifications in technical requirements or greater mutual acceptance of research and development procedures could lead to a more economical use of human, animal and material resources, without compromising safety;

- Make recommendations on practical ways to achieve harmonisation of technical requirements affecting registration of veterinary products and to implement these recommendations in the three regions. Once adopted, VICH recommendations should replace corresponding regional requirements. These recommendations should focus on the essential scientific requirements needed to address a topic and should eliminate unnecessary or redundant requirements;

- VICH should be conducted in a transparent and cost-effective manner and should provide the opportunity for public comment on recommendations at the draft stage.

VICH focuses on harmonising registration requirements for veterinary medicinal products in the EU, USA and Japan. Countries not involved in VICH are kept informed of its progress through the OIE. Consideration is given to biologicals but not, for the time being, to feed additive growth promoters.

4.2. The Steering Committee

Fundamental to the existence of VICH is the Steering Committee that is empowered to drive the harmonisation process. The Steering Committee comprises two delegates from each of the regulatory authorities and industry associations in the three regions. Australia/New Zealand (together) and Canada have observer status with one delegate representing government authorities and one representing industry associations. An additional category of interested parties has been authorised and delegates from the Committee of the Americas for the Harmonization of Registration and Control of Veterinary Medicine (CAMEVET) and the Association of Veterinary Biologics Companies (AVBC) attend Steering Committee meetings.

4.3. The Working Groups

In order to achieve harmonisation on the selected topics, the VICH Steering Committee appoints working groups to draft recommendations. Each working group normally comprises six experts – one representing each VICH full
member. Additional experts from observer countries – or even other countries – may be appointed by the Steering Committee if deemed appropriate.

A topic leader is appointed by the Steering Committee for each topic. The topic leader is responsible for initiating the working group and guiding its work. He/she normally chairs the working group and is accountable to the Steering Committee for delivering the draft harmonised guidelines.

The Steering Committee has established a specific working group to deal with harmonisation of tests used for the control of veterinary vaccines. This is the working group on biologicals quality monitoring.

4.4. VICH and the role of industry federations

IFAH\(^3\) (formally COMISA\(^4\)), the ‘industry motor’ in the VICH process, is responsible for the entire VICH secretariat. This entails the preparation of documents for meetings of VICH members, publishing Press releases, distributing working group documents and publishing recommendations adopted by the Steering Committee. IFAH also coordinates the positions of the three regional industry federations. The AHI\(^5\), IFAH (Europe) and JVPA\(^6\) have responsibility for ensuring that their regional views are properly represented. They send industry experts to each working group, liaise with their regional authorities and are responsible for organising local conferences to discuss and publicise VICH recommendations.

**CONCLUSION**

At the moment, there is a clear intention to achieve greater international harmonisation of regulatory requirements for veterinary biologicals\(^14\). Progress has already been achieved through international organisations to allow fair competition in the marketing of veterinary products. Although past efforts by international organisations have not resulted in a level of harmonisation sufficient to facilitate international trade, they have laid the groundwork for current efforts. National authorities recognise the advantages of harmonisation and are now committed to working toward this goal.

The efforts of international organisations have made the goal of harmonisation possible and have resulted in an organisation and process for proceeding toward this goal. Success in achieving this goal will depend on the willingness of participating national authorities to work together and accept the compromises that will be necessary to resolve the difficult scientific and policy issues.

**REFERENCES**


\(^3\) International Federation for Animal Health


\(^5\) Animal Health Institute (USA)

\(^6\) Japanese Veterinary Pharmaceutical Association
Chapter I.1.9. —The role of official bodies in the international regulation of veterinary biologicals


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CHAPTER 1.1.10.
LABORATORY METHODOLOGIES FOR BACTERIAL ANTIMICROBIAL SUSCEPTIBILITY TESTING

INTRODUCTION

The spread of multiple antimicrobial-resistant pathogenic bacteria has been recognised by the OIE and the World Health Organization as a serious global animal and human health problem. The emergence of antimicrobial resistance among many bacterial pathogens makes antimicrobial susceptibility testing (AST) essential when antimicrobials are used for effective therapy. Susceptibility tests are also important in studies of new antimicrobial agents. The resistance of a pathogen to an antimicrobial is highly predictive that the treatment will not be effective and the susceptibility of the pathogen is an excellent base for the choice of the antibacterial treatment. Thus, AST is an important component of effective treatment program. Additionally, AST of recovered bacterial pathogens is a component of prudent antimicrobial use guidelines in animal husbandry worldwide and the veterinarian should have these data available (1).

AST is also the basis of the epidemiological surveillance of bacterial pathogens in animals and humans. Such epidemiological surveillance provides a base to choose properly empirical treatment (first line therapy) and to detect the emergence and/or the dissemination of resistant bacterial strains or resistance determinants in different bacterial species. Standardisation and harmonisation of AST methodologies, used in epidemiological surveillance of antimicrobial drug resistance, are critical if data are to be compared among national or international surveillance/monitoring programmes of OIE Member Countries. In the absence of standardised or harmonised methods or reference procedures, susceptibility results from different laboratories cannot be reliably compared. It is essential that AST methods provide reproducible results in day-to-day laboratory use and that the data be comparable with those results obtained by an acknowledged ‘gold standard’ reference method. In the absence of standardised methods or reference procedures, susceptibility results from different laboratories cannot be reliably compared.

This Chapter provides Guidelines for AST methodologies, and includes procedures to standardise and harmonise interpretation of antimicrobial susceptibility test results.

1. Test requirements

In order to achieve standardisation of AST methods and comparability of AST results, the following requirements apply:

i) the use of standardised AST methods and the harmonisation of susceptibility data (including interpretive criteria) are essential,

ii) standardised AST methods and similar interpretive criteria should be accepted and used by all participating laboratories,

iii) all AST methods should generate reproducible data,

iv) all data should be reported quantitatively,

v) establishment of national or regional designated laboratories is essential for the coordination of AST methodologies, interpretations and quality controls,

vi) microbiological laboratories should conduct their work within an internal quality assurance system,

vii) laboratories should become accredited, where applicable, and participate in external proficiency testing programmes,
viii) specific bacterial reference/quality control strains are essential for determining intra- and inter-laboratory quality control, quality assurance and proficiency testing.

2. Antimicrobial susceptibility testing methodologies

The following requirements should be respected:

i) bacteria subjected to AST must be isolated in pure culture from the submitted sample,

ii) the bacterial identification procedure for that particular bacterium should be standardised so that the subject bacteria are consistently and correctly identified to the genus and/or species level,

iii) when possible, bacterial isolates that are considered to be the most important and a sampling of other isolates should be stored for future analysis (either lyophilisation or cryogenic preservation at –70°C to –80°C).

The following factors influencing AST methods should be standardised:

i) once the bacterium has been isolated in pure culture, the inoculum must be standardised to obtain accurate susceptibility results and should be from a fresh culture,

ii) the composition of the agar and broth media used (pH, cations, thymidine or thymine, use of supplemented media),

iii) the content of antimicrobial in the carrier (disk, strip, tablet),

iv) composition of solvents and diluents for preparation of antimicrobial stock solutions,

v) growth and incubation conditions (time, temperature, atmosphere e.g. CO₂),

vi) agar depth,

vii) the subsequent interpretive criteria.

For these reasons, special emphasis has to be placed on reference procedures and standardised methods, as sufficient reproducibility can be attained only through the use of standardised methodology.

3. Selection of antimicrobial susceptibility testing methodology

The selection of an AST methodology may be based on the following factors:

i) ease of performance,

ii) flexibility,

iii) adaptability to automated or semi-automated systems,

iv) cost,

v) reproducibility,

vi) reliability,

vii) accuracy,

viii) national and international preference.

4. Test methods

The following three methods are the only ones that consistently provide reproducible and repeatable results:

i) disk diffusion,

ii) broth dilution,

iii) agar dilution.

a) Disk diffusion method

Disk diffusion refers to the diffusion of an antimicrobial agent of a specified concentration from disks, tablets or strips, into the solid culture media, which has been seeded with a standardised bacterial inoculum. Disk
diffusion is based on the determination of an inhibition zone proportional to the bacterial susceptibility to the antimicrobial present in the disk.

The diffusion of the antimicrobial agent into the seeded culture media results in a gradient of the antimicrobial. When the concentration of the antimicrobial becomes so diluted that it can no longer inhibit the growth of the test bacterium, the zone of inhibition is demarcated. In theory, the edge of this zone of inhibition correlates with the minimum inhibitory concentration (MIC) for that particular bacterium/antimicrobial combination. In other words, the zone of inhibition correlates inversely with the MIC of the test bacterium. Generally, the larger the zone of inhibition, the lower the concentration of antimicrobial required to inhibit the growth of the organisms. However, this depends on the concentration of antibiotic in the disk and its diffusibility.

Note: Disk diffusion tests based solely on the presence or absence of a zone of inhibition without regard to the size of the zone of inhibition are not acceptable AST methodology.

- **Considerations for the use of the disk diffusion methodology**

Disk diffusion is straightforward to perform, reproducible, and does not require expensive equipment. Its main advantages are:

  i) low cost,

  ii) ease in modifying test antimicrobial disks when required.

Manual measurement of zones of inhibition may be time-consuming. Automated zone-reading devices are available that can be integrated with laboratory reporting and data-handling systems. The disks should be distributed evenly so that the zones of inhibition around antimicrobial discs in the disc diffusion test should not overlap to such a degree that the zone of inhibition cannot be determined. Generally this can be accomplished if the discs are no closer than 24 mm from centre to centre.

b) **Broth and agar dilution methods**

The aim of the broth and agar dilution methods is to determine the lowest concentration of the assayed antimicrobial that inhibits the growth of the bacterium being tested (MIC, usually expressed in mcg/ml or mg/litre). However, the MIC does not always represent an absolute value. The ‘true’ MIC is a point between the lowest test concentration that inhibits the growth of the bacterium and the next lower test concentration.

Antimicrobial ranges should:

  i) encompass both the interpretive criteria (susceptible, intermediate and resistant) and quality control reference organisms.

  ii) take into consideration the antimicrobial concentrations that are achievable in vivo for a specific bacteria/antibiotic combination.

Antimicrobial susceptibility dilution methods appear to be more reproducible and quantitative than agar disk diffusion. However, antibiotics are usually tested in doubling dilutions, which can produce inexact MIC data.

Any laboratory that intends to use a dilution method and set up its own reagents and antibiotic dilutions should have the ability to obtain, prepare and maintain appropriate stock solutions of reagent-grade antimicrobials and to generate working dilutions on a regular basis. It is then essential that such laboratories use quality control organisms (see below) to assure accuracy and standardisation of their procedures.

- **Broth dilution**

Broth dilution is a technique in which a standardised suspension of bacteria is tested against varying concentrations of an antimicrobial agent (usually doubling dilutions) in a standardised liquid medium. The broth dilution method can be performed either in tubes containing a minimum volume of 2 ml (macrodilution) or in smaller volumes using microtitre plates (microdilution). Numerous microtitre plates containing prediluted antibiotics within the wells are commercially available. The use of identical lots of microdilution plates may minimise variation that may arise due to the preparation and dilution of the antimicrobials. The use of these plates with a standardised protocol, including appropriate quality control reference strains, may facilitate harmonisation where sufficient financial resources are available.

Due to the fact that most broth microdilution antimicrobial test panels are prepared commercially, they can be considered to be less flexible than agar dilution or disk diffusion in adjusting to the changing needs of the surveillance/monitoring programme.
Because the purchase of the equipment and antimicrobial panels may be costly, this methodology may not be the choice for laboratories with limited budgets.

- **Agar dilution**

Agar dilution involves the incorporation of an antimicrobial agent into an agar medium in a geometrical progression of concentrations, followed by the application of a defined bacterial inoculum to the agar surface of the plate. These results are often considered as the gold standard for the determination of an MIC for the test bacterium/antimicrobial combination.

The advantages of agar dilution methods include:

i) a greater control of the purity of the test bacterium,

ii) the ability to test multiple bacteria on the same set of agar plates at the same time,

iii) the potential to improve the identification of MIC endpoints and extend the antibiotic concentration range,

iv) the possibility to semi-automate the method using an inoculum-replicating apparatus. Commercially produced inoculum replicators are available and these can transfer between 32 and 36 different bacterial inocula to each agar plate.

Agar dilution methods also have certain disadvantages, for example:

i) they are very laborious and require substantial economic and technical resources,

ii) once prepared they have to used within a week,

iii) the endpoints are by no means always easy to read nor is the purity of the inoculum easy to verify.

Agar dilution is often recommended as a standardised AST method for fastidious organisms (4), such as anaerobes, *Helicobacter* and *Campylobacter* species. However, at least in veterinary medicine, broth microdilution also works very well for organisms such as *Hemophilus*, *Campylobacter* and *Brachyspira* amongst others.

c) **Other bacterial AST and specific antimicrobial resistance tests**

Additionally, bacterial antimicrobial MICs can be obtained from commercially available gradient strips that diffuse a pre-formed antibiotic concentration. However, the use of gradient strips can be very expensive and MIC discrepancies can be found when compared with agar dilution results (2).

Regardless of the AST method used, the procedures should be standardised to ensure accurate and reproducible results, and appropriate quality control reference organisms should always be tested every time AST is performed in order to ensure accuracy and validity of the data.

The appropriate AST choice will ultimately depend on the growth characteristics of the bacterium in question. In special circumstances, novel test methods and assays may be more appropriate for detection of particular resistance phenotypes. For example, chromogenic cephalosporin-based tests (4) (e.g. nitrocefin) or equivalent methods may provide more reliable and rapid results for beta-lactamase determination in certain bacteria.

Similarly, extended-spectrum beta-lactamase (ESBL) (4) activity in certain bacteria can also be detected by using standard disk diffusion susceptibility test methods using specific cephalosporins (cefotaxime and ceftazidime) in combination with a beta-lactamase inhibitor (clavulanic acid) and measuring the resulting zones of inhibition. Additionally, chloramphenicol resistance attributed to production of chloramphenicol acetyl transferase can be detected in some bacteria via rapid tube or filter paper tests within 1–2 hours (4).

5. **Antimicrobial susceptibility breakpoints and zone of inhibition criteria**

The objective of in vitro AST is to predict the way in which a bacterial pathogen may respond to the antimicrobial agent in vivo. The results generated by bacterial in vitro antimicrobial susceptibility tests, regardless of whether disk diffusion or dilution methods are used, are generally reported as resistant, susceptible or intermediate to the action of a particular antimicrobial.

Antimicrobial susceptibility breakpoints are established by national standards organisations, professional societies or regulatory agencies. The relevant documents should be consulted. However, there can be notable differences in breakpoints among different countries for the same antimicrobial agent.
As mentioned previously, antimicrobial susceptibility testing results should be recorded quantitatively:

i) as distribution of MICs in milligrams per litre or mcg/ml,

ii) or as inhibition zone diameters in millimetres.

The following two primary factors enable a bacterium to be interpreted as susceptible or resistant to an antimicrobial agent:

i) the development and establishment of quality control ranges (4), using diffusion when possible and dilution testing, for quality control reference microorganisms.

This is essential for validating the specific AST method used. The quality control ranges for the quality control reference microorganisms should be established prior to determining breakpoints for susceptibility or resistance.

ii) the determination of the appropriate interpretive criteria (4).

This involves the generation of three distinct types of data:

- population distribution of MICs of relevant microorganisms,
- pharmacokinetic parameters of the antimicrobial agent,
- results of clinical trials and experience.

The interpretation of the data involves creating a scattergram from the bacterial population distribution (representative bacterial isolates), by plotting the zone of inhibition against the logarithm of the MIC for each bacterial pathogen. The selection of breakpoints is then based on multiple factors, including regression line analysis that correlates MICs and zone diameters of inhibition, bacterial population distributions, error rate bounding, pharmacokinetics, and ultimately, clinical verification.

The development of a concept known as 'microbiological breakpoints', which is based on the population distributions of the specific bacterial species tested, may be more appropriate for some antimicrobial surveillance programmes. In this case, bacterial isolates that deviate from the normal susceptible population would be designated as resistant, and shifts in susceptibility to the specific antimicrobial/bacterium combination could be monitored (5).

6. Antimicrobial susceptibility testing guidelines

A number of guidelines are currently available for antimicrobial susceptibility testing and subsequent interpretive criteria throughout the world. Amongst others, these include standards and guidelines published by:

- National Committee for Clinical Laboratory Standards (NCCLS),
- British Society for Antimicrobial Chemotherapy (BSAC),
- Comité de l’Antibiogramme de la Société française de Microbiologie (CASFM),
- Swedish Reference Group for Antibiotics (SIR),
- Deutsches Institut für Normung (DIN),
- Japanese Society for Chemotherapy (JSC),
- Werkgroep richtlijnen gevoeligheidsbepalingen (WRG system, the Netherlands).

At this time, only the NCCLS has developed protocols for susceptibility testing of bacteria of animal origin and determination of interpretive criteria (4). However, protocols and guidelines are available from a number of standards organisations and professional societies for susceptibility testing for similar bacterial species that cause infections in humans. It is possible that such guidelines can be adopted for susceptibility testing for bacteria of animal origin, but each country must evaluate its own AST standards and guidelines. Additionally, efforts focusing on harmonisation of susceptibility breakpoints on an international scale are progressing. These efforts have primarily focused on the adoption of the standards and guidelines of the NCCLS, which provide laboratories with standardised methods and quality control values enabling comparisons of AST methods and generated data (4, 7). For those OIE Member Countries that have not standardised AST methods, the adoption of NCCLS guidelines and standards would be an appropriate initial step.

As a first step towards comparability of monitoring and surveillance data, Member Countries should be encouraged to strive for harmonised and standardised programme design (6). Data from countries using different methods and study design may otherwise not be directly comparable (3, 6). Notwithstanding this, data collected over time in a given country may at least allow the detection of emergence of antimicrobial resistance or trends in prevalence of resistance in that particular country. However, if results achieved with different AST methods are to be presented side by side, then comparability of results must be demonstrated and consensus on interpretation achieved.
Note: This will be best accomplished by the use of accurate and reliable standardised AST methods in conjunction with monitoring of AST performance with defined quality control bacterial strains among participating laboratories.

7. Comparability of results

To determine the comparability of results originating from different surveillance systems, results should be reported quantitatively including information on the methods, quality control organisms and antimicrobial concentration ranges tested and interpretive criteria used.

8. Quality control and quality assurance

Adequate quality control/quality assurance systems should be established in AST performing laboratories.

The following components should be monitored:

i) precision of the AST procedure,
ii) accuracy of the AST procedure,
iii) the laboratory personnel,
iv) performance of the appropriate reagents.

The following requirements should be respected:

i) Strict adherence to standardised techniques in conjunction with quality control of media and reagents.
ii) Record keeping of:
   - lot numbers of all appropriate materials and reagents,
   - expiration dates of all appropriate materials and reagents.
iii) The appropriate quality control reference bacteria should always be tested to ensure standardisation regardless of the AST method used.
iv) Reference bacterial strains should be catalogued and characterised with stable defined antimicrobial susceptibility phenotypes. These quality control strains should also encompass resistant and susceptible ranges of the antimicrobials to be assayed.
v) Laboratories involved in AST should use the appropriate quality control reference strains.
vi) Reference strains should be kept as stock cultures from which working cultures are derived and should be obtained from national or international culture collections. Reference bacterial strains should be stored at designated centralised or regional laboratories.
vii) The preferred method for analysing the overall performance of each laboratory is to test the appropriate quality control bacterial strains on each day that susceptibility tests are performed.
   Because this may not always be practical or economic, the frequency of such quality control tests may be reduced if the laboratory can demonstrate that the susceptibility testing procedures are reproducible. If a laboratory can document the reproducibility of the susceptibility testing methods used, testing may be performed on a weekly basis. If quality control errors emerge, the laboratory has a responsibility to determine the cause(s) and repeat the tests. If the laboratory cannot determine the source of error(s), then quality control testing should be re-initiated on a daily basis.
viii) Recognised quality control strains should be tested each time a new batch of medium or plate lot is used and on a regular basis in parallel with the bacterial strains to be assayed.
ix) Appropriate biosecurity issues should be addressed in obtaining and dispersing quality control reference strains to participating laboratories. The use of such strains will allow for comparison of antimicrobial susceptibility data (run on).

9. External proficiency testing

To ensure that reported susceptibility data is accurate, OIE Member Countries should initiate external proficiency testing (e.g. third party testing). External proficiency testing can be carried out on a national basis. Laboratories in Member Countries are encouraged to participate in international inter-laboratory comparisons. All bacterial species should be included.
Countries should appoint or establish designated national laboratories that are responsible for:

i) monitoring the quality assurance programmes of laboratories participating in surveillance and monitoring of antimicrobial resistance,

ii) supplying to those laboratories a set of reference strains,

iii) creating a central database available on the internet (e.g. EARSS) that contains the different resistance profile for each bacteria species.

REFERENCES


*  *  *

NB: There is an OIE Reference Laboratory for Antimicrobial resistance (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
PART 2

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

LIST A DISEASES

CHAPTER 2.1.1.

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, 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, including 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 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 specific humoral antibody can also be used for diagnosis. Serodiagnosis is enhanced by the newly developed nonstructural protein (NSP) assays that enable detection of past or current infection, irrespective of vaccination status.

Identification of the agent: The demonstration of FMD viral antigen 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 virus-secure laboratory.

Complement fixation (CF) has been replaced in many laboratories by the enzyme-linked immunosorbent assay (ELISA), as it is more specific and sensitive and is not affected by pro- or anti-complementary factors. If the sample is inadequate or the test result inconclusive, it will be necessary to grow the virus in cell cultures or in 2–7-day old unweaned mice. The cultures should preferably be of primary bovine thyroid, but pig, lamb or calf kidney cells, or cell lines of comparable sensitivity may be used. When a cytopathic effect (CPE) appears in the cultures, the fluids can be used in CF tests or ELISAs. Similar tests can be performed on homogenised suspensions of the dissected musculo-skeletal tissues of any mice that die. In the absence of CPE or any dead mice, a further passage should be made at a 48-hour interval, with freeze–thawing of the cells, before the sample is declared to be negative.

Nucleic acid recognition tests, such as the polymerase chain reaction, are being used increasingly as rapid and sensitive diagnostic methods. Electron microscopic examination of lesion material is sometimes useful to differentiate FMD from disease caused by other viruses.
Serological tests: The demonstration of specific antibodies to structural proteins in nonvaccinated animals, where a vesicular condition is present, is sufficient for a positive diagnosis. This is particularly useful in mild cases or where epithelial tissue cannot be collected. Tests for antibodies to some NSPs of FMD virus are useful in providing evidence of previous or current viral replication in the host, irrespective of vaccination status. NSPs, unlike structural proteins, are not serotype specific and as a consequence, the detection of these antibodies is not serotype restricted.

Virus neutralisation (VN) tests and ELISAs for antibodies to structural proteins are used as serotype-specific serological tests. VN tests depend on tissue cultures and are therefore more prone to variability than ELISAs; they are also slower and subject to contamination. ELISAs for 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 cover 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 usually done using a combination of in-vitro tests on the inactivated virus preparation and in-vivo tests on the finished vaccine. 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 the vaccine producer has established a statistically significant correlation between protection and specific antibody response.

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\(^1\). The Pirbright Laboratory (see footnote 1) has dual designations as both the World Reference Laboratory and 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, 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 subtypes can be identified by biochemical and immunological tests.

In Africa, FMD viruses are maintained by cattle and African buffalo (Syncerus caffer). Available evidence indicates that although other domestic and wild species become infected, they are unable to maintain the infection for more than a few months in the absence of cattle or African buffalo. Elsewhere in the world cattle are usually the main reservoir, although in some instances the viruses involved appear to be specifically adapted to domestic pigs or sheep and goats. It is probable that these adapted viruses are able to modify their adaptation and affect other species if given the opportunity. However, the pig-adapted Cathay strain of FMD virus apparently does not infect large ruminants in the field or experimentally. Wildlife outside Africa has not, so far, been shown to be able to maintain FMD viruses. The evidence indicates that infection of deer in the past was derived from contact, direct or indirect, with infected domestic animals.

Of the domesticated species, cattle, pigs, sheep, goats and buffalo are susceptible to FMD (21). In addition, many species of cloven-hoofed wildlife, such as deer, antelope and wild pigs may become infected, although, apart from the African buffalo their involvement in the epidemiology of FMD in the domesticated species is not certain. Strains of FMD virus that infect cattle have been isolated from wild pigs and deer. For the diagnosis of FMD in wild species, procedures similar to those described for farm animals can be applied.

\(^1\) FAO World Reference Laboratory for FMD, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey GU24 0NF, United Kingdom.
Infection of susceptible animals with FMD virus leads to the appearance of vesicles on the feet, in and around the oral cavity, and on the mammary glands of females. 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 its degree of immunity (32). The signs can range from a mild or inapparent 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. Adult animals may occasionally succumb.

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 FMD virus in samples of vesicular fluid, epithelial tissue, milk, or blood is sufficient to establish a diagnosis. Diagnosis may also be established by the isolation of FMD virus from the blood, heart or other organs of fatal cases. A myocarditis may be seen macroscopically in a proportion of fatal cases.

FMD virus can replicate and be excreted from the respiratory tract of animals. Airborne excretion of virus occurs during the acute phase of infection. FMD viruses may occur in all the secretions and excretions of acutely infected animals including expired air. Transmission is generally effected by contact between infected and susceptible animals or, more rarely, exposure of susceptible animals to the excretions and secretions of acutely infected animals. Following recovery from the acute stage of infection, infectious virus disappears from all secretions and excretions with the exception, in the case of ruminants, of those of oesophageal–pharyngeal (OP) origin. Animals in which the virus persists in the OP for more than 28 days after infection are referred to as carriers. Pigs do not become carriers. Circumstantial evidence indicates that carriers are able, on rare occasions, to transmit the infection to susceptible 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. There is no information on the duration of the carrier state in another domestic buffalo, the swamp buffalo of East Asia. Domestic buffalo, sheep and goats do not usually carry FMD viruses for more than a few months.

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 virus-secure laboratory. The facility should meet the requirements for Containment Group 4 pathogens as outlined in Appendix I.1.6.1 of Chapter I.1.6. of this Terrestrial Manual. 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 the FAO WRL 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 in the use of live virus. Reagents are supplied freeze-dried or in glycerol and can remain stable at 4°C or at –20°C, respectively, for many years. The International Atomic Energy Agency2 has produced a manual that includes a recommended test and quality control protocols.

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. 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. Samples should be kept refrigerated or on ice until received by the laboratory.

2 International Atomic Energy Agency, Wagramerstrasse 5, P.O. Box 100, A-1400 Vienna, Austria.
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.

Before the collection of OP samples from cattle or large ruminants (e.g. buffaloes), 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 solid carbon dioxide (dry ice) or liquid nitrogen.

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. Of this, 2 ml is then added to the 2 ml 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.

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 solid carbon dioxide or liquid nitrogen (27).

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 be frozen by being placed either above solid carbon dioxide or liquid nitrogen. Before freezing, the containers should be carefully sealed using airtight screw caps or silicone. This is particularly important when using solid carbon dioxide, as introduction of CO₂ into the OP sample will lower its pH, inactivating any FMD virus 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.

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. Sampling methods.

1. **Identification of the agent**

   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 five times that of the epithelial sample has been added, giving a 20% suspension. This is clarified on a bench centrifuge at 2000 g for 10 minutes. Once clarified, such suspensions of field samples suspected to contain FMD virus are inoculated into cell cultures or unweaned mice. Sensitive cell culture systems include primary bovine thyroid cells and primary pig, calf or lamb kidney cells. Established cell lines, such as BHK-21 (baby hamster kidney), IB-RS-2 or BHK-21 cells, may be used but are less sensitive than primary cells for detecting low amounts of infectivity (10). 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 (38).

   b) **Immunological methods**

   - Enzyme-linked immunosorbent assay

     At the FAO WRL for FMD (see footnote 1), the preferred procedure for the detection of FMD viral antigen and identification of viral serotype is the ELISA (20, 36). 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 FMD virus. 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 FMD virus 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, indicates a

3 The pig should be properly restrained, ideally held on its back in a wooden cradle with its neck extended. Holding a swab in a suitable instrument, such as an artery forceps, the swab is pushed to the back of the mouth into the pharynx.
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 FMD virus 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.

Depending on the species affected and the geographical origin of samples, it may be appropriate to simultaneously test for swine vesicular disease (SVD) virus or vesicular stomatitis (VS) virus. 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 FMD virus (plus SVD virus 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 FMD virus (plus SVD virus if appropriate) grown on monolayer cultures of BHK-21 cells (IB-RS-2 cells for SVD virus). 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 antiserum prepared by inoculating guinea-pigs with 146S antigen of one of the seven serotypes of FMD virus (plus SVD virus 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 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 SVD virus (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 (with 20% 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 2 and 3 of rows A to H on plate 1. To well 1 of row A of plate 1 add 50 µl of control antigen type O, and to well 2 of row A add 12.5 µl of control antigen type O. Mix antigen and diluent in well 2 and transfer 12.5 µl from well 2 to well 3 of row A. Mix and discard 12.5 µl from well 3 (this gives a five-fold dilution series of antigen O). Similarly repeat with antigen A, adding 50 µl of antigen type A to well 1 of row B, and 12.5 µl of antigen type A to well 2, and then mix and transfer 12.5 µl to well 3 (as done before with antigen type O), and continue for types C, SAT 1, SAT 2, SAT 3, Asia 1 and SVD (if appropriate). 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.
  - vi) 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.
  - vii) Cover with lids and place on an orbital shaker at 37°C for 1 hour.
  - viii) Wash the plates by flooding with PBS – wash three times as before and empty residual wash fluid. Blot the plates dry.
  - ix) 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, antiserum to serotypes O, A, C, SAT 1, SAT 2, SAT 3, Asia 1 and SVD virus (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% % H₂O₂ 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.

- **Complement fixation test**

  The ELISA is preferable to the complement fixation (CF) test because it is more sensitive and it is not affected by pro- or anti-complementary factors. If ELISA reagents are not available, however, the CF test may be performed as follows:

  Antisera to each of the seven types of FMD virus are diluted in veronal buffer diluent (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% standardised sheep red blood cells (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 ≥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**

  The polymerase chain reaction (PCR) can be used to amplify the genome fragments of FMD virus in diagnostic material (2, 8). Specific primers have been designed to distinguish between each of the seven serotypes. In situ hybridisation techniques have been developed for investigating the presence of FMD virus RNA in tissue samples (44). These techniques are only in use in specialised laboratories.

  The molecular epidemiology of FMD is based on the comparison of genetic differences between virus isolates. Dendrograms showing the genomic relationship between vaccine and field strains for all seven serotypes based on sequences derived from the 1D gene have been published. Reverse-transcription PCR (RT-PCR) amplification of FMD virus RNA, followed by nucleotide sequencing, is the current preferred option for generating the sequence data to perform these comparisons. The WRL and other laboratories have developed techniques for performing these studies, and a database of over 3000 sequences is currently held.

  The recommended method is to:

  i) Extract FMD virus RNA directly from epithelial suspensions, or from a low cell culture passage.

  ii) Perform an RT-PCR of the complete VP1 gene (or if only part of the VP1 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 WRL on request or can be downloaded from the following World Wide Web URL:

  [http://www.iah.bbsrc.ac.uk/virus/picornaviridae/aphthovirus/fmd.htm](http://www.iah.bbsrc.ac.uk/virus/picornaviridae/aphthovirus/fmd.htm)

2. **Serological tests**

FMD virus infection can be diagnosed by the detection of a specific antibody response. The tests generally used are virus neutralisation (VN) and ELISA (24, 25, 41). These are also the prescribed tests for trade. The VN test is serotype specific, requires cell culture facilities and takes 2–3 days to provide results. The ELISA is a blocking- or competitive-based assay that uses serotype-specific polyclonal or monoclonal antibodies. It is therefore serotype specific, sensitive and quantitative, and has the advantage that it is quicker to perform, is less variable, and is not dependent on tissue culture systems. Low titre false-positive reactions can be expected in a small proportion of the sera in either test. An approach combining screening by ELISA and confirming the positives by the VN test
minimises the occurrence of false-positive results. The OIE has coordinated the production of reference sera to standardise the FMD serological tests; these are available from the WRL.

The detection of antibody to the nonstructural proteins (NSPs) of FMD virus has been 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. Conventionally this has been carried out by measuring antibody to the virus infection-associated antigen (VIAA; the viral RNA polymerase protein 3D) using agar gel immunodiffusion (AGID) (31). Although relatively insensitive, the test is inexpensive, easy to perform and has been used extensively in South America to detect viral activity on a population basis during FMD eradication campaigns. The VIAA test has now largely been superseded by assays that measure antibody to FMD virus NSPs 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 (11, 30, 39). In animals seropositive for antibody to 3AB or 3ABC, antibody to one or more of the other NSPs including the L, 2C, 3A, 3B or 3D protein is further confirmation of infection (9, 30, 39). The test can be used to detect FMD virus infection in vaccinated and unvaccinated populations. However, vaccine purity is an important consideration as the presence of trace amounts of NSPs in some vaccine preparations may result in false-positive reactions in animals that have been repeatedly vaccinated. Conversely, there is experimental evidence that a few animals, vaccinated and subsequently challenged with live virus and confirmed persistently infected, may not be detected in some anti-NSP tests, causing false-negative results (28). Therefore, NSP assays may be used on a herd but not on an individual animal basis to detect FMD virus circulation in vaccinated populations.

a) Virus neutralisation (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 serum (usually pig). A suitable medium is Eagle’s complete medium/LYH (Hank’s balanced salt solution with yeast lactalbumin hydrolysate) with 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 TCID\(_{50}\) (50% tissue culture infective dose) within an accepted range (e.g. 35–350 TCID\(_{50}\)).
  iii) Controls include a standard antiserum of known titre, a negative serum, 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 10\(^6\) 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% formol/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) (23). 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 at the 50% end-point, as per the Kärber method. The test is considered to be valid when the amount of virus used per well is in the range log\(_{10}\) 1.5–2.5 TCID\(_{50}\) and the positive standard serum is within twofold of its expected titre.
  ix) Interpretation of tests can vary between laboratories in regard to end-points taken. Laboratories should establish their own criteria by reference to standard reagents that can be obtained from the FAO WRL for FMD (see footnote 1). At the WRL, a titre of 1/45 or more of the final serum dilution in the serum/virus mixture is regarded as positive. Titres of 1/16 to 1/32 are considered to be doubtful, and
further serum samples are requested for testing. Animals are considered to be positive if the second sample has a titre of 1/16 or greater. A titre of 1/8 or less is considered to be negative.

b) **Solid-phase competitive enzyme-linked immunosorbent assay**  
(a prescribed test for international trade)

Rabbit antiserum to the 146S antigen of one of the seven types of FMD virus is used as the trapping antibody at a predetermined\(^4\) 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 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 and preblocked with normal bovine serum, 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.

Test sera are diluted in PBST.

Collaborative studies have shown that the solid-phase competitive ELISA is more specific but as sensitive as the liquid-phase blocking ELISA (29).

- **Test procedure**
  
i) ELISA plates are coated with 50 µl/well rabbit anti-FMD virus antigen diluted in carbonate/bicarbonate buffer, pH 9.6, and left overnight in a humid chamber at 4°C.
  
ii) The ELISA plates are washed five times with PBS.
  
iii) Then 50 µl of the FMD virus 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] normal bovine serum, 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 five 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 1/5.
  
v) Immediately 50 µl of guinea-pig anti-FMD virus 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 five times with PBS, 50 µl of anti-guinea-pig Ig conjugate 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 five times with PBS, 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.
  
ix) The reaction is stopped after 10 minutes by the addition of 50 µl of 2 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).
  
xii) **Interpretation of the results:** A percentage of inhibition is calculated for each well, either visually or using a suitable computer programme (100 – [optical density of each test or control value/mean optical density of the 0% competition] \(\times\) 100%), representing the competition between the test sera and the guinea-pig anti-FMD virus antisera for the FMD virus antigen on the ELISA plate. Greater than 60% inhibition is positive (35).

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\(^4\) A chequerboard titration of the rabbit-trapping antiserum, the guinea-pig antiserum and the anti-guinea-pig antiserum is performed. Before using the antigen-trapping ELISA or the liquid-phase blocking ELISA, each of these reagents is titrated, one against another, keeping the third reagent at a fixed concentration. In this way the optimal dilutions (for positive colour and low background colour) can be determined. These ‘predetermined’ dilutions are then used for all future tests using these particular batches of reagents.
c) **Liquid-phase blocking enzyme-linked immunosorbent assay**

Antigens are prepared from selected strains of FMD virus 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.

- **Test procedure**
  
  i) ELISA plates are coated with 50 µl/well rabbit antisera to the 14S antigen being tested for and left overnight in a humid chamber at room temperature.
  
  ii) The ELISA plates are washed five times with PBS.
  
  iii) In U-bottomed multiwell plates (carrier plates) 50 µl of a duplicate, twofold series of each test serum is prepared, starting at 1/4. To each well, 50 µl of a constant dose of viral antigen that is homologous to the rabbit antisera 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 starting serum dilution to 1/8.
  
  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) 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 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% H$_2$O$_2$ 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). 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). Titres greater than 1/40 are considered to be positive. Titres close to 1/40 should be retested using the VN test.

As mentioned previously, the AGID test to detect antibody to the VIAA (viral RNA polymerase protein 3D) has been widely used in South America (31). It has now been largely replaced by the ELISA or immunoblot.

- **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 tests (33).
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.

viii) **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]. Cut-off values, with or without suspicious zones, need to be determined by individual laboratories with consideration being given to the purpose of testing and the intended target population.

**International Standard Sera**

International standard sera are currently being developed based on the test method described above. Three standard sera: a strong positive, a weak positive and a negative are being developed according to the OIE Guidelines (34). These sera will act as reference materials for the calibration of other test methods and reagents and as prototypes for the production of national and working standards. The strong positive standard will represent the upper range of antibody detection. The weak positive standard will represent the lower range of detection or the analytical sensitivity of the test method. It is important to note that the dilution of the weak positive standard must be chosen such that it is unequivocally positive in all runs of the assay. The negative standard, used to prepare dilutions of the positive standards, will act as a baseline or background control for the positive standards.

**Enzyme-linked immunoelectrotransfer blot assay (EITB)**

The EITB assay has been widely applied in South America for serosurveillance and risk assessment associated with animal movement. Currently, the procedure is to perform an initial screening test using an indirect ELISA for antibody to 3ABC, and to follow that by a confirmatory EITB assay if samples give positive or suspect results. This combination of tests is particularly recommended when serosurveillance involves a large number of samples. Further information is available from the OIE Reference Laboratory in Brazil (see Table given in Part 3 of this Terrestrial Manual).

**Preparation of test strips containing the recombinant antigens**

i) The five bioengineered FMD virus 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 (33), whereas the rest of the proteins are obtained as fusions to the N-terminal part of the MS-2 polymerase gene (40).

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 (33).

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 (33).

**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% bromochloroindolylphosphate/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.

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 run that exhibit minimal but consistent staining for each of the four 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.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

The control of FMD is usually a national responsibility and, in many countries, the vaccine may be used only when authorised.

Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 are intended to be general in nature and may be supplemented by national and regional requirements.

Virulent FMD virus must be used to produce FMD vaccine; consequently, the FMD vaccine production facility should operate under the appropriate biosecurity procedures and practices. The facility should meet the requirements for Containment Group 4 pathogens as outlined in Appendix I.1.6.1 of Chapter I.1.6 of this Terrestrial Manual.

Routine vaccination against FMD is used in many 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 slaughter 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 (17).

FMD vaccines are chemically inactivated cell-culture-derived preparations of the virus that have been blended with a suitable adjuvant. In the case of vaccines destined for use in swine, oil adjuvants are preferred.

Because of the presence of multiple serotypes of the virus, many FMD vaccines are multivalent and it is common practice to prepare vaccines from two or more different virus strains. In areas where the disease is maintained by free-living buffalo, it is necessary to include more than one virus per serotype to ensure broad antigenic coverage against prevailing viruses.

1. Seed management

a) Characteristics of the seed

Selection of seed viruses should ideally be based on their ease of growth in cell culture, virus yield, stability and broad antigenic spectrum (37). The production strains should be characterised and distributed by the official control laboratories; they should be selected in accordance with the epidemiological importance of each variant.
b) Method of culture

Many manufacturers of FMD vaccines derive their vaccine strains from local field isolates and, for those grown in cell culture, adapt them for growth in suspension or monolayer cells by serial passage. In order to remove the risk of any contaminating lipid-containing viruses in these field isolates, it is recommended that they undergo organic solvent treatment prior to, or during, adaptation. It is preferable to keep the number of passages in cell culture to a minimum as there is evidence of antigenic 'drift' of FMD virus during this procedure.

c) Validation as a vaccine

Seed viruses must be antigenically characterised and proven to be free from all extraneous agents listed by the appropriate licensing authorities, to establish homology to the original candidate isolates, purity and effectiveness against the circulating strains for which they were developed. This often encompasses a number of methods, but to establish applicability to field strains a VN test is often used. Seed viruses may be stored at –20°C if glycerinated or at a lower temperature (e.g. –70°C) if not glycerinated. 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 at an approximate rate of 1 PFU (plaque-forming unit) per 100 cells.

2. Method of manufacture

FMD virus is usually produced in large-scale suspension cell systems under aseptic conditions. It is essential that all pipework and vessels be thoroughly sterilised ensuring that no areas in the system harbour microorganisms. In addition to general considerations of sterility, it is important to note that the virus is vulnerable to attack by proteolytic enzymes, such as those produced by microorganisms (13). Control of pH and temperature are also critical because of the acid and temperature lability of the virus (12). Optimum temperature for cell, virus growth and inactivation, normally around 37°C and 26°C, respectively, should be precisely controlled. During other stages of manufacture, the temperature should be reduced to 4–6°C. Virus should be maintained at approximately pH 7.6 and should never be below pH 7.0.

A suitable strain of the virus is used to infect a suspension of a transformed 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. As an approximation, the main culture is seeded to give an initial density of 0.2–0.5 × 10⁶ cells/ml, which is allowed to multiply to 2–3 × 10⁶ cells/ml before being infected with virus.

When the virus has reached its maximum titre, which is variously determined by infectivity, CF or other tests, the culture is clarified and filtered, often with centrifugation. The virus is subsequently inactivated by addition of ethyleneimine (EI), usually in the form of binary ethyleneimine (BEI). This is usually prepared by dissolving, to a concentration of 0.1 M, 2-bromoethyamine hydrobromide in 0.2 N sodium hydroxide solution, and incubating at 37°C for 1 hour (4, 5). The BEI formed is then added to a virus suspension held at 26°C, to give a final concentration of 3 mM. Inactivation is usually continued for 24 hours, followed by a second dose of BEI for a further 24 hours. After inactivation any residual BEI in the harvest can be neutralised by adding sodium thiosulphate solution to a final concentration of 2%. To decrease the likelihood of live virus failing to contact the EI at the second application, it is essential to transfer the vessel contents immediately to a second sterile vessel where inactivation is allowed to go to completion at 48 hours.

The inactivated virus may be concentrated by ultrafiltration, polyethylene glycol precipitin or polyethylene oxide adsorption (1, 43). These concentrated antigens can be kept at –70°C or lower temperatures for many years, if necessary, and made into vaccine when required by dilution in a suitable buffer and addition of adjuvants (15).

Conventional FMD vaccines are usually formulated in one of two ways. The vaccine most commonly used for cattle is prepared by adsorbing the virus on to aluminium hydroxide gel, one of the adjuvant constituents of the final vaccine blend. Other components of the final blend include antifoam, phenol red dye (if permitted by the country requiring vaccine), lactalbumin hydrolysate, tryptose phosphate broth, antibiotics, amino acids, vitamins and buffer salts. A second adjuvant, saponin, derived from the South American tree Quillaja saponaria mollina, is also incorporated, as well as merthiolate/chloroform as a preservative.

An alternative formulation uses mineral oils, such as Marcol and Drakeol, as adjuvants. These preparations offer a number of advantages over the standard aluminium hydroxide/saponin vaccine, not least of which is their efficacy in pigs. They are widely used for vaccinating cattle in South America because of the longer duration of immunity obtained. The mineral oil is usually premixed with an emulsifying agent, such as mannide monooleate, before the addition of an equal volume 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 Tween 80 (26).
Significant advances made in recent years have seen the introduction of alternative ‘ready-to-use’ oil adjuvants. Oils containing esters of octadecenoic acid and 2,5 anhydro-d-mannitol, for example, readily form double or mixed emulsions (water/oil/water) that are both stable and of low viscosity, without the requirement of sophisticated emulsification equipment (6, 17).

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 infectivity test, sucrose density gradient (14) 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.

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 FMD virus, e.g. BHK or bovine thyroid cells. Such cultures permit the testing of statistically meaningful samples under reproducible conditions. The log \( \frac{10}{\text{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 \text{ litres of liquid preparation at the end of the inactivation period.}

4. Batch control

a) Sterility

The bulk inactivated antigen, the adjuvants, the dilution buffers and the final formulated product should all undergo sterility testing. This may be carried out directly with components of the vaccine and the final product, but the preferred method is to collect any contaminating microorganisms by membrane filtration of the material to be examined and to detect them by incubation of the membranes with culture media. The latter 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 1997 (ref. 19; also refer to Chapter I.1.5.).

b) Safety

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 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.

c) Potency

Potency is only examined on the final formulated product (see Section C.5.b.). Antigen load can be used as an indicator of potency, if a correlation has previously been established.

d) Duration of immunity

In order to establish a satisfactory level of immunity it is usual to give a primary course of two inoculations, 2–4 weeks apart, followed by revaccination every 4–12 months. The frequency of revaccination will depend on the epidemiological situation and the type and quality of vaccine used. Where access to the animals is difficult, it is preferable to use oil adjuvanted vaccine at 4 months and 1 year of age, followed by annual revaccination.

For calves born of vaccinated dams, the first vaccination should be delayed as long as possible to allow decline of maternal antibody, but not beyond 4 months, as at that time a high proportion can be expected to respond effectively to vaccination. For calves born of nonvaccinated dams, the first vaccination may be at 1 week of age (3).

e) Stability

The shelf life of conventional FMD vaccines is usually 1–2 years at 4°C, but they are temperature labile and should neither be frozen nor stored above 4°C.
Chapter 2.1.1. – Foot and mouth disease

f) Preservatives

The most commonly used preservatives are chloroform and merthiolate. The latter is used at a final concentration of 1/30,000 (w/v).

g) Precautions (hazards)

Current FMD vaccines are innocuous and present no toxic hazard to the user. Care must be taken to avoid self-injection with oil-emulsion vaccines.

5. Tests on the final product

a) Safety

It is necessary to test FMD vaccines to ensure that the final product is noninfectious and is not unduly toxic. Some laboratories determine noninfectivity by eluting the virus from the vaccine, but this is not universally applicable to all formulations. For example, saponin influences greatly the elution of FMD from alumimum hydroxide/saponin vaccines (16). If the elution procedure is appropriate to a particular formulation, then it may be validated by seeding parallel samples of vaccine with trace amounts of live virus (7).

Toxicity and noninfectivity may be assessed simultaneously in an in-vivo test in cattle (18). Each of three healthy seronegative cattle is inoculated intradermally on the dorsal surface of the tongue with 0.1 ml of vaccine at 20 sites (four rows with five inoculation sites each). The animals are observed for no fewer than 4 days, after which three full bovine doses of vaccine are administered by the manufacturer's recommended route to each animal. The animals are observed for a further 6 days. Should any of the animals develop signs of FMD, the vaccine will fail the safety test. Equally, any undue toxicity attributable to the vaccine should be assessed and may prevent its acceptance. Ideally, vaccines prepared for species other than cattle should be safety tested in the species for which they are intended, administering a double dose of vaccine according to the manufacturer's recommended route and dose volume. The animals should be examined daily for a minimum of 7 days for evidence of toxicity or signs of FMD.

b) Potency

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 the different types of FMD virus 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 3 weeks 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 10,000 ID₅₀ (50% infectious dose) intradermally into two sites on the upper surface of the tongue (0.1 ml per site). Animals are observed for 8–10 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 (50% protective dose) content of the vaccine is calculated. There are a variety of methods for calculating PD₅₀ (22), but procedures based on Kärber are generally preferred. The vaccine should contain at least 3 PD₅₀ per dose for cattle, when employed for routine prophylactic use, although 6 PD₅₀ per dose is more commonly preferred. In some cases, vaccine of high potency will prevent the development of local tongue lesions at the site of challenge. In South American countries a variation of the potency test is performed, the PGP test (percentage of protection against generalised foot infection). A group of 16 bovines of 18–24 months of age, with the same characteristics described for PD₅₀, are vaccinated with a full vaccine dose by the route recommended by the manufacturer. These animals and a control group of two nonvaccinated animals are challenged 4 weeks 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 10,000 BID₅₀ (50% bovine infectious dose), intradermally into two sites on the upper surface of the tongue. Unprotected animals show lesions at sites other than the tongue. 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.

Potency tests in other target species, such as sheep, goats or buffalo are not common, as a successful test in cattle is considered to be sufficient to endorse its use in other species. Under circumstances where a vaccine is produced for use primarily in one particular species, it may be more appropriate to potency test the vaccine in that same species. However, in respect to the limited data for African buffalo or Asiat ic buffalo (Bubalus bubalis) and sheep, and the often inapparent nature of the disease in these species, potency results from a cattle test should be a good indicator of the vaccines applicability in these other species.
A similar protocol to the cattle test can be adopted for potency testing FMD vaccines in pigs. Using three groups of five pigs, one group is vaccinated with the full pig dose recommended by the manufacturer, one group receives a 1/4 dose, and a third group receives a 1/16 dose of vaccine. Traditionally, the response to oil vaccine is allowed longer to develop, and not until day 28 after vaccination are the three groups, plus two unvaccinated control pigs challenged. Challenge is by intradermal injection into the heel bulbs of one foot with 10,000 TCID_{50} (0.2 ml), as calculated by growth in a suitable pig cell culture, of a virulent challenge virus homologous to a strain used in the vaccine. The animals are observed daily for 10 days after challenge for clinical signs of FMD, but animals are removed as soon as they develop generalised FMD to avoid excessive challenge to those remaining. Both control animals should develop clinical signs on more than one foot. From the number of animals protected in each group, the PD_{50} content of the vaccine is calculated. There are a variety of methods for calculating PD_{50} (22), but procedures based on Kärber are generally preferred. The vaccine should contain at least 3 PD_{50} per dose for pigs. Likewise, a similar protocol to the PGP test in cattle can be adopted for pigs using a group of 16 animals vaccinated with a full vaccine dose and two nonvaccinated control animals. Challenge is by intradermal injection into the heel bulbs of one foot with 10,000 BID_{50} (0.2 ml) of a virulent challenge virus homologous to the strain used in the vaccine.

Other tests, including measurement following vaccination of virus neutralising antibodies in cell culture, or ELISA antibodies, or serum-protecting antibodies in suckling mice, may be used to assess the potency of a vaccine provided that a statistical evaluation has established a satisfactory correlation between the results obtained by the test on the relevant vaccine serotype and the potency test in cattle (42). For example, the expected percentage of protection is used to analyse the sera of a group of at least 16 vaccinated cattle and to express the probability of an animal being protected by measuring neutralising, ELISA or protecting antibodies. In a single group of animals given a full dose of vaccine, the mean individual expected percentage protection should be equal to or greater than 75% when 16 animals are used or 70% when 30 animals are used in the experimental group.

The presence of more than one serotype in a vaccine does not interfere with the induction of antibodies against another serotype or the correlation of antibody titre with protection.

c) Purity

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. Consequently, FMD vaccine or antigen that may be used in these circumstances, should be purified to reduce the NSP content. If the vaccine is being produced for a market where the NSP test will not be used, this NSP purity testing would not be required. A test method that can be used to evaluate the purity of the vaccine is to vaccinate three calves three times over 3–6 months and then test them for the presence of antibody against NSP using the tests described in Section B.2.d. of this chapter. If antibody is detected against NSP, the vaccine may be further purified before release. An alternative method is to vaccinate the calves used in the safety test two more times over 3–6 months and then test them for the presence of antibody against NSP.

REFERENCES


Chapter 2.1.1. — Foot and mouth disease


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**NB:** There are OIE Reference Laboratories for Foot and mouth disease (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.1.2.
VESICULAR STOMATITIS

SUMMARY

Vesicular stomatitis (VS) is a vesicular disease of horses, cattle and pigs caused by a vesiculovirus of the family Rhabdoviridae. This disease is clinically indistinguishable from foot and mouth disease (FMD), vesicular exanthema (VE), 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.

Although VS is transmitted directly by the transcutaneous or transmucosal route, the VS virus has been isolated from sandflies and mosquitoes, suggesting that it could be insect-borne. There is, therefore, 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 VS virus 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 virus.

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 VE when only pigs are affected.

Identification of the agent: VS virus can be readily isolated by the inoculation of several tissue culture systems, unweaned mice or embryonated chicken eggs. 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 immuno-electrophoresis.

Requirements for vaccines and diagnostic biologicals: 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) in 1926 (18) and 1927 (7) as a vesicular disease of horses, and subsequently of cattle and pigs. Vesicles are caused by the VS virus on the tongue, lips, buccal mucosa, teats and in the epithelium of the coronary band of the feet of cattle, horses, pigs, and many other species of domestic and wild animals. 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) (11).

Influenza-like signs, normally without vesicles, have been observed in humans who are in contact with animals with VS or who handle infective VS virus. All manipulations involving VS virus, including infective materials from animals, should be undertaken with using proper biosafety procedures.
There are two major immunological types of VS virus, 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. These VS viruses are represented by the Salto-Argentina/63 and Alagoas-Brazil/64 strains, which are considered to be subtypes 2 and 3, respectively, of the IND serotype (8). Strains of the serotype NJ and subtype IND-1 are identified in the endemic areas of the disease: south-eastern USA, Mexico, Central America, Panama, Venezuela, Colombia, Ecuador and Peru. The IND-2 Salto-Argentina/63 strain was isolated from horses in Argentina in 1963. This strain, along with the IND-2 Maipú-Argentina/86 and two other strains isolated in 1966 and 1979 in Brazil, and classified in the same subtype, only affected horses (2, 3). Cattle living together with the affected horses did not present antibody conversion (2). The IND-3 subtype, represented by the strain IND-3 Alagoas-Brazil/64, has been identified, sporadically, in Brazil only. Until 1977, strains of the IND-3 subtype were isolated from horses only. However, the IND-3 Espinosa-Brazil/77 was the first strain isolated from cattle. The known IND-3 strains affect cattle to a lesser degree than horses (2, 3). This finding confirms the first descriptions, in 1926 and 1927 (7, 18), of the NJ and IND serotypes in horses, and subsequently in cattle and pigs.

The mechanism of transmission of VS virus is unclear. The fact that the virus has been isolated from sandflies, mosquitoes, and other insects tends to substantiate the hypothesis that it could be transmitted by insects (6, 10, 17). There are also hypotheses that the VS virus is a plant virus present in pasture (17) 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 (20). 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 (5).

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 (16). Both NJ and IND-1 serotypes in the 1995, 1997 and 1998 US outbreaks primarily caused clinical disease in horses, although seroconversion was seen in cattle.

### B. DIAGNOSTIC TECHNIQUES

VS cannot be reliably clinically differentiated from the other vesicular diseases, such as foot and mouth disease (FMD), vesicular exanthema (VE), 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, VE and SVD, in order to facilitate the differential diagnosis of these vesicular diseases. Note: VS virus is a human pathogen and appropriate precautions should be taken when working with potentially infected tissues or the virus (see Chapter I.1.6. Human safety in the veterinary microbiology laboratory).

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. Epithelial samples should be placed in bottles of Tris-buffered tryptose broth with phenol red, pH 7.6. If complement fixation (CF) is to be carried out for antigen detection, the sample can be collected in glycerol/phosphate buffer, pH 7.2–7.6. (Note: glycerol is toxic to VS 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 insure that the CO$_2$ does not leak into the sample and destroy the virus. There are special packaging requirements for shipping samples with dry ice (see Chapter I.1.1. Sampling methods 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 (spumon) 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 VS virus should not be treated with solvents such as chloroform. Virus can be isolated from oral and nasal tissues 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. Pairs of sera from the same animals, collected 1–2 weeks apart, are preferred for checking the change in antibody titre.
Specific diagnostic reagents for VS virus 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 3 of this Terrestrial Manual), and the Institute for Animal Health, produce and distribute diagnostic reagents on request.

1. Identification of the agent

For identification of VS virus and the differential diagnosis of vesicular diseases, clarified suspensions of field samples suspected to contain VS 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 virus causes 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 virus.

The VS virus replicates and can be isolated in 8–10-day-old chicken embryos by inoculation into the allantoic sac, in 2–7-day-old unweaned mice by inoculation by any route, or in 3-week-old mice by intracerebral inoculation. In all three cases, VS 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 of the foot 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 strain 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. Similar tests can be performed on homogenate suspensions of the dissected musculo-skeletal 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 virus), picornavirus (FMD virus and SVD virus), calicivirus (VE) 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 enzyme-linked immunosorbent assay (ELISA), the CF test 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).

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. If no fluorescence is observed and no CPE is evident in the flask culture, the sample(s) are reported as negative for VS virus isolation.

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.

1 Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey GU24 0NF, United Kingdom.
b) **Enzyme-linked immunosorbent assay**

The indirect sandwich ELISA (IS-ELISA) (2, 9) 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 (2). For detection of VS virus NJ strains, a monovalent set of rabbit/guinea-pig antisera is suitable (2, 9).

- **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 refs 2 and 4), 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 30 minutes 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 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 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.

- **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.

- **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 (2) 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 VS virus.

d) **Nucleic acid recognition methods**

The polymerase chain reaction (PCR) can be used to amplify small genomic areas of the VS virus (12, 19). This technique will detect the presence of VS virus RNA in tissue and vesicular fluid samples and cell culture, but cannot determine if the virus is infectious. In general, PCR techniques are not routinely used for screening diagnostic cases for VS virus.

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 (14).

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

The liquid-phase blocking ELISA (LP-ELISA) is the method of choice for the detection and quantification of antibodies to VS virus. The use of viral glycoproteins as antigen is recommended because they are not infectious, detect neutralising antibodies and give fewer false-positive results than the VN (4).

- **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 >1.3 (1/20) 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. (1). It uses vesicular stomatitis NJ and IND-1 recombinant antigens as described by Katz et al. (15).

- **Test procedure**
  i) **Solid phase:** Antigens are diluted in carbonate/bicarbonate buffer, pH 9.6, and 50 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 60 days. The plates are thawed, antigen is decanted, and 100 µl of blocking solution is added. The plates are then incubated at 25°C for 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 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.

b) **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 NJ or IND VS virus and Vero M cells, and preformed monolayer or suspension IB-RS-2 cells to test for the presence of unneutralised virus.

- **Test procedure**
  i) *Virus:* NJ or IND VS virus are 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 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 protected. 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 VS.

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. For this purpose, twofold serum dilutions are mixed with 2 CFU$_{50}$ of known antigen and with 5% normal bovine or calf sera included in 4 CHU$_{50}$ 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 AND DIAGNOSTIC BIOLOGICALS

Attenuated virus vaccines have been tested in the field in the USA, Panama, Guatemala, Peru and Venezuela (16, 17) with unknown efficacy. At present, live or inactivated virus vaccines are not yet available commercially.

**ACKNOWLEDGEMENT**

Parts of this chapter were taken from or based on the chapter on vesicular stomatitis in previous editions of the *Terrestrial Manual*.

**REFERENCES**


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**NB:** There are OIE Reference Laboratories for Vesicular stomatitis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.1.3.

SWINE VESICULAR DISEASE

SUMMARY

Swine vesicular disease (SVD) is a contagious disease of pigs, caused by an enterovirus and characterised by vesicles on the coronary bands, heels of the feet and occasionally on the lips, tongue, snout and teats. Strains of SVD virus vary in virulence, and the disease may be subclinical, mild or severe, the latter usually only being seen when pigs are housed on abrasive floors in damp conditions. The main importance of SVD is that it is clinically indistinguishable from foot and mouth disease (FMD), and any outbreaks of vesicular disease in pigs must be assumed to be FMD until investigated by laboratory tests and proven otherwise.

Identification of the agent: Where a vesicular condition is seen in pigs, the demonstration by enzyme-linked immunosorbent assay (ELISA) of SVD viral antigen in a sample of lesion material or vesicular fluid is sufficient for a positive diagnosis. If the quantity of lesion material submitted is not sufficient (less than 0.5 g), or if the test results are negative or inconclusive, isolation of virus may be carried out by the inoculation of porcine cell cultures. If any cultures subsequently develop a cytopathic effect, the demonstration of SVD viral antigen by ELISA will suffice to make a positive diagnosis.

Serological tests: Specific antibody to SVD virus can be identified using the microneutralisation test or ELISA. Although the microneutralisation test requires 2–3 days to complete, it remains the definitive test for antibody to SVD virus. A small proportion (up to 0.1%) of normal, uninfected pigs will react positively in serological tests for SVD. These singleton reactors can only be differentiated from infected pigs by resampling of the positive animal and its cohorts.

Requirements for vaccines and diagnostic biologicals: There are currently no commercial vaccines available against SVD. Diagnostic and standard reagents are available from regional reference laboratories.

A. INTRODUCTION

Swine vesicular disease (SVD) can be a subclinical, mild or severe vesicular condition depending on the strain of virus involved, the route and dose of infection, and the husbandry conditions under which the pigs are kept. Clinically, SVD is indistinguishable from foot and mouth disease (FMD) and this is its main importance. It is therefore urgent that cases of SVD be distinguished from FMD by laboratory investigation.

The incubation period for SVD is between 2 and 7 days, after which a transient fever of up to 41°C may occur. Vesicles then develop on the coronary band, typically at the junction with the heel. More rarely, vesicles may also appear on the snout, particularly on the dorsal surface, on the lips, tongue and teats, and shallow erosions may be seen on the knees. Affected pigs may be lame and off their feed for a few days. Abortion is not a typical feature of SVD. Recovery is usually complete in 2–3 weeks, with the only evidence of infection being a dark, horizontal line on the hoof where growth has been temporarily interrupted. The clinical signs vary according to the age of pigs affected, the conditions under which they are kept and the strain of SVD virus involved (8). Disease caused by mild strains may remain unobserved, particularly in pigs kept on grass or housed on deep straw. Younger animals are more severely affected, although mortality due to SVD is very rare, in contrast with FMD in young stock. Nervous signs have been reported, but are unusual. Affected pigs may excrete virus from the nose and mouth and in the faeces up to 48 hours before the onset of clinical signs. Most virus is produced in the first 7 days after infection, and virus excretion from the nose and mouth normally stops within 2 weeks. Virus may continue to be shed for up to 3 months in the faeces. The SVD virus is extremely resistant to inactivation in the environment, and is stable in the pH range 2.5–12.0 (9). This is in contrast to the FMD virus, which is very labile outside the pH range 6.0–8.0.
Because SVD may be mild or subclinical, it is essential when submitting samples from suspect clinical cases that serum samples from both the suspect pigs and other apparently unaffected animals in the group be included. It is possible for SVD to circulate unnoticed until it affects a particularly susceptible group, and therefore, in order to ascertain how long infection has been present, it is necessary to look for seroconversion to SVD virus in apparently healthy animals.

SVD is clinically very similar to FMD. Samples for virus isolation or antigen detection must be handled and submitted as though they contained FMD virus and must be transported in phosphate buffered saline (PBS) mixed with glycerol (1/1), pH 7.2–7.6, with antibiotics such as (final concentration per ml) penicillin (1000 International Units [IU]), neomycin sulphate (100 IU), polymyxin B sulphate (50 IU), and mycostatin (100 IU) (6).

SVD virus has been classified as a pig enterovirus, in the family Picornaviridae. Antigenically it is related to the human virus coxsackievirus B5. There are reports of seroconversion to SVD virus in laboratory workers handling the agent. Clinical disease was reported to be mild with the exception of a single case of meningitis associated with SVD virus infection. However, there have been no reported cases of seroconversion or disease in farmers or veterinarians working with infected pigs. Under experimental conditions, it has not been possible to show transmission of coxsackievirus B5 between pigs.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Any vesicular condition in pigs may be FMD. If FMD has been eliminated, the diagnosis of SVD requires the facilities of a specialised laboratory. Countries that lack such a facility should send samples for investigation to the FAO World Reference Laboratory (WRL) for Foot and Mouth Disease1. In the Americas, parallel testing for vesicular stomatitis viral antigen should also be conducted.

Investigation should start with the examination of a 10% suspension of lesion material in PBS by enzyme-linked immunosorbent assay (ELISA) using antiserum specific for SVD and FMD viruses. This suspension should also be inoculated on to monolayers of IB-RS-2 porcine cells (or other susceptible porcine cells), primary calf thyroid cells, and primary (or secondary) calf kidney cells. FMD virus will grow in all three tissue culture systems. Generally SVD virus will grow in cells of porcine origin only, however there is a report that the virus can be isolated in secondary lamb kidney cells. SVD virus can also be isolated from faecal samples.

a) Culture

A portion of the clarified epithelial suspension is inoculated on to monolayers of IB-RS-2 cells or other susceptible porcine cells. For differential diagnosis (e.g. FMD) bovine cell culture systems should also be employed. A culture medium found to be satisfactory is 50/50 Eagle's complete medium/yeast lactalbumin hydrolysate (LYH). For cell growth, add 10% bovine serum; for maintenance, add 3% bovine serum; and for virus isolation, add antibiotics only; it is preferable not to add serum.

Cultures are examined twice daily. If a cytopathic effect (CPE) is observed, the supernatant fluid is harvested and used as antigen in the ELISA for virus identification. Negative cultures are blind-passaged after 48 or 72 hours, and observed for a further 3 days. When isolating virus from faeces in which the amount of virus present may be low, a third tissue culture passage may be required.

b) Immunological methods

• Enzyme-linked immunosorbent assay

The detection of SVD viral antigen by an indirect sandwich ELISA has replaced the complement fixation test as the method of choice. The test is the same as that used for FMD diagnosis. Duplicate rows in multwell ELISA plates are coated with rabbit antiserum to SVD virus. This is the capture serum. Test sample suspensions are added to each of the rows. Appropriate controls are also included. Guinea-pig detection serum is added at the next stage followed by rabbit anti-guinea-pig serum conjugated to horseradish peroxidase. Extensive washing is carried out between each stage to remove unbound reagents. A positive reaction is indicated if there is a colour reaction on the addition of chromogen (orthophenylenediamine) and substrate (H₂O₂). With strong positive reactions this will be evident to the naked eye, but results can also be read spectrophotometrically at 492 nm, in which case an absorbance reading ≥0.1 above background indicates a positive reaction. As an alternative to guinea-pig and rabbit antisera, suitable monoclonal

1 FAO (Food and Agriculture Organization of the United Nations) World Reference Laboratory for FMD, Institute for Animal Health, Pirbright, Woking, Surrey GU24 0NF, United Kingdom (also an OIE Reference Laboratory for FMD).
antibodies (MAbs) can be used, coated to the ELISA plate as the capture antibody, or peroxidase conjugated as tracing antibody.

An MAb-based ELISA can also be used to study antigenic variation among strains of SVD virus. Tissue-culture grown viral antigens are trapped by a rabbit hyperimmune antiseraum to SVD virus adsorbed to the solid phase. Appropriate panels of MAbs are then reacted and the binding of MAbs to field strains is compared with the binding of MAbs to the parental strains. Strong binding indicates the presence of epitopes shared between the parental and the field strains (1).

c) Faecal samples

i) Resuspend faecal material (approximately 20 g) in a minimal amount of phosphate buffer (0.04 M phosphate buffer or PBS).

ii) Leave shaking, or on a rotary mixer, overnight at 4°C.

iii) Clarify by centrifugation at 10,000 rpm for 30 minutes in a high speed centrifuge.

iv) Harvest the supernatant and inoculate five tubes of IB-RS 2 cells with 0.2 ml per tube. Leave in a stationary position to adsorb for 1 hour at 37°C. Wash the tubes three times with PBS. Add serum-free maintenance medium (2 ml/tube) and place in a rolling rack, or suitable alternative that allows agitation of the culture media, at 37°C.

v) Centrifuge the remaining supernatant at 28,000 rpm for 3 hours. This will concentrate any virus present in the supernatant in low amounts.

vi) Discard the supernatant and resuspend the pellet in 2 ml of PBS by brief sonication. Add an equal volume of Freon and vortex 2–3 ml. Spin at 4000 rpm for 10 minutes on a bench centrifuge. Remove the supernatant fluid.

vii) Inoculate five tissue culture tubes as in step iv above.

viii) Incubate the tubes at 37°C for 3 days inspecting daily for evidence of CPE.

ix) After 3 days, if there is no evidence of CPE, freeze–thaw the cell cultures and perform a blind passage into fresh tubes of IB-RS-2 cells. Incubate for a further 3 days, inspecting the tubes daily as before.

x) Harvest the supernatant of any tubes showing CPE and confirm the presence of SVD virus by ELISA (or other appropriate test).

xi) If no CPE is evident after the second passage the sample will be recorded as NVD (no virus detected).

d) Nucleic acid recognition methods

Nucleic acid recognition methods can be used to detect SVD viral genome in clinical material using reverse transcription followed by the polymerase chain reaction (PCR) and to establish relationships between isolates of SVD virus by determining the nucleotide sequence of part of the genome. By sequencing approximately 200 nucleotides within the 1D gene, which codes for the major structural protein VP1, it has been possible to group strains of SVD virus according to their sequence homology, and to relate epidemiologically strains causing disease in different regions or at different times (2). Techniques using the PCR have been developed to improve the sensitivity of diagnosis. A PCR has been described that combines extraction of RNA using commercially available silica gel columns with a reverse-transcription PCR (RT-PCR) using primers corresponding to highly conserved regions in the 1C and 1D genes (7). The technique is rapid, detects all genotypes of SVD virus, and is sufficiently sensitive for use on samples collected from cases of suspect clinical disease. Where subclinical infection is suspected, or when samples are collected after the resolution of clinical disease, a more sensitive nested RT-PCR can be combined with a more elaborate RNA extraction method to produce a detection system at least as sensitive, and considerably more rapid, than multiple passage on tissue culture. Several laboratories have developed alternative PCRs using a variety of protocols (3, 10, 11).

2. Serological tests

SVD is often diagnosed solely on the evidence of serological tests. Because of the subclinical or mild nature of the disease, it is often first suspected following routine serology for disease surveillance or export certification. The virus neutralisation (VN) test, the double immunodiffusion test, the radial immunodiffusion test, the counter immunoelectrophoresis test and the ELISA have all been used for the detection of antibodies to SVD virus (1, 4, 5). The VN test and the ELISA are used most frequently. The VN test is the accepted standard test, but has the disadvantage that it takes 2–3 days to complete and requires tissue culture facilities. The ELISA is more rapid and can be more easily standardised. A small proportion of sera from animals with no previous exposure to SVD virus will react positively in serological tests for antibody to SVD virus. The 5B7 MAb competitive ELISA (MAC-ELISA) has been a reliable technique for detecting SVD antibody (1). Results from up to approximately 1% of
sera from normal pigs are borderline or positive by the MAC-ELISA and should be retested by the VN test. Up to approximately 10% of these sera will also be positive by the VN test (i.e. 0.1% of the original population). Animals positive by ELISA, but negative by VN test can be regarded as uninfected. Repeat samples should be collected from animals positive in both tests and from cohorts. A constant or declining titre in the positive animal and the absence of antibody to SVD virus in cohorts confirms the status of the positive animal as a ‘singleton reactor’. The factors responsible for ‘singleton reactors’ are unknown. Serological cross-reactivity with SVD virus might arise due to infection with another, as yet unidentified, picornavirus or may be due to other nonspecific factors present in the serum. Identification of the isotype of antibody present in positive sera (1) can be helpful as sera from infected pigs usually contain specific IgG alone or both IgG and IgM, whereas sera from ‘singleton’ reactors contain exclusively IgM.

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

The quantitative VN microtest for antibody to SVD virus is performed using IB-RS-2 cells (or suitable susceptible porcine cells) in flat-bottomed tissue-culture grade microtitre plates.

Virus is grown on IB-RS-2 cell monolayers and stored at –20°C after the addition of an equal volume of glycerol. SVD 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. A suitable medium is Eagle’s complete medium/LYH with antibiotics.

The test is an equal volume test in 50 µl volumes:

i) Starting from a 1/4 dilution, sera are diluted in a twofold dilution series across the plate, two rows of wells per serum.

ii) Previously titrated virus is added; each 50 µl unit volume of virus suspension contains about 100 TCID₅₀ (50% tissue culture infective dose).

iii) Controls include a strong positive serum, a weak positive serum and a negative serum, 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 prepared in medium containing 10% bovine serum for cell growth. 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 5% carbon dioxide at 37°C for 2–3 days.

vii) Microscopic 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; staining is done by immersion in 0.05% methylene blue in 10% formalin for 30 minutes. The plates are rinsed in tapwater.

viii) Positive results are blue-stained cell sheets; the negative wells are empty. Titres are expressed as the final dilution of serum present in the serum/virus mixture at the 50% end-point. The test is considered to be valid when the amount of virus actually used per well is between 10¹⁵ and 10²⁵ TCID₅₀ and the positive standard sera are within twofold of their expected titre.

ix) Interpretation of the results: At the OIE/FAO WRL for FMD (see footnote 1), VN titres less than or equal to 1/11 are considered to be negative. Titres of 1/16 to 1/32 are doubtful and VN titres of 1/45 or more are regarded as positive. However, as titres depend on the cell system used, laboratories should establish their own criteria by reference to standard reagents available from the OIE/FAO WRL for FMD.

b) Enzyme-linked immunosorbent assay

In the ELISA developed by Brocchi et al. (1), the SVD viral antigen is trapped to the solid phase using the MAb 5B7. The ability of test sera to inhibit the binding of peroxidase-conjugated MAb 5B7 to the trapped antigen is then evaluated. Finally, the amount of conjugated MAb bound is detected by the addition of substrate and chromogen.

i) ELISA plates are coated with 50 µl/well of MAb 5B7 at a dilution of 10 µg/ml in carbonate/bicarbonate buffer, pH 9.6, by overnight incubation at 4°C.

ii) The plates are washed three times with PBS containing 0.05% Tween 20, and 50 µl of SVD antigen (SVD virus grown in IB-RS-2 cells, clarified, filtered and inactivated) at a predetermined optimal dilution, is added to each well. The optimal dilution of antigen is determined by checkerboard titrations of antigen and conjugated MAb that define the working dilution giving an absorbance on the upper part
of the linear region of the antigen titration curve (between 1.5 and 2.0 optical density units). Plates are then incubated for 1 hour at 37°C.

iii) After three additional washes, 50 µl of diluted test sera (not inactivated) and control sera are incubated with the trapped antigen for 1 hour at 37°C. Three-fold dilution series of sera are obtained directly in ELISA wells by adding 10 µl of serum to 65 µl of buffer (1/7.5 dilution) then transferring 25 µl to sequential wells containing 50 µl of buffer, mixing, and finally discarding 25 µl.

iv) After incubation for 1 hour, 25 µl of an optimal dilution of peroxidase-conjugated MAb 5B7 (see step ii above) is added to each well and the plates are incubated at 37°C for a further 1 hour.

v) After a final series of washes, the colorimetric reaction is developed by distributing 50 µl per well of the substrate solution (0.5 mg/ml orthophenylene-diamine in phosphate/citrate buffer, pH 5, containing 0.02% H2O2).

vi) The reaction is stopped after 10 minutes by adding 50 µl of 2 N H2SO4. The absorbance is read at 492 nm using a microplate reader.

Antigen, sera and conjugate are diluted in PBS, pH 7.4, containing 0.05% Tween 20 and 1% yeast extract; the dilution buffer for sera contains, in addition, 1.0% mouse serum to prevent nonspecific binding of pig serum to MAb 5B7 either coated to the plate or conjugated to peroxidase.

vii) Controls: Four wells on each plate containing all reactants except test serum confirm the maximum absorbance reading for the antigen; convalescent pig serum at four selected dilutions; negative pig serum; a low positive standard pig serum.

viii) Interpretation of the results: Reactions are expressed as the percentage inhibition by each test serum of the MAb reaction with the SVD antigen. Sera are considered to be strongly positive if the mean percent inhibition at both 1/7.5 and 1/22.5 dilutions is more than 70%. Sera registering a mean of more than 70% inhibition at the 1/7.5 dilution but less than 70% inhibition at the 1/22.5 dilution are considered to be low positive or borderline. Sera showing less than 70% inhibition at both dilutions are considered negative. All positive and borderline sera should be confirmed using the VN test.

**STANDARD REFERENCE SERA FOR SVD SEROLOGY**

The FAO/OIE WRL for FMD maintains a panel of reference sera that have been extensively validated by the National SVD Reference Laboratories of the Member States of the European Union.

**C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS**

No commercial SVD vaccines are currently available. Standard sera can be obtained from the OIE/FAO WRL for FMD (see footnote 1). MAb 5B7 is available from the OIE Reference Laboratory for swine vesicular disease in Italy (see Table given in Part 3 of this Terrestrial Manual).

**REFERENCES**


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**NB:** There are OIE Reference Laboratories for Swine vesicular disease (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.1.4.

RINDERPEST

SUMMARY

Classical rinderpest is an acute, viral disease of domestic cattle, buffaloes and yaks characterised by high morbidity and mortality rates. Sheep, goats, pigs and wild ungulates may also be affected. Clinically this form of the disease is characterised by pyrexia, the progressive development of shallow erosions on the gums, tongue, cheeks and hard palate, together with serous or mucopurulent ocular and nasal discharges. Alimentary tract involvement is marked by the development of diarrhoea or dysentery, leading to severe dehydration and depression. Rinderpest conforming to this description is now rarely seen, but a milder form of the disease, with the potential to regain classical characteristics, still occurs in East Africa.

Three genetically distinct lineages of rinderpest virus have been recognised as causing disease in Africa and Asia in the last decade. Lineage 1 was limited to Ethiopia and Sudan, Lineage 2 to East Africa and Lineage 3 to Asia. Until recently, both southern Sudan and West Asia were infected, but both are now considered to be rinderpest free. The Food and Agriculture Organisation of the United Nations (FAO) launched a Global Rinderpest Eradication Programme (GREP) in 1992, calling for eradication of the virus by the year 2010. The success of this programme may be judged by the fact that two of the three rinderpest lineages have now been globally eradicated.

Identification of the agent: Clinical confirmation of classical rinderpest is based on the finding of individual or small groups of animals showing pyrexia, inappetance, depression, 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, recumbency and possibly death. 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. It is particularly important to isolate the virus if a geographical extension or significant animal health deterioration has occurred. Following the successes of global eradication, rinderpest-free countries may now confirm the presence of peste des petits ruminants (PPR) in sheep or goats based on the clinical appearance of affected animals and the presence of precipitating antigens, even though both the clinical signs and the virus-induced antigens are common to both viruses.

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 are PPR in sheep and goats, and bovine viral diarrhoea/mucosal disease and malignant catarrhal fever in cattle; differentiation of these diseases requires the use of appropriate laboratory methods.

Serological tests: The OIE has developed a set of Recommended Standards for Epidemiological Surveillance for Rinderpest (the ‘OIE Pathway’) that governs the actions of Member Countries wishing to demonstrate that they have achieved freedom from infection. To this end, both competition and indirect enzyme-linked immunosorbent assays are available that will determine the presence of rinderpest antibodies in animals that have been infected with field virus or with rinderpest vaccine. The selected test should be sensitive with respect to the lineage of virus likely to be present. Neutralising antibody estimations may be used for the same purpose. Member Countries may wish to seek expert advice from an OIE Reference Laboratory with regard to the selection of the test most appropriate for their purpose.

Requirements for vaccines and diagnostic biologicals: A live attenuated cell culture rinderpest vaccine is available. In recent years its use has been restricted to intensified focal vaccination
Chapter 2.1.4. – Rinderpest

A. INTRODUCTION

In recent years the Global Rinderpest Eradication Programme (GREP) of the Food and Agriculture Organisation of the United Nations (FAO) has made enormous progress in organising and documenting the decline of rinderpest (15). Historically, the virus was widely distributed throughout Europe, Africa, and Asia; recently however, it has only occurred in Africa and Asia. Gene sequence analysis has shown that all known rinderpest isolates fall into one of three non-overlapping phylogenetic lineages, and in recent years it has been possible to describe the virus’ distribution in lineage-specific terms. Thus, the so-called Asian lineage (lineage 3) was only ever recorded in Afghanistan, India, Iran, Iraq, Kuwait, Oman, Pakistan, Russia, Saudi Arabia, Turkey, Sri Lanka and Yemen. As a result of concerted and coordinated vaccination and surveillance campaigns, this virus lineage has failed to resurface since September 2000 (Pakistan). Although evaluations are not yet complete, it is almost certain that this virus has been successfully eradicated.

Rinderpest virus lineages 1 and 2 have only been recorded from Africa. Lineage 1 appears to have been distributed from Egypt to southern Sudan and eastwards into Ethiopia and into northern and western Kenya. On the other hand, lineage 2 has been recorded from both East and West Africa and at one time may have been distributed in a sub-Saharan belt running across the whole of the continent (16). Now however, as the result of further coordinated vaccination and surveillance programmes (Pan African Rinderpest Campaign in particular), neither West nor Central Africa have reported rinderpest for the past 15 years. Until recently both lineages were being reported from eastern Africa (lineage 1 in southern Sudan in 1998 and lineage 2 in Kenya in 2001). The failure to find more recent evidence that lineage 1 is still being transmitted supports the belief that this lineage has also been eradicated.

Reappearing in 1994, 1996 and 2001 in wildlife, Lineage 2 is still being transmitted within the Somali pastoral ecosystem (12) where its continued presence is causing considerable concern (13). In 1994, this virus reappeared in south-east Kenya where its effects were expressed most dramatically in buffaloes in Tsavo National Park (3) thereby illustrating its ability to engage in cryptic persistence for a period of at least 30 years, during which time it is likely to have been transmitted with a low level of virulence among susceptible cattle. Although this virus is now seen as having evolved to the point where it has been possible for it to escape veterinary attention in remote areas, its presence did not go unnoticed by the nomadic pastoralists whose cattle it infected. Most recently, in 2003 mild rinderpest was reported in cattle in Kenya close to the border with Somalia; the virus has not been isolated but phylogenetic studies identified viral RNA closely related to the ancestral RBOK strain. This enigma needs to be resolved urgently. The danger posed to cattle elsewhere in Africa by viruses circulating in what is almost certainly the last reservoir of rinderpest is shown by their ability to spread within Kenya and to neighbouring Tanzania in the early 1980s and again in the mid-1990s (18), regaining virulence for cattle in the process.

Rinderpest is caused by a negative-strand RNA virus of the Morbillivirus genus within the family Paramyxoviridae. Classic descriptions of rinderpest refer to it as a highly fatal disease of domestic cattle, buffaloes and yaks. The virus also affects sheep, goats, some breeds of pigs and a very large variety of wildlife species within the order Artiodactyla, although not always in a clinically apparent form.

In describing rinderpest in contemporary terms it is perhaps best to consider that for the most part it is a mild, nonfatal, infectious disease of cattle, but one with two very dangerous attributes. The first is an almost certain ability to undergo virulence modulations. The second is an ability to infect game animal species and, in buffaloes, eland, giraffe, lesser kudu and warthog, to cause an acute infection associated with high levels of mortality.

The incubation period of the lineage 2-associated mild form of rinderpest is between 1 and 2 weeks, and the ensuing clinical disease can be seen as a subacute febrile attack and little more. The fever is not invariable; it is short-lived (3–4 days) and not very high (38–40°C). The depression that characterises more acute forms of
rinderpest is absent from mildly affected animals and, as they do not lose their appetite, they will probably continue to graze, water and trek as well as unaffected animals. These animals do not usually develop diarrhoea. On close examination there may be some slight congestion of the visible mucous membranes and small, focal areas of raised, whitish epithelial necrosis may be found on the lower gum – sometimes no larger than a pin head – along with a few eroded cheek papillae. Some animals may escape the development of such erosions, the appearance of which is fleeting. Other animals may show a slight, serous, ocular or nasal secretion but, in contrast to the more severe forms of the disease, these do not progress to become mucopurulent.

From time to time the virulence of mild lineage 2 rinderpest may increase, in which case the following description of classical rinderpest is applicable. After 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, nonhaemorrhagic 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, the breath is fetid and mucopurulent ocular and nasal discharges develop.

Deaths will occur but the mortality rate will be variable and may be expected to rise as the virus gains progressive access to large numbers of susceptible animals. Initial mortality rates will probably be in the order of 10–20% and, 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 and diarrhoea, others after a sharp fall in body temperature, often to subnormal values. Alternatively, 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.

Typically the carcass of dead animals 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 ileocelecal 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’.

Even though infections with lineage 2 may pass unnoticed in cattle, the virus is highly infectious for wildlife species, and among those generally regarded as highly susceptible (buffalo, eland, and lesser kudu) it causes fever, a nasal discharge, typical erosive stomatitis, gastroenteritis, and death. Kock (10) observed that in addition, buffaloes infected with lineage 2 showed enlarged peripheral lymph nodes, plaque-like keratinised skin lesions and keratoconjunctivitis. Lesser kudus 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, under the present 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.

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

In view of the efforts now being directed towards the eradication of lineage 2, any rinderpest outbreak assumes a high level of epidemiological significance. Consequently, samples from all outbreaks diagnosed as rinderpest on
clinical or pathological grounds must be routinely submitted for laboratory confirmation. A variety of suitable laboratory tests is available, but at this time it is of paramount importance to isolate the virus, identify its lineage and assess its virulence in experimental cattle (1). While not a definitive diagnostic test, a rapid chromatographic strip test (Penside Test; ref. 6) has proved a useful tool to assist field personnel in investigating outbreaks of rinderpest in the final stages of eradication. It was found particularly useful in Pakistan (9) and Yemen. 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.

To isolate the virus from blood, uncoagulated blood is centrifuged at 2500 g for 15 minutes to produce a Buffy 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 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 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.

b) Antigen detection by agar gel immunodiffusion

The agar gel immunodiffusion (AGID) tests may be conducted in Petri dishes or on glass microscope slides (7). 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 micropip (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

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1 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.
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

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 a negative-sense RNA probe to the rinderpest N protein gene (5).

d) Lineage identification using the reverse-transcription polymerase chain reaction

The reverse-transcription polymerase chain reaction (RT-PCR) (8) produces DNA suitable for gene sequence analysis. Viral RNA can be purified from spleen (not ideal due to 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 solution², 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. 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 the two morbilliviruses. 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 3 of this Terrestrial Manual), can advise on use of the technique for field sample analysis.

• Differential immunocapture

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 must be used. Rapid differentiation can be achieved using a differential immunocapture ELISA test (11). This test employs MAb 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.

2 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.
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.

2. Serological tests

a) The competitive enzyme-linked immunosorbent assay (the prescribed test for international trade)

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. The antigen is concentrated from the infected cell culture supernate by ammonium sulphate precipitation. The MAb was obtained by fusing the splenocytes of hyperimmunised mice with the NSO myeloma cell line, and then shown to be rinderpest specific (2); 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 3 of this Terrestrial Manual). Kits are 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 MAb 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 MAb control. Inhibition values of 50% or more are considered to be positive and values below 50% are considered to be negative.

ix) Currently GREP is analysing the ‘H’ competitive ELISA at two cut-off values (50% and 35%) in an effort to increase sensitivity.

An indirect ELISA method has been developed and might be useful for rinderpest surveillance programmes, especially in areas in which lineage II rinderpest virus could be present (20). However, the performance characteristics of the test indicate a problem with specificity and therefore its use will require confirmatory testing.
b) Virus neutralisation

The ‘gold standard’ virus neutralisation (VN) test should be undertaken in roller-tube cultures of primary calf kidney cells following the method of Plowright & Ferris (14) on the basis that the results obtained with this test were validated in cattle. In the roller tube procedure, unactivated sera are diluted at intervals of 1 in 10 and then, starting with undiluted serum, mixed with an equal volume of virus containing approximately 10\(^{3.0}\) TCID\(_{50}\) (50% tissue culture infective dose) per ml of the attenuated Kabete ‘O’ vaccine strain. 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 it falls within the range 10\(^{1.8}\) to 10\(^{2.8}\) TCID\(_{50}\)/tube; serum dilutions are considered to double after mixing with the virus dose. 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\(_{50}\) (17). Following a 45-minute or an overnight incubation period, between 1 and 2 \(\times\) 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 AND DIAGNOSTIC BIOLOGICALS

Many countries have used rinderpest vaccine to reduce the incidence of rinderpest to zero, and then followed the OIE Pathway in order to have their rinderpest-free status internationally recognised. In order to obtain this status, the process of annual rinderpest vaccination has largely been replaced by active and passive clinical and serological surveillance. Intensive focal vaccination (immunosterilisation) has been retained for dealing with emergency campaign management (18).

The live attenuated tissue culture rinderpest vaccine (TCRV) described in previous editions of this Terrestrial Manual was developed by Plowright by the serial passage of the virulent bovine rinderpest strain Kabete ‘O’ (RBOK) in primary bovine calf kidney cells. In view of the success of the Global Rinderpest Eradication Programme it is believed that most vaccine manufacturers no longer make this product, although a number of them may be storing considerable stocks. Vaccination with TCRV induces a life-long immunity that unfortunately cannot be easily distinguished from the similarly enduring antibody response induced by the wildtype virus. Due to the conflicting demands of retaining serosurveillance as the most reliable tool for determining the field distribution of mild lineage 2 virus and the possible need to simultaneously immunise animals with a view to interrupting transmission, there is an urgent need to replace TCRV with a marker rinderpest vaccine. Candidate vaccines have been described (4, 19) but have still to be registered with the national authorities by a producer.

Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 are intended to be general in nature and may be supplemented by national and regional requirements.

As stated previously, production of TCRV has virtually stopped and future use appears unlikely. However, the description published in the previous edition of the Terrestrial Manual will be repeated here so that it is available if conditions change.

1. Seed management

a) Characteristics of the seed

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, that retains its attenuated characteristics during at least five back
Chapter 2.1.4. — Rinderpest

passages in cattle, and that lacks the ability to spread by contact. Substrains of RBOK used in the manufacture of TCRV must be identifiable by written historical records, which must include information on the origin of the strain and of its subsequent manipulations.

b) Method of culture

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 \(-20^\circ\text{C}\) 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.

c) Validation as a vaccine

Seed lots must be shown to be:

i) **Pure**: Free from contamination with viruses, bacteria, fungi or mycoplasmas.

ii) **Safe**: Inducing no abnormal clinical reaction on inoculation into rinderpest-susceptible cattle.

iii) **Efficacious**: Inducing an immunity to rinderpest in rinderpest-susceptible cattle.

2. Method of manufacture

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. To facilitate long-term storage and cold-chain distribution, this fluid is freeze dried in the presence of a cryoprotectant consisting of 5% lactalbumin hydrolysate and 10% sucrose. 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 noninfected 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. Two harvests are permissible from the same set of cultures and may be pooled to form a bulk suspension. Written records must accompany all stages of vaccine manufacture.

3. In-process control

**Cells**: Primary cells, serially cultivated primary cells 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. Whatever cells are committed for vaccine production, uninfected control cultures must be maintained using the same media and incubation conditions as the rinderpest-infected cells. They must be subjected to frequent microscopic observations. After harvesting the vaccine, the control cultures should be washed to remove ox serum and re-incubated for 10 days in media containing ox serum substitutes. They are again subject to frequent microscopic observations for evidence of cytopathic change. Simultaneously a sample of the cultures should be examined for the presence of noncytopathic BVD virus using an immunofluorescence or immunoperoxidase test or RT-PCR. The serum used in the culture media must come from rinderpest-susceptible animals.

**Virus**: 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. A similar titration must be undertaken on the final bulk. Virus should be derived from cultures maintained in roller bottles and may not be harvested more than 10 days after the date that these cultures were infected. The harvest should be clarified by low-speed centrifugation before mixing with cryoprotectant. Prior to lyophilisation it may be held for not more than 5 days at \(4^\circ\text{C}\) but for considerably longer if frozen at \(-20^\circ\text{C}\) to \(-60^\circ\text{C}\). As adventitious viral contamination may arise during a manufacturer's manipulations or from the use of contaminated media, rabbit hyperimmune serum should be used to neutralise the rinderpest content of the bulk suspension, after which the mixture should be used to infect calf kidney or Vero cells, which are handled as described above. The final bulk must be tested for freedom from bacteria, fungi and mycoplasmas.

4. Batch control

a) **Identity**

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.
b) Sterility
Tests for sterility and freedom of contamination of biological materials may be found in Chapter 1.1.5.

c) 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 and one ox with a volume equivalent to 1/10th of a cattle field dose. These animals are maintained in close contact with an uninoculated 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. This test is not a potency test. Each vaccine lot must also be tested for innocuity in small animals.

d) 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.

e) Duration of immunity
It is unnecessary to routinely establish the duration of immunity to TCRV. Reported results indicate that lifelong immunity can be expected following the successful vaccination of cattle free of all vestiges of maternal immunity.

f) Stability
TCRV is highly stable when correctly freeze-dried and will keep for long periods at either +4 or –20°C provided the product is stored under vacuum. Recent evidence indicates that 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 thermolabile. 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; this can be determined by National Control Authorities, but a universal period of 4 hours can be recommended.

g) Preservatives
TCRV contains lactalbumin hydrolysate and sucrose which are added as cryoprotectants; otherwise it contains no specific chemical preservative.

h) Precautions (hazards)
There are no known hazards associated with the manufacture or field use of TCRV.

REFERENCES


**NB:** There are OIE Reference Laboratories for Rinderpest (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.1.5.

PESTE DES PETITS RUMINANTS

SUMMARY

Peste des petits ruminants (PPR), is an acute contagious disease caused by a Morbillivirus in the family Paramyxoviridae. It affects small ruminants, especially goats, which are highly susceptible, and occasionally wild animals. PPR occurs in countries in Africa lying between the Equator and the Sahara, the Arabian Peninsula, throughout most of the Middle Eastern countries, and south-west Asia.

The clinical disease resembles rinderpest in cattle. It is usually acute and characterised by serous ocular and nasal discharges. PPR is characterised by severe pyrexia, which can last for 3–5 days, erosive lesions, which occur in the mouth, diarrhoea and pneumonia. At necropsy, characteristic zebra markings may occur in the large intestine, but are not a consistent finding. Lesions also occur in the lungs showing congestion or bronchopneumonia when associated with bacterial infection.

The disease must be differentiated from rinderpest, bluetongue, foot and mouth disease and other exanthemous conditions.

Identification of the agent: The collection of specimens at the correct time is important to achieve diagnosis by virus isolation and they should be obtained in the acute phase of the disease when clinical signs are still apparent. The specimens can be swabs of conjunctival discharges, nasal secretions, buccal and rectal mucosae, and unclotted blood.

Rapid diagnosis is done by immunocapture enzyme-linked immunosorbent assay (ELISA), counter immunoelectrophoresis and agar gel immunodiffusion. Polymerase chain reaction may also be used.

Serological tests: Serological tests routinely used include virus neutralisation and competitive ELISA. Others, such as counter immunoelectrophoresis, indirect fluorescent antibody tests and a precipitinogen inhibition test, may be employed.

Requirements for vaccines and diagnostic biologicals: Vaccination has been carried out using rinderpest tissue culture vaccine because an antigenic relationship between PPR and rinderpest viruses exists. A homologous vaccine has been developed, tested in field trials and is commercially available. The use of this PPR vaccine is strongly recommended to avoid confusion with rinderpest during serological surveys.

A. INTRODUCTION

Peste des petits ruminants (PPR) is an acute viral disease of small ruminants characterised by fever, oculonasal discharges, stomatitis, diarrhoea and pneumonia. Infected animals present clinical signs similar to those of rinderpest in cattle, from which it must be differentiated. PPR virus (PPRV) causes clinical disease in sheep and goats but, whereas it will infect cattle, it produces no clinical signs. It is transmitted by aerosols between animals living in close contact (17).

On the basis of its similarities to the viruses of rinderpest, canine distemper and measles, the PPRV has been classified within the genus Morbillivirus in the family Paramyxoviridae (14). Virus members of this group have six structural proteins: the nucleocapsid protein (Np), which encapsulates the virus genomic RNA, the phosphoprotein (P), which associates with the polymerase (L for large protein), the matrix (M) protein, the fusion (F) and the haemagglutinin (H). The matrix protein, intimately associated with the internal face of the viral envelope, makes a link between the nucleocapsid and the virus external glycoproteins: H and F, which are responsible for the attachment and the penetration of the virus into the cell to be infected. PPR was first
described in Côte d’Ivoire (13), but it occurs in most African countries south of the Sahara and north of the equator (17), and in nearly all Middle Eastern countries up to Turkey (12, 18, 23, 31). PPR is also wide-spread in India and south-west Asia (27).

The natural disease affects mainly goats and sheep, but it is usually more severe in goats where it causes heavy losses and is only occasionally severe in sheep. It is generally admitted that cattle can only be infected subclinically. However, in poor conditions it might be possible that cattle develop lesions following PPRV infection, clinical signs of which would be ascribed to rinderpest. Indeed, in the 1950s, disease and death were recorded in calves experimentally infected with PPRV-infected tissue (27). Moreover, PPRV was isolated from an outbreak of rinderpest-like disease in buffaloes in India in 1995 (15). PPRV antigen and PPRV nucleic acid were detected in some pathological samples collected during that outbreak, but no live virus was isolated. A case of clinical disease has been reported in wildlife resulting in deaths of gazelles (Gazella dorcas), ibex (Capra ibex nubiana), gemsbok (Oryx gazella) and Laristan sheep (Ovis orientalis laristanica) (12). The American white-tailed deer (Odocoileus virginianus) can be infected experimentally (16).

The incubation period is 4–6 days, but may range between 3 and 10 days. The clinical disease is acute, with a pyrexia up to 41°C that can last for 3–5 days; the animals become depressed, anorexic and develop a dry muzzle. The serous oculonasal discharges become progressively mucopurulent and, if death does not ensue, persist for around 14 days. Within 4 days of the onset of fever, the gums become hyperaemic, and erosive lesions develop in the oral cavity with excessive salivation. These lesions may become necrotic. A watery blood-stained diarrhoea is common in the later stage. Pneumonia, coughing, pleural rales and abdominal breathing also occur. The morbidity rate can be up to 100% and, in severe outbreaks, with 100% mortality. In milder outbreaks, the mortality rate may not exceed 50%. A tentative diagnosis of PPR is made on these clinical signs, but where rinderpest exists in cattle, laboratory confirmation is required.

At necropsy, the lesions are very similar to those observed in cattle affected with rinderpest. Erosive lesions may extend from the mouth to the reticulo–rumen junction. Characteristic linear haemorrhages or zebra stripes occur in the large intestine, commonly at the caeco–colic junction, but they are not a consistent or common finding; necrotic or haemorrhagic enteritis is usually present. Lymph nodes are enlarged, the spleen may show necrotic lesions, and there is an apical pneumonia.

There are no known health risks to humans working with PPRV as no report of human infection with the virus exists.

**B. DIAGNOSTIC TECHNIQUES**

1. Identification of the agent

   a) Collection of samples

   In live animals, swabs are made of the conjunctival discharges and from the nasal and buccal mucosae. During the very early stage of the disease, whole blood is also collected in anticoagulant for virus isolation, polymerase chain reaction (PCR) and haematology. At necropsy (two to three animals), lymph nodes, especially the mesenteric and bronchial nodes, lungs, spleen and intestinal mucosae should also be collected aseptically, chilled on ice and transported under refrigeration. Fragments of organs collected for histopathology are placed in 10% formalin. At the end of the outbreak, blood can be collected for serological diagnosis.

   b) Agar gel immunodiffusion

   Agar gel immunodiffusion (AGID) is a very simple and inexpensive test that can be performed in any laboratory and even in the field. Standard PPR viral antigen is prepared from mesenteric or bronchial lymph nodes, spleen or lung material and ground up as 1/3 suspensions in buffered saline. These are centrifuged at 500 \( g \) for 10–20 minutes, and the supernatant fluids are stored in aliquots at −20°C. The cotton material from the cotton bud used to collect eye or nasal swabs is removed using a scalpel and inserted into a 1 ml syringe. With 0.2 ml of phosphate buffered saline (PBS), the sample is extracted by repeatedly expelling and filling the 0.2 ml of PBS into an Eppendorf tube using the syringe plunger. The resulting eye/nasal swab extracted sample, like the tissue ground material prepared above, may be stored at −20°C until used. They may be retained for 1–3 years. Negative control antigen is prepared similarly from normal tissues. Standard antiserum is made by hyperimmunising sheep with 1 ml of PPRV with a titre of 10^6 TCID\(_{50}\) (50% tissue culture infective dose) per ml given at weekly intervals for 4 weeks. The animals are bled 5–7 days after the last injection (7). Standard rinderpest hyperimmune antiserum is also effective in detecting PPR antigen.
i) Dispense 1% agar in normal saline, containing thiomersal (0.4 g/litre) or sodium azide (1.25 g/litre) as a bacteriostatic agent, into Petri dishes (6 ml/5 cm dish).

ii) Wells are punched in the agar following a hexagonal pattern with a central well. The wells are 5 mm in diameter and 5 mm apart.

iii) The central well is filled with positive antiserum, three peripheral wells with positive antigen, and one well with negative antigen. The two remaining peripheral wells are filled with test antigen, such that the test and negative control antigens alternate with the positive control antigens.

iv) Usually, 1–3 precipitin lines will develop between the serum and antigens within 18–24 hours (9, 29). These are intensified by washing the agar with 5% glacial acetic acid for 5 minutes (this procedure should be carried out with all apparently negative tests before recording a negative result). Positive reactions show lines of identity with the positive control antigen.

Results are obtained in 1 day, but the test is not sensitive enough to detect mild forms of PPR due to the low quantity of viral antigen that is excreted.

c) Immunocapture enzyme-linked immunosorbent assay

The immunocapture enzyme-linked immunosorbent assay (ELISA) (19), using several anti-N monoclonal antibodies (MAb), allows a rapid differential identification of PPR or rinderpest viruses, and this is of great importance as the two diseases had until recently a similar geographical distribution and may affect the same animal species.

i) Microtitre ELISA plates (e.g. high adsorption capacity Nunc Maxisorb) are coated with 100 µl of a capture MAb solution (diluted according to the instructions of the Reference Laboratory providing the kit). This MAb reacts with both rinderpest virus and PPRV.

ii) After washing, 50 µl of the sample suspension is added to four wells, and control wells are filled with buffer.

iii) Immediately, 25 µl of a detection biotinylated MAb for PPR and 25 µl of streptavidin/peroxidase are added to two wells, and 25 µl of a detection MAb for rinderpest and 25 µl of streptavidin/peroxidase are added to the two other wells.

iv) The plates are incubated at 37°C for 1 hour with constant agitation.

v) After three vigorous washes, 100 µl of ortho-phenylenediamine (OPD) in hydrogen peroxide is added, and the plates are incubated for 10 more minutes at room temperature.

vi) The reaction is stopped by the addition of 100 µl of 1 N sulphuric acid, and the absorbance is measured at 492 nm on a spectrophotometer/ELISA reader.

The cut-off above which samples are considered to be positive is calculated from each blank (PPR blank and rinderpest blank) as three times the mean absorbance values.

A sandwich ELISA can also be performed: the sample is first allowed to react with the detection MAb and the immunocomplex is then captured by the second MAb adsorbed on to the ELISA plate.

The test is very specific and sensitive (it can detect $10^{0.6}$ TCID$_{50}$/well for the PPRV and $10^{2.2}$ TCID$_{50}$ for the rinderpest virus). The results are obtained in 2 hours.

d) Nucleic acid recognition methods

cDNA $^{32}$P labelled clones have been used to differentiate PPR and rinderpest (5), but their use in routine diagnosis is not recommended due to the short half-life of the $^{32}$P and the need for special equipment to protect the users.

A PCR technique based on the amplification of the Np and F protein genes has been developed for the specific diagnosis of PPR (4, 11). This technique is very sensitive compared with other tests and results are obtained in 5 hours, including the RNA extraction. The OIE and FAO$^1$ Reference Laboratory for PPR in France (see Table given in Part 3 of this Terrestrial Manual) can advise on the use of this technique.

e) Counter immuno-electrophoresis

Counter immuno-electrophoresis (CIEP) is the most rapid test for viral antigen detection (9, 21). It is carried out on a horizontal surface using a suitable electrophoresis bath, which consists of two compartments

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1 Food and Agriculture Organization of the United Nations.
connected through a bridge. The apparatus is connected to a high-voltage source. Agar or agarose (1–2%, [w/v]) dissolved in 0.025 M barbitone acetate buffer is dispensed on to microscope slides in 3-ml volumes. From six to nine pairs of wells are punched in the solidified agar. The reagents are the same as those used for the AGID test. The electrophoresis bath is filled with 0.1 M barbitone acetate buffer. The pairs of wells in the agar are filled with the reactants: sera in the anodal wells and antigen in the cathodal wells. The slide is placed on the connecting bridge and the ends are connected to the buffer in the troughs by wetted porous paper. The apparatus is covered, and a current of 10–12 milliamperes per slide is applied for 30–60 minutes. The current is switched off and the slides are viewed by intense light: the presence of 1–3 precipitation lines between pairs of wells is a positive reaction. There should be no reactions between wells containing the negative controls.

f) Culture and isolation methods

Even when diagnosis has been carried out by rapid techniques, the virus should always be isolated from field samples in tissue cultures for further studies (9, 17).

PPRV may be isolated in primary lamb kidney or in African green monkey kidney (Vero) cell tissue cultures. Monolayer cultures are inoculated with suspect material (swab material, buffy coat or 10% tissue suspensions) and examined daily for evidence of cytopathic effect (CPE). The CPE produced by PPRV can develop within 5 days and consists of cell rounding and aggregation culminating in syncytia formation in lamb kidney cells. In Vero cells, it is sometimes difficult to see the syncytia. If they exist, they are very small. However, in stained, infected Vero cells, small syncytia are always seen. Syncytia are recognised by a circular arrangement of nuclei giving a ‘clock face’ appearance. Cover-slip cultures may give a CPE earlier than day 5. There are also intracytoplasmic and intranuclear inclusions. Some cells are vacuolated. Similar cellular changes may be seen in stained histopathological sections of infected tissues. After 5–6 days, blind passages should always be carried out as CPE may take time to appear.

g) Other virus detection techniques

Other virus detection techniques have potential benefits, but they are not yet widely used. While virus isolation needs pathological samples to be kept in cold conditions until the start of their processing, it is possible to keep them at ambient temperature in a formalin-fixed solution and later analyse them directly by immunofluorescence (IF) or immunohistochemical test (2, 3, 28). IF has been used successfully on conjunctival smears and tissues collected at necropsy; the smears are fixed in cold acetone. It has now been demonstrated that unlike the rinderpest virus but like the measles virus, PPRV has haemagglutination capability. This characteristic has been used for specific, rapid and inexpensive diagnosis of PPR infection (15, 33).

2. Serological tests

Goats and sheep infected with PPRV develop antibodies that may be demonstrated to support a diagnosis by the antigen-detection tests. Tests that are routinely used are the virus neutralisation (VN) test and the competitive ELISA. Other tests, such as CIEP (10), AGID (31), precipitinogen inhibition and indirect fluorescent antibody test (8), have been described, but remain of little interest compared with the VN and ELISA.

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

This test is sensitive and specific, but it is time-consuming. The standard neutralisation test is carried out in roller-tube cultures of primary lamb kidney cells, or Vero cells when primary cells are not available.

i) Dilute 1 ml of inactivated serum in a twofold dilution series and mix with a stock virus suspension containing approximately $10^5$ TCID$_{50}$/ml.

ii) Incubate the virus/serum mixtures either for 1 hour at 37°C or overnight at 4°C.

iii) Inoculate 0.2 ml of the mixture into each of five roller tubes, followed immediately by 1 ml of Vero cell suspension in growth medium at a rate of $2 \times 10^5$ cells/ml.

iv) Incubate the sloped tubes for 3 days at 37°C.

v) Discard the tubes showing virus-specific CPE; replace the medium in the remaining tubes with maintenance medium, and roll the tubes for a further 7 days. The virus-challenge dose is acceptable if it falls between $10^{1.8}$ and $10^{2.8}$ TCID$_{50}$/tube. Any detectable antibody at a dilution of 1/8 is considered to be positive.

Usually, a cross-neutralisation test is carried out with rinderpest virus and a serum is considered to be positive for PPR when the neutralisation titre is at least twofold higher for PPR than for rinderpest.
b) **Competitive enzyme-linked immunosorbent assay**

Competitive ELISA based on the use of monoclonal anti-nucleoprotein and a recombinant nucleoprotein produced in the baculovirus has been described (20).

i) Coat microtitre plates (e.g. high adsorption capacity Nunc Maxisorb) with 50 µl of a predetermined dilution of N-PPR protein (produced by a recombinant baculovirus) for 1 hour at 37°C with constant agitation.

ii) Wash the plates three times and blot dry.

iii) Distribute 45 µl of blocking buffer (PBS + 0.5% Tween 20 + 0.5% fetal calf serum) to all wells, and then add 5 µl of test sera to test wells (at a final dilution of 1/20) and 5 µl of the different control sera (strong positive, weak positive and negative serum) to control wells.

iv) Add 50 µl of MAb diluted 1/100 in blocking buffer, and incubate at 37°C for 1 hour.

v) Wash the plates three times and blot dry.

vi) Add 50 µl of anti-mouse conjugate diluted 1/1000, and incubate at 37°C for 1 hour.

vii) Wash the plates three times.

viii) Prepare OPD in hydrogen peroxide solution. Add 50 µl of substrate/conjugate mixture to each well. Stop the reaction after 10 minutes with 50 µl of 1 M sulphuric acid.

ix) Read on an ELISA reader at 492 nm.

   The absorbance is converted to percentage inhibition (PI) using the formula:
   
   \[ \text{PI} = 100 - \frac{(\text{absorbance of the test wells/absorbance of the MAb control wells})}{100} \times 100 \]

Sera showing PI greater than 50% are positive.

Two other competitive ELISA techniques, based on the use of monoclonal anti-haemagglutinin (H), have also been described (1, 26).

C. **REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS**

Sheep and goats that recover from PPR develop an active immunity against the disease. Antibodies have been demonstrated 4 years after infection (8), but immunity is probably life long. A homologous PPR vaccine is available. In 1998, the OIE International Committee endorsed the use of this vaccine in countries that have decided to follow the ‘OIE pathway’ for epidemiological surveillance for rinderpest in order to avoid confusion when serological surveys are performed.

1. **Seed management**

   a) **Characteristics of the seed**

   Homologous vaccine against PPR is a live vaccine cultured in Vero cells. The original strain of virus for the homologous vaccine against PPR is strain PPR 75/1, isolated in Nigeria in 1975 (30). This is attenuated by passage in Vero cell cultures (6). The strain provided for vaccine production is the 70th passage in Vero cells (PPRV 75/1 I.K6 BK2 Vero 70). It is stored in freeze-dried form at –20°C and may be obtained from Reference Laboratories (see Table given in Part 3 of this Terrestrial Manual). Tests of vaccine activity show that it retained the ability to protect (at a dose of 10^3 TCID<sub>50</sub>) up to the 120th passage in Vero cells, the latest passage tested so far.

   b) **Method of culture**

   • **Cells**

   PPR vaccine is produced in Vero cells, which must be free from all bacterial, fungal and viral contamination.

   • **Culture medium**

   The culture medium consists of minimal essential medium (MEM) supplemented with antibiotics (for example penicillin + streptomycin at final concentrations of 100 IU [International Units]/ml and 100 µg/ml, respectively), and an antifungal agent (nystatin [Mycostatin] at a final concentration of 50 µg/ml). The medium is enriched with 10% fetal calf serum (complete medium) for cell growth. This proportion of serum is reduced to 2% for maintenance medium when the cell monolayer is complete.
• **Primary seed batch of vaccine virus**

This consists of virus in its 70th passage in Vero cells (PPRV 75/1 LK6 BK2 Vero 70). The freeze-dried contents of a flask from the seed bank are reconstituted with 2 ml of sterile water (or cell culture medium without serum). This liquid is mixed with Vero cells suspended in complete culture medium to provide at least 0.001 TCID<sub>50</sub> per cell. Cell culture dishes are filled with this virus/cell mixture (around 2 × 10<sup>7</sup> Vero cells in a 175 cm<sup>2</sup> dish), and are incubated at 37°C. The cultured cells are examined regularly to detect a CPE. The medium is renewed every 2 days, reducing the proportion of serum to 2% once the cell monolayer is complete. Virus is first harvested when there is 40–50% CPE. This viral suspension is stored at −70°C. Successive harvesting is made every 2 days until the CPE reaches 70–80%, which is the time for final freezing of the culture dishes (in general, at least two further harvestings can be made before final freezing of the culture dishes). All suspensions of virus collected are submitted to two freeze–thaw cycles, then added to form a single batch, which serves as the primary seed batch. This is divided into small volumes in bottles and stored at −70°C. All suspensions of virus collected are submitted to two freeze–thaw cycles, then added to form a single batch, which serves as the primary seed batch. This is divided into small volumes in bottles and stored at −70°C. Five samples are thawed and titrated (minimum titre required: 10<sup>5</sup> TCID<sub>50</sub>/ml). It is best to freeze-dry this seed in order to store it at −20°C. In this case it will be necessary to titrate the freeze-dried virus (five bottles). A batch made up in this way must pass all tests for sterility.

When preparing seed batches, it is important to avoid infecting the cells with too strong a dose of virus (high multiplicity of infection), as this will lead to accumulation of defective particles in the viral suspension produced, which will diminish the titre of subsequent products. On the other hand, very weak multiplicity of infection (e.g. 0.0001) will prolong the culture time.

• **Preparation of the working seed batch**

This is done under the same conditions as for the primary seed batch. A large stock of virus is formed, from which the final vaccine will be produced. This batch is distributed into receptacles and stored at −70°C. It must satisfy tests for sterility. Five samples are titrated (minimum titre required: 10<sup>6</sup> TCID<sub>50</sub>/ml).

c) **Validation as a vaccine**

It is necessary to confirm or rule out the presence of PPRV in the product under test. For this purpose, anti-PPR serum is used to neutralise the virus in cell culture.

• **Test procedure**

  i) Mix the contents of two vaccine bottles with sterile double-distilled water to provide a volume equal to the volume before freeze-drying.

  ii) Make tenfold dilutions of the reconstituted vaccine in serum-free culture medium (0.5 ml of viral suspension + 4.5 ml of medium).

  iii) Make two series of mixtures for virus dilutions from each bottle on a 96-well plate as follows:

      | Series 1 | Dilutions of viral suspension | −1 | −2 | −3 | −4 |
      |-----------|-------------------------------|-----|-----|-----|-----|
      | Viral suspension (in µl)   | 50                           | 50  | 50  | 50  | 50  |
      | Culture medium (in µl)     | 50                           | 50  | 50  | 50  | 50  |

      | Series 2 | Dilutions of viral suspension | −1 | −2 | −3 | −4 |
      |-----------|-------------------------------|-----|-----|-----|-----|
      | Viral suspension (in µl)   | 50                           | 50  | 50  | 50  | 50  |
      | PPR antiserum (in µl)      | 50                           | 50  | 50  | 50  | 50  |

      (Note: PPR antiserum used for this purpose is prepared in goats and freeze-dried. It is reconstituted with 1 ml of sterile double-distilled water in a dilution of 1/10.)

  iv) Incubate the mixtures at 37°C for 1 hour

  v) Add to each well 100 µl of cells suspended in complete culture medium (30,000 cells/well).

  vi) Incubate the microplate at 37°C in the presence of CO<sub>2</sub>.

  vii) Read the plate after 10–15 hours of incubation.

Normally a CPE is present only in the wells containing cells infected with the mixture of virus and culture medium. If it is detected in the wells of Series 2, it will be necessary to identify PPRV by immunofluorescence, using a PPR MAb, or by immunocapture (specific PPR MAb, and the immunocapture test kit are available from the OIE Reference Laboratory for PPR in France [see Table given in Part 3 of this Terrestrial Manual]). If this identification confirms the presence of PPRV, the PPR antiserum used must have been too weak, or the batch must be changed. If immunofluorescence or immunocapture is negative, a viral contaminant must be present, and the material under test must be destroyed.
2. **Method of manufacture**

   a) **Vaccine production**

   This operation is performed on a larger scale. Cells can be infected with virus at a multiplicity of infection as before or with high doses, e.g. up to 0.01. Products of the various harvests, after two freeze–thaw cycles, are brought together (to form the final product) and stored at –70°C pending the results of titration and tests for sterility. If these results are satisfactory, the vaccine is freeze-dried.

   b) **Freeze-drying**

   The freeze-drying medium (Weybridge medium) is composed of 2.5% (w/v) lactalbumin, 5% (w/v) sucrose and 1% (w/v) sodium glutamate, pH 7.2.

   This medium is added to an equal volume of viral suspension for freeze-drying (which may have been diluted beforehand to provide the desired number of vaccine doses per bottle). The resulting mixture is kept cool, homogenised, then distributed into bottles and freeze-dried. At the end of a freeze-drying cycle, the probe is adjusted and kept at 35°C for 4 hours. Once this operation has been completed, the bottles are capped under vacuum. Randomly selected samples (e.g. 5%) of this final batch are submitted to tests for innocuousness, efficacy and sterility, and residual moisture is estimated by the Karl Fisher method (optimum 3.5%). If the tests give unsatisfactory results, the entire batch is destroyed.

3. **In-process control**

   Cells used in cultures must be checked for normal appearance and shown to be free from contaminating viruses, especially bovine viral diarrhoea virus. A virus titration must be undertaken on the seed lot: using MEM (serum-free) medium, a series of tenfold dilutions is made (0.5 ml virus + 4.5 ml diluent) down to 10^-6 of the product to be titrated. Vero cells from one flask are trypsinised and suspended in complete culture medium at 300,000/ml. They are distributed on a 96-well plate (30,000 cells per well, equivalent to 100 µl of cell suspension). Then, 100 µl of virus diluted tenfold is added to the cells (dilutions ranging from 10^-2 to 10^-6). One row of wells serves as a control for uninfected cells to which virus-free culture medium (100 µl) is added. The plate is incubated at 37°C in the presence of CO2. The plates are read (by examining for CPE) 10–15 days after infection.

   Virus titre is determined by the Spearman–Kärber method. The minimum titre per dose is 10^2.5.

4. **Batch control**

   a) **Identity**

   The contents of one container from each filling lot must be checked for identity by culture after neutralisation with specific antiserum.

   b) **Sterility**

   This consists of testing for viral, bacterial or fungal contaminants. It is done on cells and sera before their use in vaccine production, and on the seed stock and the vaccine before and after freeze-drying. Any product that fails this test for sterility is destroyed.

   Tests for sterility and freedom from contamination of biological materials may be found in Chapter I.1.5.

   c) **Safety**

   This test is done in rodents in order to detect any nonspecific toxicity associated with the product. The test requires reconstituted vaccine in solvent (mixed contents of five bottles), six guinea-pigs, each weighing 200–250 g; ten unweaned mice (17–22 g, Swiss line or similar).

   Vaccine, 0.5 ml, is injected intramuscularly into a hind limb of two guinea-pigs, 0.5 ml into the peritoneal cavity of two guinea-pigs, and 0.1 ml into the peritoneal cavity of six mice. Two guinea-pigs and four mice are kept as uninoculated controls. The animals are observed for 3 weeks. If one guinea-pig or two mice die, the test must be repeated. Dead animals undergo post-mortem examination to ascertain the cause of death. At the end of 3 weeks of observation, all animals are killed for post-mortem examination. All the results are recorded. The vaccine is considered to be satisfactory if, during the first or second test, at least 80% of animals remain in good health during the period of observation, and no significant post-mortem lesion is found.
d) **Potency and efficacy in small ruminants**

This test requires the following: vaccine reconstituted with normal saline (the mixed contents of five bottles) to provide 100 doses and 0.1 dose/ml; six goats and six sheep, all approximately 1-year old and free from antibodies to rinderpest or PPR; sterile syringes and needles; and pathogenic PPRV previously titrated in goats, diluted with sterile normal saline to provide $10^3$ of the 50% lethal dose for goats (LD$_{50}$).

Vaccinate two goats and two sheep subcutaneously with 100 doses per animal; vaccinate two goats and two sheep subcutaneously with 0.1 dose per animal; keep the remaining animals as in-contact controls. The animals are observed and temperature measurements are recorded daily for 3 weeks. At the end of this period, blood is taken from all animals for the preparation of sera. All animals are challenged by subcutaneous injection of a 1 ml suspension of pathogenic PPRV ($10^3$ LD$_{50}$ per animal). The animals are observed and their body temperature measurements are recorded daily for 2 weeks.

The vaccine is considered to be satisfactory if all vaccinated animals resist the challenge infection, while at least half of the in-contact controls develop signs of PPR. The serum neutralisation test must be positive for PPR antibody (in serum diluted at least 1/10) in vaccinated animals only in samples taken 3 weeks after vaccination. If any of the controls are also positive, the experiment must be repeated using another batch of pathogenic PPRV. The batch of vaccine is destroyed if vaccinated animals react to the virulent challenge.

- **Titration of neutralising PPR antibody**

This test requires the following: cell suspensions at 600,000/ml; 96-well cell culture plates; sera to be titrated (inactivated by heating to 56°C for 30 minutes); complete cell culture medium; PPRV diluted to give 1000, 100, 10 and 1 TCID$_{50}$/ml.

Dilute the sera at 1/5, then make a twofold dilution in cell culture medium. Mix 100 µl of virus at 1000 TCID$_{50}$/ml (to give 100 TCID$_{50}$ in each well) and 100 µl of a given dilution of serum (using six wells per dilution) in the wells of the cell culture plate. Arrange a series of control wells for virus and uninfected cells as follows: six wells with 100 TCID$_{50}$ (100 µl) per well; six wells with 10 TCID$_{50}$ (100 µl) per well; six wells with 1 TCID$_{50}$ (100 µl) per well; six wells with 0.1 TCID$_{50}$ (100 µl) per well; and six wells with 200 µl of virus-free culture (control cells) per well.

Make the wells containing the virus controls up to 100 µl with complete culture medium, and incubate the plates for 1 hour at 37°C. Add 50 µl of cell suspension to each well. Incubate the plates at 37°C in the presence of CO$_2$. Read the plates after 1 and 2 weeks of incubation. The results should be as follows: 100% CPE in virus control wells of 100 and 10 TCID$_{50}$, 50% CPE for the 1 TCID$_{50}$ dilution, no CPE for the 0.1 TCID$_{50}$ dilution, no CPE in wells where the virus had been neutralised by serum during the test, and CPE in wells where the virus had not been neutralised by serum during the test.

e) **Duration of immunity**

Duration of immunity is at least 3 years.

f) **Stability**

Freeze-dried vaccine can be kept for at least 2 years at 2–8°C (although storage at −20°C is better), provided it is stored under vacuum and protected from light. Recently, it has been demonstrated that this vaccine, suspended in medium containing trehalose and submitted to the ultra rapid method of dehydration, can resist at 45°C for a period of 14 days with minimal loss of potency (32).

5. **Tests on the final product**

a) **Safety**

See Section C.4.c.

b) **Potency**

See Section C.4.d.

REFERENCES


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NB: There are OIE Reference Laboratories for Peste des petits ruminants (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
**CHAPTER 2.1.6.**

**CONTAGIOUS BOVINE PLEUROPNEUMONIA**

**SUMMARY**

Contagious bovine pleuropneumonia (CBPP) is a disease of cattle caused by Mycoplasma mycoides subsp. mycoides SC (bovine biotype) (MmmSC; SC = small colonies). It is manifested by anorexia, fever and respiratory signs such as dyspnoea, polypnoea, cough and nasal discharges. Diagnosis depends on the isolation of the aetiiological agent. The main problems for control or eradication are the frequent occurrence of subacute or asymptomatic 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 with 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 3–10 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, breakdown of glucose, absence of arginine hydrolysis, and the absence of phospha- tase 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 the prescribed test for international trade. However, it has significant limitations regarding sensitivity and specificity. The competitive enzyme-linked immunosorbent assay was designated as an alternative test by the OIE International Committee in May 2000 and is being evaluated to determine if it should be made an OIE prescribed test for international trade. Please consult the OIE Web site for the most recent version of this chapter. An immunoblotting test has undergone evaluation and is reported to be highly specific and sensitive.

**Requirements for vaccines:** One attenuated strain is now recommended for vaccine production: the T1/44. The minimal recommended titre is $10^7$ viable mycoplasmas per vaccinal dose.

**A. INTRODUCTION**

Contagious bovine pleuropneumonia (CBPP) is a contagious disease of cattle caused by Mycoplasma mycoides subsp. mycoides SC (bovine biotype) (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 and in Southern Europe; 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. MmmSC (bovine biotype) has been isolated from buffaloes in Italy (*Bubalus bubalus*) (25), and from sheep and goats in Africa and more recently in Portugal. 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 in
experimental conditions, the mortality rate may be as high as 50% in the absence of antibiotic treatment. This mortality rate may be much lower in the field; however, sometimes when an outbreak first occurs in an area, the mortality will be higher. Clinical signs are not always evident. Subacute or asymptomatic forms occur frequently as affected animals recover partially. In that 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 naïve 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, persistence of chronic carriers and impact on human health, have not been evaluated yet. The M. mycoides cluster consists of six mycoplasma species or groups of strains, originating from bovines and goats (9, 21, 28). 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 (9) with standard techniques. Specific identification of MmmSC can now be achieved by polymerase chain reaction (PCR) or the use of specific monoclonal antibodies (MAbs). 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 (17). Quite interestingly, the strains of European origin can be clearly differentiated from African ones (8, 32). Recent European strains are characterised by the deletion of a long DNA fragment and are not able to oxidise glycerol, which may account for an apparent lower pathogenicity (33). However, the European strains isolated in 1967 did not exhibit such a deletion. African strains seem to be more diverse. There is no doubt that further technical development will allow for a finer characterisation of strains.

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 (14). 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. Samples should be frozen if they cannot be processed on the day that they are collected.

a) Culture

MmmSC needs appropriate media to grow (24). In attempting isolation, 2–3 blind passages may be required. 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.
Chapter 2.1.6. – Contagious bovine pleuropneumonia

After grinding in broth with 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 every day for 10 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 MmmSC (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 can be performed.

b) Biochemical tests

Biochemical tests are to be regarded as confirmatory tests, to be carried out by reference laboratories. For routine field use, the immunological tests are sufficient, but where these give dubious results and in all cases of identification of first isolates, biochemical tests should be confirmed by a reference laboratory. 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 antibiotics. Once this test is done and after cloning (at least three colonies should be selected), the organism can be identified using biochemical tests (2, 12).

MmmSC 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). 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 must 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

Radiolabelled or enzyme probes have been developed, but their use is restricted, mainly for safety reasons.

On the other hand, the PCR is sensitive, highly specific, rapid, and not too difficult to perform. Primers specific for the M. mycoides cluster (26) and for MmmSC (10, 20, 31) have been reported and PCR assays have been developed (5, 10, 20), including a new technique that permits the specific identification of the T1 vaccinal strains (18). Using samples such as lung exudate, the PCR can be applied directly after denaturation in boiling water, without any DNA extraction. However, boiling of samples is less sensitive than DNA extraction and should not be used as a routine technique. It can even be applied to dry samples on filter paper. Lung fragments have first to be ground and submitted to a low speed centrifugation before denaturation and PCR. 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 characterisation of and differentiation between members of the M. mycoides cluster and MmmSC.

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.

i) Indirect fluorescent antibody test

The IFA test can be performed on smears from clinical material using hyperimmune rabbit serum against MmmSC 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 (12). 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 (13). 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 (0.2 g/litre) in PBS, pH 7.2 (1000 ml). The test is considered to lack sensitivity and little is known about its specificity, but it has served as a screening test and only positive reactions should be taken into account. The results are better when the plate is incubated at 37°C and can be read within 24 hours. A simpler field test has been developed using impregnated paper discs instead of wells (23).

v) Dot immunobinding on membrane filtration

The MF-dot test can be used for routine testing in the laboratory. This technique may be performed with polyclonal antisera adopting a quantitative procedure (22), however, cross-reactions within the mycoides cluster can occur. Specific MAbs have been developed that may overcome this problem (7).

vi) Immunohistochemistry

*Mmm*SC immunoreactive sites can be detected in lung lesions using the peroxidase–antiperoxidase method on sections of paraffin-embedded material (11). Because the isolation of the agent is not always achieved from chronic cases and after treatment with antimicrobial drugs, this test is only supplementary to the diagnosis of CBPP (6); 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 the 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 (24). The CF test, as a micromethod, has been harmonised in the European Union (19). For antigen titration and harmonisation purposes, international standard positive sera were prepared (840 and 845, PS\textsubscript{1} and PS\textsubscript{2}, respectively) and are available from the Laboratório Nacional de Investigação Veterinária, Lisboa, Portugal (19). 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 \textit{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 (HD\textsubscript{50} [50% haemolysing 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 HD\textsubscript{50}. 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 two positive standard sera, PS\textsubscript{1} and PS\textsubscript{2}, are bovine sera from naturally infected animals with a low and a high titre, respectively, for CF test harmonisation and control, and antigen titration (PS\textsubscript{2}). They are negative to \textit{Brucella}, \textit{Mycobacterium paratuberculosis}, \textit{Chlamydia}, \textit{Coxiella burnetii}, \textit{Leptospira}, infectious bovine rhinotracheitis/infectious pustular vulvovaginitis, \textit{Mycoplasma bovis} and bovine leukosis virus antibodies.
  
  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.

  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% haemolysis at 1/10;
  - Doubtful results: 25, 50 or 75% 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 99.5%, 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 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 3 of this *Territorial Manual* (16), has undergone evaluation (3). An indirect ELISA based on lipoprotein is currently being validated by the IAEA (1). In May 2000, The C-ELISA was designated an ‘alternative test’ by the OIE International Committee. At the time of publication, the C-ELISA had been proposed as a prescribed test for international trade. Please consult the OIE Web site for the most recent version of this chapter. Compared with the CF test, the C-ELISA has equal sensitivity and greater specificity. The C-ELISA is a herd test that is easier to perform than the CF test, but its performance characteristics have not yet been fully assessed (16). The reagents for the C-ELISA can be obtained as kits.

Validation tests (3, 16) 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.

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. 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. In an afflicted herd, many animals will exhibit positive results.

- **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 3 of this *Territorial Manual*).
  iii) The conjugate is DAKO P260 diluted 1/1500 in PBS with 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 H2O2 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.

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1 International Atomic Energy Agency, Wagramerstrasse 5, P.O. Box 100, A-1400 Vienna, Austria or CIRAD/EMVT, Santé Animale, Campus International de Baillarguet, TA30/G, 34398 Montpellier cedex 5, France.
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 considered to be 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, was considered to be 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. It is of particular value where disease control or eradication policies are being implemented.

- 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.

d) Other tests

A rapid field slide agglutination test (SAT) with either whole blood or serum (30) 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 (4).

For CBPP, the CF test and ELISAs can be used in screening and eradication programmes, but the highly specific IB test should 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.

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 \textit{Mmm}SC 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 (34, 35). The 44th egg-passage is sufficiently attenuated to protect cattle without post-vaccinal severe reactions. Post-vaccination reactions may occur at the inoculation site. 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; however, as the vaccine is to be injected subcutaneously, this should not create a serious disease problem (15).

   The identity of the strain can be verified with the insertion sequence profile or by the specific PCR assay (18).

   The master seed strain is kept in freeze-dried form at –20°C.

   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 either Gourlay’s medium or \textit{F}_{60} medium: they both contain the same basic ingredients, but \textit{F}_{60} medium is more complex (24). The media are sterilised by filtration, have a pH of 7.8–8.0, and should be used within a few days of preparation. Samples of medium should be distributed in tubes and incubated at 37°C for 48 hours to check sterility before use.

   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 100 ml of medium.

2. Method of manufacture
   The media used for vaccine production, either Gourlay’s medium or \textit{F}_{60} medium, are described above in Section C.1.b. They should be distributed in 1 litre flasks (500 ml per flask) and 10 litre flasks (5 litres per flask), and incubated at 37°C for 48 hours to check sterility before use.

   To obtain high bacterial counts, the culture should be harvested just before the end of the logarithmic growth phase before the peak is reached, as the number of viable mycoplasmas will decrease quickly afterwards. Therefore, a kinetic curve should be drawn in each production laboratory. The contents of a vial of the working seed is used in a tenfold dilution series, in tubes. If no contamination occurs after incubation, the second tube is used, when the culture is still in the exponential rate of growth, to inoculate 1 litre flasks. The 10 litre flasks are heavily inoculated with a 48-hour-old culture of the 1 litre flasks (10 ml/litre of medium) and incubated at 37°C. After 24 hours of incubation, slow magnetic stirring is recommended. Just before the peak is reached (between 60 and 70 hours after incubation), the culture is prepared for freeze-drying.

   All the following steps should be carried out on melting ice:
   - Addition of the freeze-drying protective medium: dried skimmed milk (4.5% i.e. 225 g per 5 litres).
   - Distribution into vials (the volume depends on the number of doses required per vial and on the steps of lyophilisation).
For freeze-drying, shelf freeze-driers are recommended so that the different steps can be controlled and monitored. The vials must be sealed in the freeze-drying machine in order to avoid leakage.

3. In-process control

Microscopic examination of Gram-stained preparations of the broth or blood agar plate cultures of the broth must be performed at every step to check for the absence of bacterial contamination.

A rapid examination should also be carried out to check for the presence of \textit{Mmm}SC by phase-contrast microscopic examination of the harvested medium, accompanied by FAT examination with a labelled hyper-immune antiserum.

Samples of the freeze-dried vaccine are inoculated on to mycoplasma agar or into broth to carry out growth-inhibition tests (11).

4. Batch control

a) Sterility

Bacteriological broths are inoculated, together with blood agar, and incubated at 37°C. All media should remain sterile (24). Tests for sterility may be found in Chapter I.1.5.

b) Titration

The minimum titre is \(10^7\) mycoplasmas per vaccinal dose, but higher titres, at least \(10^9\), are recommended. Titration is performed after reconstitution of the freeze-dried vaccine in the diluent recommended for vaccination. Titrations should be performed on at least five vials per batch.

\textit{Classic technique}: Tenfold dilutions are made in Gourlay's medium. The use of an agitator (vortex type) is useful to homogenise the suspension in each tube. First, 5 ml from the tubes with dilutions from \(10^{-6}\) to \(10^{-12}\) are inoculated in five further tubes of medium (1 ml per tube, five tubes per dilution). All the tubes are then incubated for 4 days at 37°C and growth is detected by colour change of the indicator. The titre is obtained using MacCrady's 'Most Probable Number' table or by modification of the Reed & Muench method. This technique is simple and easy to perform, but lacks precision (±0.5 log).

A microtitration method that is more accurate has been described (27) and should be used when possible.

Alternative titration procedures include the use of preliminary tenfold dilutions as in the classical technique but the seeding of twenty 100-µl aliquots, for each dilution, is made in a microplate already containing 'glucose medium' with a pH indicator. Four dilutions can be tested in a microplate, leaving two columns for medium control. The titre is calculated by a modification of the Reed and Muench method that consists of subtracting 0.25 log to the final titre in order to get a 'number of viable organisms' and not a 50% infective dose. The final precision should be in the order of 0.2 to 0.25 log.

Comparative titrations can be obtained by seeding 20-µl aliquots of each of the primary dilutions on convenient agar medium. The titres are then obtained in CFUs and should not differ significantly from titres obtained in liquid medium. Titration on solid medium requires more skill than titration in liquid medium.

c) Safety

After reconstitution in cold buffer, 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

At least ten cattle are injected subcutaneously at the recommended site with one dose of vaccine, as stated on the label. At least ten similar animals are kept as unvaccinated controls. After no fewer than 2 months, all the cattle are challenged by placing them in contact with experimentally infected donor cattle, in an enclosed space, using at least one donor for every three cattle being challenged. The cattle are kept in contact for 3 months. After this time, all animals are killed and post-mortem examinations are carried out. The vaccinated cattle should show no more than mild clinical signs of disease, and no post-mortem lesions of pleuropneumonia. At least 80% of the control animals must show typical lesions at post-mortem. This potency test need only be carried out once; subsequent batches do not need to be retested provided they
are produced according to standard protocols (34, 35). Any modification to the standard protocol, such as increasing the number of in-vitro passages, should be strictly prohibited. Other modifications, such as production in fermentors with continuous adjustment of pH or addition of reagents, should always be followed by a potency test in order to ensure that the protection induced has not been altered.

e) **Duration of immunity**

Strain T1/44 confers protection for approximately 1 year (14), 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 (24), viability may even be conserved for many years without loss of titre allowing for the constitution of emergency stocks. The titres of these stock naturally need to be controlled regularly.

g) **Preservatives**

For lyophilisation, dried skimmed milk is added: 45 g/litre of culture medium. For reconstitution of a freeze-dried vaccine normal saline is 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 (24). The purity of the salts used is important.

h) **Precautions (hazards)**

Procedures for use in the field and reconstitution of freeze-dried vaccines have been described by Provost et al. (24).

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 (29).

The general sensitivity of a given bovine population should be first tested by vaccinating sample groups (24).

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**


* *

NB: There are OIE Reference Laboratories for Contagious bovine pleuropneumonia (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.1.7.
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 because it causes reduced production, particularly in dairy herds. It also causes damage to the hide. LSD is caused by strains of capripoxvirus that are antigenically indistinguishable from strains causing sheep pox and goat pox. However, LSD has a different geographical distribution to 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. Until 1988 LSD was confined to sub-Saharan Africa, but then spread into Egypt. There has been only one laboratory-confirmed outbreak of LSD outside Africa, in Israel in 1989, which was eliminated by slaughter of all infected and in-contact cattle, and vaccination. Outbreaks reported in Bahrain and Reunion in 1993 were not confirmed by virus isolation.

Identification of the agent: Laboratory confirmation of LSD is most rapid 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 cells. Capripoxvirus causes a characteristic cytopathic effect and intracytoplasmic inclusion bodies, and is distinct from the virus of pseudo-LSD (Allerton – herpes mammillitis), 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 has been developed. Genome detection using capripoxvirus-specific primers for the fusion protein gene and attachment protein gene has also been reported.

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 antigen, expressed by a suitable vector, in an ELISA offers the prospect of an acceptable and standardised serological test.

Requirements for vaccines and diagnostic biologicals: 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 (15), 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 (24). 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. In 1988 LSD became established in Egypt, and in 1989 a single outbreak was reported in Israel. LSD must be considered to have the potential to become established outside Africa. The principle method of transmission is mechanical by arthropod vector (6, 9).

The severity of clinical signs of LSD, (Neethling or knopvelsiekte), depends on the strain of capripoxvirus and the breed of host. 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 (7). There may be failure of the virus to infect the whole group, depending on vector prevalence.

In the acutely infected animal, there is an initial pyrexia, which may exceed 41°C and persist for 1 week. The incubation period under field conditions has not been reported, but following inoculation is 6–9 days until the onset of fever. A rhinitis and conjunctivitis develop, and in lactating cattle there is a marked reduction in milk yield. Nodules of 2–5 cm in diameter develop over the body, particularly on the head, neck, udder and perineum. These nodules involve the dermis and epidermis and may initially exude serum, but over the following 2 weeks may become necrotic plugs that penetrate the full thickness of the hide. All the superficial lymph nodes are enlarged, the limbs may be oedematous and the animal is reluctant to move. The nodules on the mucous membranes of the eyes, nose, mouth, rectum, udder and genitalia quickly ulcerate, and by then all secretions contain LSD virus. On the appearance of clinical signs, the discharge from the eyes and nose becomes mucopurulent, and keratitis may develop. Nodules may also develop in the mouth, subcutis and muscle, in the trachea and alimentary tract, particularly the abomasum, and in the lungs, resulting in primary and secondary pneumonia. Pregnant cattle may abort, and there are reports of aborted fetuses being covered in nodules. Bulls may become permanently or temporarily infertile. 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 (21).

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 (12). Samples for genome detection by polymerase chain reaction (PCR) may be collected when neutralising antibody is present. However, the virus can be demonstrated for up to 35 days in old lesions. 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 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) (2). Lesion material for virus isolation and antigen detection is minced using sterile scissors and forceps and then
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ground in a sterile pestle and 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 (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. 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 growth medium, such as Glasgow’s modified Eagle’s medium (GMEM). After centrifugation at 600 g for a further 15 minutes, the resulting 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) Culture

LSD virus will grow in tissue culture of bovine, ovine or caprine origin, although primary or secondary culture of lamb testis (LT) cells are considered to be the most susceptible, particularly those derived from a breed of wool sheep. Sample material prepared as above, i.e. 1 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 14 days for evidence of cytopathic effect (CPE) and the medium is replaced if it appears to be cloudy. 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 herpes virus of pseudo-LSD produces a Cowdry type A intranuclear inclusion body. Formation of syncytia is not a feature of capripoxvirus infection, 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.

- 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 pileiform-carbon substrate activated by glow discharge in pentyamine vapour, on to a drop of the suspension placed on paraffilm 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 (19).

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).
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b) Immunological methods

• 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, due to antibodies to cell culture, can cause problems.

• 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 resuspended 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 (18).

• 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). These reagents have facilitated the development of a highly specific ELISA (5). 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.

c) Nucleic acid recognition methods

It is not possible to distinguish between strains of capripoxvirus from cattle, sheep or goats using serological techniques. However, strains can be characterised by comparing the genome fragments generated by HindIII digestion of their purified DNA (1, 3, 17). 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 (14).

The PCR can be used to detect the capripoxvirus genome in biopsy or tissue culture samples. Primers for the viral attachment protein gene and the viral fusion protein gene are specific for capripoxvirus, and the nature of the PCR products can be confirmed using restriction enzyme recognition sites (16). The LSD virus genome contains 156 putative genes (23).

2. Serological tests

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 TCID\(_{50}\), the neutralisation index is the preferred method. 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 10⁵ 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 0240 KSGP vaccine strain of capripoxvirus, the final reading is taken on day 9, and the titre of virus in each duplicate titration is calculated according to Kärber (1931). 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 ≥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.
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), glycine (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 again thoroughly 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

A capripoxvirus antibody ELISA has been developed using the expressed structural protein P32 of capripoxvirus and MAbs raised against the P32 protein (8).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Two live attenuated strains of capripoxvirus have been used as vaccines specifically for the control of LSD (3, 4): a strain of Kenya sheep and goat pox virus passaged 18 times in LT or fetal calf muscle cells, and a 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 (10). In 1989 and 1990 the Romanian strain of sheep pox vaccine was used to help control the LSD outbreak in Egypt (20). 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. Both strains of capripoxvirus used routinely as vaccines can produce a large local reaction at the site of inoculation in Bos taurus breeds, 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.

A new generation of capripox vaccines is being developed that uses the capripoxvirus genome as a vector for the genes of other ruminant pathogens, for instance genes of rinderpest and peste des petits ruminants viruses. The recombinant vaccine will provide protection against LSD and rinderpest in a single vaccination (22).
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1. Seed management

a) Characteristics of the seed

A strain of capripoxvirus used for vaccine production must be accompanied by a history describing its origin and tissue culture or animal passage. It must be safe to use in all breeds of cattle for which it is intended, including pregnant animals. It must also be nontransmissible, remain attenuated after further tissue culture passage, and provide complete protection against challenge with virulent field strains for a minimum of 1 year. A quantity of master seed vaccine virus should be prepared and stored in order to provide a consistent working seed for regular vaccine production.

b) Method of culture

Vaccine seed should be lyophilised and stored in 2 ml vials at –20°C. It may be stored wet at –20°C, but when wet, it is more stable at –70°C or lower. The virus should be cultured in primary or secondary LT cells of wool sheep origin for maximum yield. Vero cells may also be used.

c) Validation as a vaccine

Seed lots must be shown to be:

i) Pure: 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.

ii) Safe: Produce minimum clinical reaction in all breeds of cattle when given by the recommended route.

iii) Efficacious: Stimulate complete immunity to LSD in all breeds of cattle for at least 1 year.

The necessary tests are described in Section C.4.

2. Method of manufacture

Vaccine batches are produced on fresh monolayers of secondary LT cells. A vial of seed virus is reconstituted with GMEM and inoculated on to 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. After 4–6 days, there will be extensive (50–70%) CPE. The culture is freeze–thawed three times, and the suspension is removed 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 10⁶ doses, the yield from five 175 cm² flasks is required).

The procedure is repeated and the harvests from individually numbered flasks are each mixed separately with an equal volume of sterile, chilled 5% lactalbumin hydrolysate and 10% sucrose, 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.

3. In-process control

Cells: 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 bovine viral diarrhoea or border disease viruses by immunofluorescence or immunoperoxidase techniques. If possible, cells should be prepared and screened prior to vaccine production, and a stock stored in sterile DMSO (dimethyl sulfoxide) in liquid nitrogen (1–2 ml aliquots containing 20 × 10⁶ cells/ml). Serum used in the growth medium must be free from antibody to capripoxvirus and contamination with pestivirus.

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 log₁₀ 3.5 TCID₅₀, although the minimum protective dose is log₁₀ 2.0 TCID₅₀. 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 log₁₀ 2.5 sheep infective doses (SID₅₀), and the recommended dose for cattle of the RM65-adapted strain of Romanian sheep pox vaccine is log₁₀ 3 TCID₅₀ (11). 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 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.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination with biological materials may be found in Chapter I.1.5.

b) Safety and efficacy

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. Two cattle are inoculated with 100 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 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. 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 due to the vaccine.

c) Potency tests

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.

d) Duration of immunity

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 confusing the results.

e) Stability

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.

f) Preservatives

No preservatives other than a protectant, such as sucrose and lactalbumin hydrolysate, are required for the freeze-dried preparation.
g) Precautions (hazards)
There are no precautions other than those described above for sterility and freedom from adventitious agents. Strains of LSD virus are not a hazard to human health.

5. Tests on the final product
a) Safety
Safety tests should be carried out on the final product of each batch as described in Section C.4.b.

b) Potency
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.

REFERENCES
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NB: There are OIE Reference Laboratories for Lumpy skin disease (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.1.8.

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 genotypes. 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.

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 (13, 15). 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 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 (9).

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 (CER: cells developed by Tsunemasa Motohashi at the Nippon Institute for Biological Science, Tokyo, Japan), 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 immunosassay 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.
African animals may have only inapparent infections, while exotic or other breeds suffer severe clinical disease in sheep, goats and cattle. The susceptibility of different breeds to RVF varies considerably. Some indigenous following heavy rains and flooding, and is characterised by high rates of abortion and neonatal mortality, primarily together with disease in humans, is characteristic. RVF has a short incubation period: 12–36 hours in lambs. A

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 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 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 (6). 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 diffusely and in sheep, icterus is more common than in lambs (26).

In humans, RVF infections are usually inapparent or associated with a moderate to severe, nonfatal, influenza-like illness (15). 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 I.1.6. Human safety in the veterinary microbiology laboratory).

No significant antigenic differences have been demonstrated between RVF isolates and laboratory-passaged strains from many countries, but differences in pathogenicity have been shown (27).

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 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) (3) 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. This is to avoid the production of defective particles, which follows the use of very high virus inocula. 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 (23) using an 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. 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 (22).

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 (27). In suspected cases, the OIE Reference Laboratory for RVF (see Table given in Part 3 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 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 mousebrain strain of highly attenuated RVF virus (25), also referred to as modified live virus and adapted to cell culture, is used as antigen. The antigen is stored at 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% CO$_2$ 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

An indirect ELISA employing an inactivated, cell-culture-produced antigen and Protein G-peroxidase conjugate has been developed and extensively tested (21). The RVF ELISA antigen is prepared from Madin–Darby cell cultures and the negative control antigen from uninoculated monolayers. Cell pellets are extracted with a sucrose-acetone method (4) and inactivated with beta-propriolactone (24).

- **Test procedure**
  
  Unless otherwise stated, volumes used are 50 µl/well, all dilutions are made with 3% (w/v) dried milk buffer, and all washes executed three times.

  i) Coat half the plate with 50 µl/well positive antigen and half with negative antigen at a predetermined dilution. Incubate overnight at 4°C. Wash the plate.

  ii) Add 100 µl/well blocking buffer, incubate for 1 hour at 37°C. Wash the plate.

  iii) Add test and control sera at a predetermined dilution in duplicate wells to positive and negative antigen. Incubate for 1 hour at 37°C. Wash the plate.

  iv) Add Protein G/horseradish-peroxidase conjugate at a working dilution. Incubate for 1 hour at 37°C. Wash the plate.

  v) Add substrate tetramethylbenzidine as supplied and leave the plate for 20 minutes at room temperature (22°C) in the dark (developing time).

  vi) Add stop solution (2 M sulphuric acid) and read the plate at optical density 450/650 nm.

  vii) The plate layout is as shown:

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A, B, C and D = Ag positive; E, F, G and H = Ag negative


c) Haemagglutination inhibition

The HI test adapted to a microtechnique is based on Clarke & Casals (4). 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 (27).

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, while inactivated vaccines for use in
pregnant animals and in RVF-free countries are prepared from virulent field strains (1, 2). 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 I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 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 (17). MV P12 was protective in young lambs (10, 14) and in cattle (18, 19). In further more extensive testing in sheep, the vaccine, when used after 28 days of pregnancy, resulted in abortion and severe fetal teratology (12).

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 (20). In further trials it was non-abortogenic in pregnant sheep and gave more than 80% protection from virulent challenge (11). 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 thiglycollate and two tubes of soybean casein digest medium. The thiglycollate 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 disqualify 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 sterility testing.

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 sterility tests in thioglycollate and soybean casein digest medium (see Section C.1.c. and Chapter I.1.5. 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 I.1.5. of this Terrestrial Manual).

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 families of infant mice, 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 (1, 2).

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%.

- **Acknowledgement**

Parts of this chapter were taken from or based on the chapter on RVF in the previous editions of this Terrestrial Manual.

**REFERENCES**


* * *

**NB:** There is an OIE Reference Laboratory for Rift Valley fever (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.1.9.

BLUETONGUE

SUMMARY

Bluetongue (BT) is an infectious, noncontagious, insect-borne viral disease of sheep and domestic and wild ruminants, such as goats, cattle, deer, bighorn sheep, most species of African antelope and various other Artiodactyla. The outcome of infection ranges from inapparent in the vast majority of infected animals to fatal in a proportion of infected sheep, goats, deer and wild ruminants. Clinical signs of disease, when they appear in domestic and wild ruminants, include a febrile response characterised by inflammation and congestion, facial oedema and haemorrhages, and ulceration of the mucous membranes. In severe cases the tongue may show intense hyperaemia, and become oedematous and protrude from the mouth. Hyperaemia may extend to other parts of the body particularly the groin, axilla and perineum. There is often severe muscle degeneration. Dermatitis may cause wool breaks. Sheep may become lame as a result of coronitis, inflammation of the coronary band of the hoof, or skeletal myopathy. A similar severe disease of wild ruminants is caused by epizootic haemorrhagic disease virus (EHDV), which, like BT virus (BTV), is a member of the Orbivirus genus, but is classified in a separate serogroup. EHD may occasionally cause clinical signs in cattle that appear to be similar to bluetongue.

Identification of the agent: BTV is a member of the Orbivirus genus of the family Reoviridae. Within the genus there are 14 serogroups. The BT serogroup contains 24 serotypes. The former are differentiated by immunological tests that detect viral proteins that are conserved within each serogroup. Most serogroups appear to be immunologically distinct, but there is considerable cross-reaction between members of the BT and EHD serogroups. The serotype of individual viruses in each serogroup is identified on the basis of neutralisation tests. Complete BTV particles are double-shelled and the outer layer contains two proteins, one of which is the major determinant of serotype specificity. The inner icosahedral core contains two major and three minor proteins and ten species of double-stranded RNA. VP7 is a major core protein possessing the serogroup-specific antigens. Virus identification traditionally requires isolation and amplification of the virus in tissue culture, and the subsequent application of serogroup- and serotype-specific tests. Recently, the application of polymerase chain reaction (PCR) technology has permitted very rapid amplification of BTV RNA in clinical samples, and PCR-based procedures are now available to provide information on virus serogroup and serotype.

Serological tests: Serological responses in ruminants appear some 7–14 days after BTV infection and are generally long-lasting. Until recently, tests such as agar gel immunodiffusion and indirect enzyme-linked immunosorbent assay (ELISA) were used to detect BT serogroup-specific antibody, although these tests had the major drawback of being unable to consistently distinguish between antibodies to viruses in the BT and EHD serogroups. A monoclonal antibody-based competitive ELISA has solved this problem and competitive ELISAs to specifically detect anti-BTV antibodies are recommended. Current procedures to determine the serotype-specificity of antibodies in sera are cumbersome because they require determination of the capacity of test sera to inhibit the infectivity of panels of known virus serotypes in time-consuming neutralisation tests.

Requirements for vaccines and diagnostic biologicals: Live, attenuated vaccines that are serotype-specific are used in several countries of the world, such as South Africa, where nonvaccination may lead to outbreaks of disease. Attenuated viruses are prepared by serial passage of field virus in embryonated chicken eggs or cultured cells. Following serial passage, virulence is attenuated and, concomitantly, viruses replicate to lower titres in sheep. Attenuated virus vaccines are teratogenic and should not be administered to pregnant sheep during the first half of pregnancy as this may cause fetal death and abnormalities. In determining an appropriate
degree of attenuation for vaccine purposes, a compromise is sought between a level of replication sufficient to reduce virulence but stimulate protective immunity in sheep, and a need to reduce the titre of virus in the blood in an attempt to prevent infection of feeding insects. Procedures to test vaccine efficacy and teratogenic potential in sheep are easily performed. In contrast, few studies have been carried out to determine whether or not attenuated virus can be transmitted by insects from vaccinated sheep to other animals. The fact that attenuated viruses are teratogenic makes determination of transmissibility very important especially if live virus vaccines are used in countries for the first time.

A. INTRODUCTION

Midges of the genus Culicoides transmit bluetongue virus (BTV) to and from susceptible animals, having become infected by feeding on viraemic vertebrates. After a replication period of 6–8 days, and following its appearance in the salivary gland, the virus can be transmitted to a vertebrate host during a blood meal. Infected midges remain infective for life. The central role of the insect in BT epidemiology ensures that prevalence of the disease is governed by ecological factors, such as high rainfall, temperature, humidity and soil characteristics, which favour insect survival (6). In many parts of the world therefore, the disease has a seasonal occurrence (13).

BT is an infectious, noncontagious disease of sheep and other domestic and wild ruminants, such as goats, cattle, deer, bighorn sheep, most species of African antelope and other Artiodactyla. The outcome of infection ranges from inapparent in the vast majority of infected animals to fatal in a proportion of infected sheep, deer and some wild ruminants. Although the frequency of BTV infection of cattle is generally higher than in sheep, overt disease in cattle is rare and the signs, when they occur, are much milder than those observed in sheep. In nondomestic ruminants, the disease can vary from an acute haemorrhagic disease with high mortality, as observed in white-tailed deer (Odocoileus virginianus), to an inapparent disease as seen in the North American elk (Cervus canadensis). Epizootic haemorrhagic disease virus (EHDV) can produce a disease in wild ruminants with clinical manifestations identical to those observed in response to BTV infection.

Clinical signs of disease in domestic and wild ruminants range from subclinical in the vast majority of cases to an acute febrile response characterised by inflammation and congestion, leading to oedema of the face, eyelids and ears, and haemorrhages and ulceration of the mucous membranes. Extensive erosions can develop in the cheeks and on the tongue opposite molar teeth. 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 groin, axilla and perineum. There is often severe muscle degeneration. Dermatitis may cause wool breaks. Coronitis with haemorrhage of the coronary band of the hoof is common and may cause lameness. When sheep die as a result of acute BT disease, the lungs may show interalveolar hyperaemia, severe alveolar oedema and the bronchial tree may be filled with froth. The thoracic cavity may contain several litres of plasma-like fluid and the pericardial sac may show many petechial haemorrhages. Most cases show a distinctive haemorrhage near the base of the pulmonary artery (11).

BTV is a member of the Orbivirus genus, currently one of nine genera classified in the family Reoviridae. Within the Orbivirus genus, 14 groups are differentiated on serological grounds. The best-studied Orbiviruses are in the BT, EHD and African horse sickness (AHS) serogroups. Within the serogroups, individual members are differentiated on the basis of neutralisation tests, and 24 serotypes of BTV have been described to date. There is significant immunological cross-reactivity between members of the BT and EHD serogroups (16). Details of EHD-specific tests will not be provided in this chapter.

BTV 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. It is also responsible for haemagglutination and the binding of BTV to mammalian cells. The ability of a MAb to VP5 to neutralise AHS virus (AHSV) and react with the equivalent protein of BTV and EHDV confirms a role for VP5 in neutralisation of Orbiviruses and highlights the extent of immunological cross-reactivity between members of the different Orbivirus serogroups (24). 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 antibody. VP7 can also mediate attachment of BTV to insect cells (39). VP7 subunits consist of two domains.
B. DIAGNOSTIC TECHNIQUES

1. Identification of the 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 are in common use, but two of the most efficient are embryonated chicken eggs (ECE) and sheep. Identification of BTV following inoculation of sheep may 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. Within a virus population not all BTV particles are identical at the genetic and amino acid level and only a small, perhaps minute, proportion of viruses present in the blood of infected animals may have appropriate amino acid sequences in key viral proteins to bind to and replicate in cells in culture. This may be the reason why direct inoculation on to cultured cells of viraemic blood that contains a relatively small number of virus particles is an inefficient way to isolate BTV. A high-titre virus preparation, and one more likely to contain virus that has the ability to replicate in tissue culture, is most readily generated by one or at most two passages in ECE. Cell culture has proven to be a more sensitive technique for isolation of EHDV.

- Isolation in embryonated chicken eggs
  i) Blood is collected from febrile animals into an anticoagulant such as heparin; EDTA (ethylamine diame tetra-acetic acid) or sodium citrate, and the blood cells are washed three times with sterile phosphate buffered saline (PBS). Washed cells are resuspended 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 (10). If samples can be frozen, they should be collected in buffered lactose peptone or 10% dimethyl sulphoxide (36) 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 resuspended in distilled water or sonicated in PBS and 0.1 ml amounts inoculated intravascularly into 6–12 ECE that are 9–12 days old. This procedure is difficult to perform and requires practise. Details are provided by Clavijo et al. (8).
  v) The eggs are incubated in a humid chamber at 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 often 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 specific organs such as the liver, are homogenised and the debris is removed by centrifugation.
  vii) Virus in the supernatant may be identified either directly by antigen-capture ELISA (18), 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 repassaged in ECE or in cell culture.

- Isolation in cell culture

Virus may also be added to mouse L, baby hamster kidney (BHK)-21, African green monkey kidney (Vero) or Aedes albopictus (AA) cells in culture. The efficiency of isolation is often significantly lower following direct addition to cultured cells compared with that achieved in ECE. Greatest efficiency of isolation in cell culture is achieved by first passing ECE homogenates in AA cells, followed by either antigen detection procedures or additional passages in mammalian cell lines, such as BHK-21 or Vero. A cytopathic effect (CPE) is not necessarily observed in AA 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 serological methods described below, including antigen-capture ELISA, immunofluorescence, immunoperoxidase, or virus neutralisation (VN) tests.
• Isolation in sheep

  i) Sheep are inoculated with washed cells from 10 ml up to approximately 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 for antibody using the agar immunodiffusion (1) test or C-ELISA as described below.

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 (3, 22). In contrast to serogrouping, the usual method of serotyping is by VN testing using methods described later. Commonly used methods for the identification of viruses to serogroup level are as follows.

  i) **Immunofluorescence**

     Monolayers of BHK or Vero cells on 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 and standard immunofluorescent procedures.

  ii) **Antigen capture enzyme-linked immunosorbent assay** (27)

     Virus in ECE lysates, culture medium and infected insects may be detected directly. In this technique, virus and/or core particles are captured by antibody adsorbed to an ELISA plate and bound virus is 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 (18).

  iii) **Immunospot test** (14)

     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 BT serogroup-reactive MAb, bound antibody is detected using horseradish peroxidase-conjugated anti-mouse IgG.

• Serotyping by virus neutralisation

Neutralisation tests are type specific for the currently recognised 24 BTV 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 serotypes should 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 antibody. Cell lines commonly used are BHK, Vero and L929. Four methods to serotype BTV are outlined briefly below. BTV 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 to ensure that an effective level of reference antiserum is used against comparable and standardised titres of reference and untyped virus.

  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 individual standard antisera to a panel of BTV serotypes. Virus/antiserum mixtures are added to monolayers of cells and the virus titre is determined by plaque assay. 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 $5 \times 10^4$ 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 TCID$_{50}$ (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 10$^5$ 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 (5)

This rapid and simple neutralisation assay 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) Polymerase chain reaction (a prescribed test for international trade)

Primer-directed amplification of viral nucleic acid has revolutionised BT diagnosis (8, 26, 37). Polymerase chain reaction (PCR) techniques have allowed the rapid identification of BT viral nucleic acid in blood and other tissues of infected animals. Regarding international trade, PCR has allowed the identification of BT antibody-positive animals that are negative for viral nucleic acid, permitting their importation. PCR can also be used to ‘serogroup’ Orbiviruses and may ultimately be used to ‘serotype’ BTV within a few days of receipt of a clinical sample, such as infected sheep blood. Traditional approaches, which rely on virus isolation followed by virus identification serologically, may require at least 3–4 weeks to generate information on serogroup and serotype.

Oligonucleotide primers used so far have been derived from RNA 7 (VP7 gene) (37), RNA 6 (NS1 gene) (9), RNA 3 (VP3 gene) (30), RNA 10 (NS3 gene) (4) and RNA 2 (VP2 gene) (26). 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 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 (19).

The nucleic acid sequence of cognate BTV genes may differ with the geographical area of virus isolation (17). This has provided a unique opportunity to complement studies of BT 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 3 and RNA 6 may provide information on whether the virus came from Australia, North America or South Africa. 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. Genotypes specific to geographical locations were not as clearly defined by PCR analyses of RNA genome segment 7 (38) as they appeared to be using RNA genome segment 3 (17). The development of topotyping as an epidemiological tool thus depends on the acquisition of sequence data for BTV isolates from many and diverse regions of the world and availability of the data in readily accessible data banks. In principle, given a large enough RNA 2 sequence database, it should ultimately be possible to determine rapidly virus serotype by PCR amplification of RNA 2. To facilitate this process new sequence data derived from both characterised and uncharacterised BTV isolates should be made widely available by submitting the data to web sites such as:

http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ and

The web site http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/btv2-segment-2-tree.htm provides phylogenetic tree analyses of BTV isolates based on the sequence of RNA2. These compiled data will provide a resource for epidemiological studies, the identification of new isolates and the design of new primers for further reverse transcription (RT) PCR and possibly serotype-specific assays for BTV.
It has been observed that BTV nucleic acid can be detected by PCR from the blood of infected calves and sheep at least 30 days, and sometimes over 90 days, after the virus can be isolated. When blood that was positive for virus isolation (infectious) and blood that was negative for virus isolation but positive by PCR (PCR-detectable only) were inoculated into or fed to the vector, *Culicoides sonorensis*, it was shown that the virus was amplified and transmitted only by vectors exposed to infectious blood. Vectors exposed to PCR-detectable only blood did not amplify or transmit the BTV (23). Because of this, PCR-based diagnostics should be interpreted with caution. The PCR procedure will detect virus-specific nucleic acid, but this does not necessarily indicate the presence of infectious virus.

The capacity of 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. Latex 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 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.

The 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 (Orca Research, Bothell, Washington, United States of America [USA]). The reagents provided with the kit are numbered and their use is indicated in the protocol below. Other kits are available and one, TRIZOL (Life Technologies, Grand Island, New York, USA), 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 PCR. In the procedure described below, the Superscript™ Preamplification System (Life Technologies) is used to transcribe viral RNA, and reagents from Perkin-Elmer are used for the PCR. Equivalent kits and reagents are available from other sources. The final step of the process is the analysis of the 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 10³ to 10⁷ 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.
  
  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 PCR primers (to amplify RNA 6 from nucleotide 11 to 284)
      - Primer A: 5'-GTT-CTG-CTC-TAG-TTG-GCA-ACC-ACC-3'
      - Primer B: 5'-AAG-CCA-GAC-TGT-TTC-CCG-AT-3'

    - Nested 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 PCR reaction are prepared by mixing equal volumes of A and B. Primers for the nested PCR reaction are prepared by mixing equal volumes of C and D. Small aliquots of pooled primer mixes are frozen at −20°C.
  
  v) 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.
  
  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 PCR tubes (38).
  
  viii) To each 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 Superscript™Preamplification System (Life Technologies).

    - 10 × Superscript™ buffer (200 mM Tris/HCl, pH 8.4, and 500 mM KCl): 2.0 µl
    - MgCl₂ (25 mM): 2.0 µl
    - dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP): 1.25 µl
    - Dithiothreitol (DTT) (0.1 M): 2.0 µl
    - Reverse transcriptase (200 units/µl): 0.75 µl

  x) Then, 8.0 µl of the mix is added to each PCR tube to a final volume of 20.0 µl.
  
  xi) The PCR tubes are placed in a thermal cycler, such as GeneAmp™ PCR System 9600, which is programmed for reverse transcription as follows:

    - Hold 44°C: 50 minutes
    - Hold 4°C: Forever

  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:

    - Hold 37°C: 20 minutes
    - Hold 98°C: 4 minutes
    - Hold 4°C: Forever
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 available from Perkin-Elmer. The amount given is per sample.

- RNase-free water: 62.0 µl
- 10 × PCR Perkin-Elmer buffer (100 mM Tris/HCl, pH 8.3, and 500 mM KCl): 7.0 µl
- MgCl₂ (25 mM): 7.0 µl
- dNTP mix (2.5 mM each dATP, dCTP, dGTP, dTTP): 4.0 µl
- Taq DNA polymerase (5 units/µl): 0.85 µl

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:

- One cycle: Hold 95°C 3 minutes
- Hold 58°C 20 seconds
- Hold 72°C 30 seconds
- 40 cycles: Hold 95°C 20 seconds
- Hold 58°C 20 seconds
- Hold 72°C 20 seconds
- One cycle: Hold 95°C 20 seconds
- Hold 58°C 20 seconds
- Hold 72°C 5 minutes
- Hold 4°C Forever

xvi) PCR reaction tubes are prepared for the nested reaction in a ‘clean’ hood 15 minutes before cycling is complete, and held on ice:

- RNase-free water: 17 µl per tube
- Nested primer mix (C+D): 4.0 µl per tube
- Wax bead

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 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:

- Hold 98°C 4 minutes
- Hold 4°C Forever

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.

- RNase-free water: 17.0 µl
- 10 × PCR buffer: 5.0 µl
- MgCl₂: 3.5 µl
- dNTP mix: 4.5 µl
- Taq DNA polymerase: 0.5 µl

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:

- One cycle: Hold 95°C 3 minutes
- Hold 58°C 20 seconds
- Hold 72°C 30 seconds
- 40 cycles: Hold 95°C 20 seconds
- Hold 58°C 20 seconds
- Hold 72°C 20 seconds
- One cycle: Hold 95°C 20 seconds
- Hold 58°C 20 seconds
- Hold 72°C 5 minutes
- Hold 4°C Forever
Electrophoretic analysis of PCR product

i) First, 1 x TBE buffer (0.045 mM Tris/borate, pH 8.6, and 1.5 mM EDTA) is prepared from a x10 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 (FMC Bioproducts, Rockland, Maine, USA) 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. Ethidium bromide is added to a concentration of 0.5 µg/ml to both the agarose and the tank buffer. Ethidium bromide is a mutagen and is toxic. Gloves, protective clothing, and eye-wear must always be worn.

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:

- Gel-loading solution (Cat. G-2526, Sigma, St Louis, Missouri, USA) 5.0 µl
- Amplified DNA from each of the PCR tubes and an extra tube is set up for a DNA ladder 15.0 µl
- Gel-loading solution (Cat. G-2526, Sigma, St Louis, Missouri, USA) 5.0 µl
- 100 base-pair ladder (Cat. 15268-019, Life Technologies, Grand Island, New York, USA) 1.0 µl

vi) Samples are loaded into the appropriate wells in the gel and run at 65–75 volts for 1–1.5 hours 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 bag (Ameresco, Solon, Ohio, USA) 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.

Kits and reagents for two prescribed serological tests – the agar gel immunodiffusion (AGID) test and the C-ELISA – are available from three licensed manufacturers in the USA (VMRD, P.O. Box 502, Pullman, Washington 99163, USA; or Veterinary Diagnostic Technology, 4980 Van Gordon Street, Suite 101, Wheat Ridge, Colorado 80033, USA; or Diagxotics, 27 Cannon Road, Wilton, Connecticut 06897, USA). The C-ELISA reagents are available from the European Union ‘Community Reference Laboratory’ for BTV (Pirbright Laboratory, Ash Road, Pirbright, Woking GU24 0NF, United Kingdom).

2. Serological tests

Anti-BTV antibody generated in infected animals can be detected in a variety of ways that depend on the sensitivity and type of test used. 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 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. There are two explanations for this phenomenon. First, several serotypes share monoclonal MAb-defined neutralisation epitopes. Secondly, serotypes also share a large number of epitopes that are present in a neutralising conformation in one serotype, but in non-neutralising conformations in other serotypes.

a) Complement fixation

A complement fixation (7) 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.

b) Agar gel immunodiffusion (a prescribed test for international trade)

The AGID test to detect anti-BTV antibodies is simple to perform and the antigen used in the assay relatively easy to generate. Since 1982, the test has been the standard testing procedure for international movement of ruminants. However, one of the disadvantages of the AGID used for BT is its lack of specificity.
in that it can detect antibodies to other Orbiviruses, particularly those in the EHD serogroup. Thus AGID positive sera may have to be retested using a BT 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) Three positive and three test sera are placed in alternate wells surrounding antigen in the 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 (1, 3, 22, 28, 31). The specificity is the result of using one of a number of BT serogroup-reactive MAbs, such as MAb 3-17-A3 (3) or MAb 20E9 (22) or MAb 20E9 (21). 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**
  
i) First, 96-well microtitre plates are coated at 4°C overnight or 37°C for 1 hour with 50–100 µl of either tissue culture-derived sonicated cell culture antigen (3) of the major core antigen VP7 expressed in either baculovirus (29) or yeast (25) 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 (1) or 1/10 (22) 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-sulphonic 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.

\[
\% \text{ inhibition} = 100 - \frac{[(\text{Mean absorbance test sample})/(\text{Mean absorbance MAb control})] \times 100.}
\]

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.
Percentage inhibition values >50% are considered to be positive. Inhibition between 40% and 50% is considered to be suspicious. The results of the duplicates of test sera can vary as long as they do not lie either side of the chosen inhibition value.

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.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Of several vaccine options available, namely live attenuated, killed or recombinant, only attenuated virus vaccines are in current use in several countries. In South Africa, for example, they have been used for over 40 years and are known to induce an effective and lasting immunity (11). Although the efficacy of inactivated virus vaccines has been investigated in some laboratory studies (15), they do not appear to have been used in the field. There are several options for the development of recombinant BTV vaccines, including live virus delivery of BTV neutralisation antigens and the virus-like particles (VLP) generated in infected insect cells by recombinant baculoviruses expressing the four major BTV coat proteins VP2, 3, 5 and 7. Only the latter has shown significant promise (33). However there is still much to determine, such as the longevity of the neutralising response generated to VLP, the need for multiple VLPs for different serotypes and the commercial scale up of VLP production in a cost effective and efficient process. The following description applies to attenuated virus vaccines.

1. Seed management

a) Characteristics of seed

The master or primary virus seed is prepared from a single plaque of serially passaged, attenuated BTV. Secondary seed lots, which are used as inocula for vaccine production, are usually not more than three passages beyond the primary seed lot. Primary seed virus must be free of contaminating bacteria, viruses, fungi and mycoplasmas, particularly pestivirus contamination, and must be shown to have the desired serotype specificity. 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.

b) Method of culture

The first BT vaccines were propagated in ECE (2). More recently, several different cells 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. Cells used for attenuation must be thoroughly checked for the presence of contaminating viruses. Not only may continuous cell lines harbour oncogenic viruses, but primary cells may also contain a number of inapparent or latent virus infections, such as pestivirus contamination. For the latter, particular attention should be paid to the fetal bovine serum used in cell cultures, as it may be contaminated. Vaccine viruses have been attenuated by either passage in ECE, tissue culture cells or a combination of both.

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 transmitted from such animals by insect vectors. The latter criterion is very important because insect-mediated transmission of attenuated virus from vaccinated to nonimmune 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.

i) Avirulence

A number of sheep, seronegative by BT C-ELISA, are inoculated with either the primary seed stock or an equal volume of tissue culture medium. 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 viraemia and antibody responses. The test shall be valid if all of the sheep inoculated with vaccine show evidence of virus growth and do not display signs of disease other than mild transient illness. In South Africa, a clinical reaction index (33) is calculated for each animal between days 4 and 14 and must be below a specific standard value.
ii) **Safety**

Safety tests for attenuated vaccines do not address the issue of their teratogenicity (34). 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 death (20).

iii) **Efficacy**

Vaccinated and unvaccinated sheep are challenged with virulent virus of the same serotype and animals are monitored for clinical signs of BT. Rectal temperatures are taken twice daily. Unvaccinated control sheep should show clinical signs of BT. 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 presence of neutralising antibody.

iv) **Transmissibility**

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 (35). 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. Due to the fact that the titre of attenuated virus in the blood of vaccinated sheep is low, very large numbers of *Culicoides* would 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. However, in South Africa it is estimated that the minimum titre of virus circulating in the bloodstream of an animal must be at least $10^3$ before feeding *Culicoides* become infected, although it has also been suggested that a lower titre may sometimes be infective. To select a suitable attenuated virus strain, whole blood is collected between days 4 and 14 after vaccination, and the virus titre is determined. Only attenuated viruses that generate titres under $10^3$ are deemed to be acceptable as vaccines.

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 (32). The implications of these observations for virus transmissibility are unclear.

v) **Reversion to virulence**

Validation studies confirm that attenuated viruses do not revert to virulence in vaccinated sheep. Consequently, if insects do not transmit attenuated viruses from vaccinated to unvaccinated animals, reversion to virulence becomes a theoretical possibility only. 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.

2. **Method of manufacture**

Attenuation of field isolates of BTV was first achieved by serial passage in ECE. 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.

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.
4. Batch control

a) Sterility

Every batch of vaccine should be tested for the presence of viable bacteria, extraneous viruses, fungi or mycoplasmas, particularly pestivirus 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 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, the batch is disqualified.

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. In order 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 (13). Many serotypes of BTV are present in endemic areas of South Africa, and polyvalent vaccines are used. The inclusion of 15 serotypes in the vaccine 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 (12).

e) Stability

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.

f) Precautions (hazards)

The polyvalent vaccine is safe except if used in ewes during the first half of pregnancy. Lambs possessing colostral immunity cannot be effectively vaccinated before 6 months of age.

5. Tests on the final product

a) Safety

See C.4.b.

b) Potency

See C.4.c.

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REFERENCES


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**NB:** There are OIE Reference Laboratories for Bluetongue (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.1.10.

SHEEP POX AND GOAT POX

SUMMARY

Sheep pox and goat pox are viral diseases of sheep and goats characterised by fever, generalised papules or nodules, vesicles (rarely), internal lesions (particularly in the lungs), and death. Both diseases are caused by strains of capripoxvirus, all of which can infect sheep and goats, and although most of the strains examined cause more severe clinical disease in either sheep or goats, some strains have been isolated that are equally pathogenic in both species. It has been proposed that the malignant pox diseases of sheep and goats caused by capripoxvirus and including Kenyan sheep and goat pox, Indian goat dermatitis and north African stone pox of sheep and goats be referred to as capripox.

Capripox is endemic in Africa north of the Equator, the Middle East, Turkey, Iran, Afghanistan, Pakistan, India, Nepal, parts of the people’s Republic of China and, since 1984, Bangladesh. Recently, it has made frequent incursions into southern Europe.

Identification of the agent: Laboratory confirmation of capripox is most rapid by the demonstration of typical capripox virions using the transmission electron microscope in combination with a clinical history of generalised capripox infection. The capripox virion is distinct from that of the other poxvirus commonly infecting sheep and goats – a parapoxvirus that causes orf or contagious pustular dermatitis. A precipitating antigen can be identified by an agar gel immunodiffusion test (AGID) using lymph gland biopsy material taken from an early case of capripox and specific immune sera; however, there is a cross-reaction with parapoxvirus. Capripoxvirus will grow on tissue culture of ovine, caprine or bovine origin, although field isolates may require up to 14 days to grow or require one or more additional tissue culture passage(s). The virus causes intracytoplasmic inclusions, clearly seen using haematoxylin and eosin staining. The antigen can also be detected in tissue culture using specific sera and immunoperoxidase or immunofluorescence techniques. Capripoxvirus antigen and inclusion bodies may be seen in stained cryostat or paraffin sections of biopsy or post-mortem lesion material.

An antigen-detection enzyme-linked immunosorbent assay (ELISA) using a polyclonal detection serum raised against a recombinant immunodominant antigen of capripoxvirus has been developed. Genome detection using capripoxvirus-specific primers for the fusion protein gene and attachment protein gene has also been reported.

Serological tests: The virus neutralisation test is the most specific serological test, but because immunity to capripox infection is predominantly cell mediated, the test is not sufficiently sensitive to identify animals that have had contact with the virus and developed only low levels of neutralising antibody. The AGID and indirect immunofluorescence tests are less specific due to cross-reactions with antibody to other poxviruses. Western blotting using the reaction between the P32 antigen of capripoxvirus with test sera is both sensitive and specific, but is expensive and difficult to carry out. The use of this antigen, expressed by a suitable vector, in an ELISA offers the prospect of an acceptable and standardised serological test.

Requirements for vaccines and diagnostic biologicals: Live and inactivated vaccines have been used for the control of capripox. All strains of capripoxvirus so far examined share a major neutralisation site and will cross protect. Inactivated vaccines give, at best, only short-term immunity.
A. INTRODUCTION

Sheep pox and goat pox (capripox) are endemic in central and north Africa, the Middle East and India. Capripox is caused by strains of capripoxvirus and produces a characteristic clinical disease in fully susceptible breeds of sheep and goats that cannot be confused with any other disease. In indigenous animals, generalised disease and mortality are less common, although they are seen where disease has been absent from an area or village for a period of time, when intensive husbandry methods are introduced, or in association with other disease agents, such as peste des petits ruminants virus or foot and mouth disease virus.

Capripox is a major constraint to the introduction of exotic breeds of sheep and goats, and to the development of intensive livestock production. Strains of capripoxvirus that cause lumpy skin disease (such as Neethling), are also found in cattle, but there is no evidence that these strains will naturally cause disease in sheep and goats. The geographical distribution of lumpy skin disease differs from that of sheep pox and goat pox.

Strains of capripoxvirus do pass between sheep and goats, although most cause more severe clinical disease in only one species; recombination also occurs between these strains, producing a spectrum showing intermediate host preferences and a range of virulence. Some strains are equally pathogenic in both sheep and goats. Capripox has the potential to spread and become established in countries outside its normal distribution. In 1983 it spread into Italy, in 1985 and 1989 into Cyprus, and in 1988 and numerous subsequent occasions into Greece, but did not become established in these countries. In 1984, however, it spread into Bangladesh where it has persisted. During the past decade there have been further frequent incursions into Greece and Bulgaria.

The incubation period is between 8 and 13 days following contact between an infected and susceptible animals. It may be as short as 4 days following experimental infection by intradermal inoculation or mechanical transmission by insects. Some breeds of European sheep, such as Soay, may die of acute infection before the development of skin lesions. In other breeds there is an initial rise in rectal temperature to above 40°C, followed in 2–5 days by the development of, at first, macules – small circumscribed areas of hyperaemia, which are most obvious on unpigmented skin – and then of papules – hard swellings of between 0.5 and 1 cm in diameter – which may cover the body or be restricted to the groin, axilla and perineum. Papules may be covered by fluid-filled vesicles, but this is rare. A flat haemorrhagic form of capripox has been observed in some breeds of European goat, in which all the papules appear to coalesce over the body; this form is always fatal.

Within 24 hours of the appearance of generalised papules, affected animals develop rhinitis, conjunctivitis and enlargement of all the superficial lymph nodes, in particular the prescapular lymph nodes. Papules on the eyelids cause blepharitis of varying severity. As the papules on the mucous membranes of the eyes and nose ulcerate, so the discharge becomes mucopurulent, and the mucosa of the mouth, anus, and prepuce or vagina become necrotic. Breathing may become laboured and noisy due to pressure on the upper respiratory tract from the swollen retropharyngeal lymph nodes and to the developing lung lesions.

If the affected animal does not die in this acute phase of the disease, the papules start to become necrotic from ischaemic necrosis following thrombi formation in the blood vessels at the base of the papule. In the following 5–10 days the papules form scabs, which persist for up to 6 weeks, leaving small scars. The skin lesions are susceptible to fly strike, and secondary pneumonia is common. Anorexia is not usual unless the mouth lesions physically interfere with feeding. Abortion is rare.

On post-mortem examination of the acutely infected animal, the skin lesions are often less obvious than on the live animal. The mucous membranes are necrotic and all the body lymph nodes are enlarged and oedematous. Papules, which may be ulcerated, can usually be found on the abomasal mucosa, and sometimes on the wall of the rumen and large intestine, on the tongue, hard and soft palate, trachea and oesophagus. Pale areas of approximately 2 cm in diameter may occasionally be seen on the surface of the kidney and liver, and have been reported to be present in the testicles. Throughout the lungs, but particularly in the diaphragmatic lobes, there are numerous hard lesions of up to 5 cm in diameter.

The clinical signs and post-mortem lesions vary considerably with breed of host and strain of capripoxvirus. Indigenous breeds are less susceptible and frequently show only a few lesions, which could be confused with insect bites or contagious pustular dermatitis. However, lambs that have lost their maternally derived immunity, animals that have been kept isolated and animals brought into endemic areas from isolated villages, particularly if they have been subjected to the stress of moving long distances and mixing with other sheep and goats and their pathogens, can often be seen with generalised and sometimes fatal capripox. Invariably there is high mortality in unprotected imported breeds of sheep and goats following capripoxvirus infection. Capripox is not infectious 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 papules, 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. Samples for genome detection by polymerase chain reaction (PCR) may be collected when neutralising antibody is present. Buffy coat from blood collected into heparin or EDTA (ethylene diamine tetra-acetic acid) during the viraemic stage of capripox (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 temperatures. Tissues for virus isolation, antigen detection and genome 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/detection.

   Material for histology should be prepared by standard techniques and stained with haematoxylin and eosin (H&E). Lesion material for virus isolation and antigen detection is minced using sterile scissors and forceps and then ground in a sterile pestle and 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 (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. 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 growth medium, such as Glasgow’s modified Eagle’s medium (GMEM). After centrifugation at 600 g for a further 15 minutes, the resulting pellet is suspended in 5 ml of fresh GMEM. Alternatively, the buffy coat may be separated from a heparinised sample using a Ficoll gradient.

a) Culture

   Capripoxvirus will grow in tissue culture of bovine, ovine or caprine origin, although primary or secondary cultures of lamb testis (LT) or lamb kidney (LK) cells are considered to be the most susceptible, particularly those derived from a wool sheep breed. Either 1 ml of buffy coat cell suspension or 1 ml of clarified biopsy preparation supernatant is inoculated on to a 25 cm² tissue culture flask of 90% confluent LT or LK cells, and allowed to absorb for 1 hour at 37°C. 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 or LK cells and a flying cover-slip, or tissue culture microscope slides, are also infected.

   The flasks should be examined daily for 14 days for evidence of cytopathic effect (CPE), and the medium is replaced if it appears to be cloudy. 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 4 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 or LK 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. Syncytia formation is not a feature of capripoxvirus infection. The CPE can be prevented or delayed by inclusion in the medium of specific anti-capripoxvirus serum. Some strains of capripoxvirus have been adapted to grow on African green monkey kidney (Vero) cells, but these are not recommended for primary isolation.

   • Electron microscopy

   Before centrifugation, material from the original suspension is prepared for examination under the transmission electron microscope by floating a 400-mesh hexagon electron microscope grid, with pileoform-
carbon substrate activated by glow discharge in pentyamine vapour, on to a drop of the suspension placed on parafilm 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 $290 \times 270$ nm. A host-cell-derived membrane may surround some of the virions, and as many as possible should be examined to confirm their appearance (16).

The virions of capripoxvirus are indistinguishable from those of orthopoxvirus, but, apart from vaccinia virus, no orthopoxvirus causes lesions in sheep and goats. The virions of parapoxvirus that cause contagious pustular dermatitis are smaller, oval in shape, and each is covered in a single continuous tubular element, which appears as striations over the virion.

- **Histology**

Following preparation, staining with H&E, and mounting of the formalin-fixed biopsy material, a number of sections should be examined by light microscopy. On histological examination, the most striking aspects of acute-stage skin lesions are a massive cellular infiltrate, vasculitis and oedema. Early lesions are characterised by marked perivascular cuffing. Initially infiltration is by macrophages, neutrophils and occasionally eosinophils, and as the lesion progresses, by more macrophages, lymphocytes and plasma cells. A characteristic feature of all capripoxvirus infections is the presence of variable numbers of 'sheep pox cells' in the dermis. These sheep pox cells can also occur in other organs where microscopic lesions of sheep and goat pox are present. These cells are large, stellate cells with eosinphillic, poorly defined intracytoplasmic inclusions and vacuolated nuclei. Vasculitis is accompanied by thrombosis and infarction, causing oedema and necrosis. Epidermal changes consist of acanthosis, parakeratosis and hyperkeratosis. Changes in other organs are similar, with a predominant cellular infiltration and vasculitis. Lesions in the upper respiratory tract are characterised by ulceration.

- **Animal inoculation**

Clarified biopsy preparation supernatant (see Section B.1.a. Culture) may also be used for intradermal inoculation into susceptible lambs. These lambs should be examined daily for evidence of a skin reaction.

### b) Immunological methods

- **Fluorescent antibody tests**

Capripoxvirus antigen can also be identified on 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 sheep or goat sera is subject to high background colour and nonspecific reactions. However, a direct conjugate can be prepared from sera from convalescent sheep or goats or from rabbits hyperimmunised with purified capripoxvirus. Uninfected tissue culture should be included as a negative control because cross-reactions, due to antibodies to cell culture antigens, can cause problems. The fluorescent antibody tissue section technique has also been used on cryostat-prepared slides.

- **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 ($76 \times 26$ 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 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 humidified 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. This test will not, however, distinguish between capripox infection and contagious pustular dermatitis (orf).

To prepare antigen for the AGID, one of two 125 cm$^2$ flasks of LT or LK 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 resuspended 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 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 (14).

- **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). These reagents have facilitated the development of a highly specific ELISA (4). 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 trapped 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.

c) **Nucleic acid recognition methods**

It is not possible to distinguish between strains of capripoxvirus from cattle, sheep or goats using serological techniques. However, strains can be characterised by comparing the genome fragments generated by HindIII digestion of their purified DNA (1, 13). This technique has identified differences between isolates from the different species, but these are not consistent and there is evidence for the movement of strains between species and recombination between strains in the field (9).

The PCR can be used to detect the capripoxvirus genome in biopsy or tissue culture samples. Primers for the viral attachment protein gene and the viral fusion protein gene are specific for capripoxvirus, and the nature of the PCR products can be confirmed using restriction enzyme recognition sites (10, 11).

2. Serological tests

a) **Virus neutralisation**

A test serum can either be titrated against a constant titre of capripoxvirus (100 TCID\(_{50}\) [50% tissue culture infectious 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 TCID\(_{50}\), the neutralisation index is the preferred method. 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 of 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 toxicity 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 0240 KSGP vaccine strain of capripoxvirus, the final reading is taken on day 9, and the titre of virus in each duplicate titration is calculated.
according to Kärber (1931). 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 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.

b) **Agar gel immunodiffusion**

The AGID test cannot be recommended as a serological test for the diagnosis of capripox because of the cross-reaction with antibody to contagious pustular dermatitis virus, which is the main differential diagnosis. A consequence of this cross-reaction is many false-positive 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 \(-20^\circ C\) for 10 minutes and stored at \(4^\circ C\). Dilutions of test sera are made in PBS, starting at 1/5, and positives are identified using an anti-sheep gamma-globulin conjugated with fluorescein isothiocyanate (7). Cross-reactions can occur with orf, bovine papular stomatitis virus 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 (6).

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 then 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), glycine (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. As an alternative to tissue culture antigen, it is possible to use purified virus or expressed recombinant P32 (5, 10).

Molecular weight markers should be run concurrently with the protein samples. The separated proteins in the SDS/PAGE gel should then be transferred by electroblotting to a nitrocellulose membrane (NCM). After blotting, 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 \(4^\circ C\) 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 can be 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 thoroughly washed and incubated (in blocking buffer) with anti-species immunoglobulin horseradish-peroxidase-conjugated immunoglobulins at a dilution predetermined by titration. After further incubation at room temperature for 1.5 hours, the membrane is washed and stained with a solution of 0.04% dianinobenzidin 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 (orf virus) will react with some of the capripoxvirus proteins, but not the 32 kDa protein that is specific for capripoxvirus.
e) Enzyme-linked immunosorbent assay

A capripoxvirus antibody ELISA has been developed using the expressed structural protein P32 of capripoxvirus and MAbs raised against the P32 protein (5, 10).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

A variety of attenuated live and inactivated capripoxvirus vaccines has been used to provide protection to sheep and goats against capripox (see refs 3 and 12 for reviews). All strains of capripoxvirus of ovine, caprine or bovine origin examined so far share a major neutralising site, so that animals recovered from infection with one strain are resistant to infection with any other strain (2). Consequently, it is possible to use a single strain of capripoxvirus to protect both sheep and goats against all field strains of virus, regardless of whether their origin was in Asia or Africa (15, 17).

There are two antigenic forms of capripoxvirus, the intact virion covered in short tubular elements, and the intact virion additionally covered in a host-cell-derived membrane. The latter is the form usually produced by the infected animal, whereas the former is that seen when virus is produced by freeze–thawing infected tissue culture. Dead vaccines produced from tissue culture are almost entirely naked virions, and when used as a vaccine do not stimulate immunity to the membrane-bound virion. This in part explains the poor success of inactivated vaccines. An additional factor is that inactivated vaccines are less effective than live, replicating vaccine virus in stimulating the cell-mediated immune response, which is the predominant protective response to poxvirus infection. Dead capripox vaccines provide, at best, only temporary protection. A number of strains of capripoxvirus have had widespread use as live vaccines (8), for example the 0240 Kenya sheep and goat pox strain used in sheep and goats, the Romanian and RM-65 strains used mainly in sheep, and the Mysore and Gorgan strains used in goats. Immunity in sheep and goats against capripox following vaccination with the 0240 strain lasts over a year, and will probably provide lifelong protection against lethal challenge. The 0240 strain should not be used in *Bos taurus* breeds of cattle.

A new generation of capripox vaccines is being developed that uses the capripoxvirus genome as a vector for the genes of other ruminant pathogens, for instance genes of rinderpest and peste des petits ruminants (PPR) viruses. The recombinant vaccine will provide protection against capripox, rinderpest and PPR in a single vaccination (18).

1. Seed management

a) Characteristics of the seed

A strain of capripoxvirus used for vaccine production must be accompanied by a history describing its origin and tissue culture or animal passage. It must be safe to use in all breeds of sheep and goats for which it is intended, including pregnant animals. It must be nontransmissible, remain attenuated after further tissue culture passage, and provide complete protection against challenge with virulent field strains for a minimum of 1 year. A quantity of master seed vaccine virus should be prepared and stored in order to provide a consistent working seed for regular vaccine production.

b) Method of culture

Vaccine seed should be lyophilised and stored in 2 ml vials at −20°C. It may be stored wet at −20°C, but when wet, is more stable at −70°C or lower. The virus should be cultured in primary or secondary LT or LK cells of wool sheep origin for maximum yield. Vero cells may also be used.

c) Validation as a vaccine

Seed lots must be shown to be:

i) **Pure**: 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.

ii) **Safe**: Produce no clinical reaction in all breeds of sheep or goats when given by the recommended route.

iii) **Efficacious**: Stimulate complete immunity to capripox in all breeds of sheep and goats for at least 1 year.

The necessary tests are described in Section C.4.
2. Method of manufacture

Vaccine batches are produced on fresh monolayers of secondary LT or primary LK cells. A vial of seed virus is reconstituted with GMEM and inoculated on to an LT or LK 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. After 4–6 days, there will be extensive (50–70%) CPE. The culture should be examined for any evidence of nonspecific CPE, medium cloudiness or change in medium pH. The culture is freeze–thawed three times, the suspension removed and centrifuged at 600 g for 20 minutes. 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 from individually numbered flasks are each mixed separately with an equal volume of sterile and chilled 5% lactalbumin hydrolysate and 10% sucrose, 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.

Inactivated vaccines are produced, usually from unattenuated field strains of capripoxvirus, grown in tissue culture as described above, inactivated with 0.03% formaldehyde, and mixed with an equal volume of alhydrogel as adjuvant. Formaldehyde is no longer considered to be a suitable inactivant for certain other viral vaccines because its mode of action cannot be guaranteed to be totally effective in inactivating all the live virus. This has not been fully investigated for capripoxvirus.

3. In-process control

**Cells:** Cells should be obtained from the testis or kidney 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 bovine virus diarrhoea or border disease viruses by immunofluorescence or immunoperoxidase techniques. If possible, cells 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 x 10^6 cells/ml). Serum used in the growth medium must be free from antibody to capripoxvirus or contamination with pestivirus.

**Virus:** Seed virus and final vaccine must be titrated in tissue culture tubes or microtitre plates. 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 tested negative for antibody to pestivirus to prevent the vaccine virus itself interfering with the test. The vaccine bulk can be held at –20°C until all sterility tests and titrations have been completed, at which time it should be freeze-dried in 1 ml aliquots in vials sufficient for 100 doses. The vaccine harvest diluted with lactalbumin hydrolysate and sucrose should have a minimum titre log_{10} 4.5 TCID_{50} per ml after freeze-drying, equivalent to a field dose of log_{10} 2.5 TCID_{50}. A further titration is carried out on five randomly chosen vials of the freeze-dried preparation to confirm the titre.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter I.1.5.

b) Safety and efficacy

Four sheep and four goats of known susceptibility to capripox are placed in a high containment level animal unit and serum samples are collected. Five randomly chosen vials of the freeze-dried vaccine are reconstituted in 1 ml of sterile PBS each, and pooled. One sheep and one goat are inoculated intradermally with 0.2 ml of the concentrated vaccine. The remaining vaccine is diluted 20 times with sterile PBS and two sheep and two goats are inoculated subcutaneously with 0.2 ml – the recommended field dose. The remaining sheep and goat are control animals. The animals are clinically examined daily and rectal temperatures are recorded. On day 21 after vaccination, the eight animals are again serum sampled and 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 capripox, whereas there should be no local or systemic reaction in the vaccines other than a delayed-type hypersensitivity reaction, which will disappear within 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 day 0 and 30 samples are compared to confirm the absence of antibody to pestivirus.
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 due to the vaccine.

c) Potency tests
Less than 1 TCID$_{50}$ of the 0240 strain is sufficient to immunise a sheep or goat. However potency tests must be undertaken for other 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 wool or 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.

d) Duration of immunity
Immunity to virulent field virus following vaccination of sheep or goats with the 0240 strain lasts over 1 year, and protection against generalised infection following intradermal challenge lasts at least 3 years and is effectively lifelong. The duration of immunity produced by other vaccine strains should be ascertained in both sheep and goats by undertaking controlled trials in an environment in which there is no possibility of field strains of capripoxvirus confusing the results.

The inactivated vaccines provide immunity for under 1 year, and for the reasons given at the beginning of this section, may not give immunity to the form of capripoxvirus usually associated with natural transmission.

e) Stability
Properly freeze-dried preparations of capripox vaccine, particularly those that include a protectant, such as sucrose and lactalbumin hydrolysate, are stable for over 25 years when stored at $-20^\circ$C and for 2–4 years stored at 4°C. There is evidence that they are stable at higher temperatures, but no long-term controlled experiments have been reported.

The inactivated vaccines must be stored at 4°C, and their shelf life is usually given as 1 year.

f) Preservatives
No preservatives other than a protectant, such as sucrose and lactalbumin hydrolysate, are required for the freeze-dried preparation.

g) Precautions (hazards)
There are no precautions other than those described above for sterility and freedom from adventitious agents. The 0240 vaccine strain should not be used in $Bos taurus$ breeds of cattle.

Capripoxvirus is not infectious to humans.

5. Tests on the final product

a) Safety
Safety tests should be carried out on the final product of each batch as described in Section C.4.b.

b) Potency
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.
REFERENCES


* *

**NB:** There are OIE Reference Laboratories for Sheep pox and goat pox (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
Chapter 2.1.11.

African Horse Sickness

Summary

African horse sickness (AHS) is an infectious but noncontagious viral disease affecting all species of equidae caused by an orbivirus of the family Reoviridae and characterised by alterations in the respiratory and circulatory functions. AHS is transmitted by at least two species of Culicoides. Nine different serotypes have been described.

All serotypes of AHS occur in eastern and southern Africa. Only AHS serotype 9 and 4 have been found in West Africa from where they occasionally spread into countries surrounding the Mediterranean. Examples of outbreaks that have occurred outside Africa are: in the Middle East (1959–1963), in Spain (serotype 9, 1966, serotype 4, 1987–1990), and in Portugal (serotype 4, 1989).

Laboratory diagnosis of AHS is essential. Although the clinical signs and lesions are characteristic, they can be confused with those of other diseases.

As a viral disease, the laboratory diagnosis of AHS can be based on the identification of infectious virus, virus nucleic acid, viral antigens or specific antibodies. Over the past few years, a wide variety of laboratory tests have been adapted for the detection of both AHS virus (AHSV) and specific antibodies.

Identification of the agent: it is important to perform virus isolation and serotyping whenever outbreaks occur outside the enzootic regions.

AHSV can be isolated from blood collected during the early febrile stage. For virus isolation, the other tissues of choice for diagnosis are spleen, lung, and lymph nodes, collected at necropsy. Sample preparations can be inoculated in cell cultures, such as baby hamster kidney-21 (BHK-21), monkey stable (MS) or African green monkey kidney (Vero), and intracerebrally in newborn mice. Several enzyme-linked immunosorbent assays (ELISAs) for the rapid detection of AHSV antigen in spleen tissues and supernatant from infected cells have been developed. Identification of AHSV RNA has also been achieved using a reverse-transcription polymerase chain reaction method. Virus isolates can be serotyped by a type-specific serological test such as virus neutralisation (VN) and by reverse-transcription polymerase chain reaction.

Serological tests: Horses that survive natural infection develop antibodies against the infecting serotype of AHSV within 8–12 days post-infection. This may be demonstrated by several serological methods, such as complement fixation test, ELISA, immunoblotting and VN. The latter test is used for serotyping. Other tests that have been described are immunodiffusion and haemagglutination inhibition.

Requirements for vaccines and diagnostic biologicals: Attenuated (monovalent and polyvalent) live vaccines for use in horses, mules and donkeys, are currently available. A monovalent inactivated vaccine has been produced commercially, but is no longer available. New vaccines, including a subunit vaccine, have been evaluated experimentally.

A. Introduction

African horse sickness (AHS) (Peste equina africana, Peste equine) is an infectious, noncontagious arthropod-borne disease of equidae, caused by a double-stranded RNA orbivirus belonging to the family Reoviridae. The genus Orbivirus also includes bluetongue virus and epizootic haemorrhagic disease virus, which have similar morphological and biochemical properties with distinctive pathological and antigenic properties as well as host
ranges. The genome of AHS virus (AHSV) is composed of ten double-stranded RNA segments, which encode seven structural proteins (VP1-7), most of which have been completely sequenced for AHSV serotypes 4, 6 and 9 (25, 31, 34) and four nonstructural proteins (NS1, NS2, NS3, NS3A) (9, 17). Proteins VP2 and VP5 form the outer capsid of the virion, and proteins VP3 and VP7 are the major inner capsid proteins. Proteins VP1, VP4 and VP6 constitute minor inner capsid proteins. Recently it was indicated that NS3 proteins are the second most variable AHSV proteins (32), the most variable being the major outer capsid protein, VP2. This protein, VP2, is also the principal responsible for AHSV serotypes and, together with VP5, for virus neutralisation activity (23). Nine antigenically distinct serotypes of AHSV have been identified by virus neutralisation, but no cross-reactions with other known orbiviruses have been observed.


The disease has both a seasonal (late summer/autumn) and a cyclical incidence with major epizootics in southern Africa during warm-phase events (1). Mortality due to AHS is related to the species of equidae affected and to the strain or serotype of the virus. At least two field vectors are involved: Culicoides imicola and C. bolitinos. Among the equidae family, horses are the most susceptible to AHS with a mortality rate of 50–95%, followed by mules with mortality around 50%. In enzootic regions of Africa, donkeys are very resistant to AHS and experience only subclinical infections. In European and Asian countries, however, donkeys are moderately susceptible and have a mortality rate of 10%. Zebras are also markedly resistant with no clinical signs, and may have extended viraemia (up to 40 days).

### B. DIAGNOSTICS TECHNIQUES

Laboratory diagnosis is essential. Although some clinical signs and lesions are characteristic, AHS can be confused with other diseases. For example, the supraorbital swelling, which is often present in horses with subacute AHS, is, in combination with an appropriate history, sufficient for a tentative diagnosis. Other signs and lesions are less specific for AHS, and other diseases such as equine encephalitis, equine infectious anaemia, equine morbillivirus pneumonia, equine viral arteritis, babesiosis and purpura haemorrhagica should be excluded. There are four classical forms of AHS: pulmonary, cardiac, mixed, and horse sickness fever (6).

The peracute or pulmonary form, which has a short incubation period (3–5 days), is characterised by very marked by severe dyspnoea and progressive respiratory involvement. An acute febrile reaction, lasting 1–2 days and reaching a maximum of approximately 40–41°C, may be the only sign. This is followed by various degrees of respiratory distress – respiratory rate may increase to 60 or even 75 breaths/minute. The animal may be observed to stand with its forelegs spread apart, its head extended and its nostrils fully dilated. Profuse sweating is common and spasmodic coughing may be observed terminally, with frothy fluid exuding from the nostrils. Death usually occurs within a few hours after the first clinical signs are observed, the animal having literally drowned in its own serous fluid. The pulmonary form is usually observed in completely susceptible animals, animals infected with a highly virulent strain of virus, or animals that are worked during the febrile stage of the disease. Recovery from this form is very rare, occurring in <5% of cases.

The incubation period of the subacute, oedematous or cardiac form varies from about 7 to 14 days, and the onset of clinical disease is marked by a febrile reaction (39–41°C) that lasts for 3–6 days. Shortly before the decline of the fever, characteristic oedematous swellings may appear. These initially involve the temporal or supraorbital fossae and the eyelids, and later extend to the lips, cheeks, tongue, intermandibular space and laryngeal region. Subcutaneous oedema sometimes extends a variable distance down the neck towards the chest and, in severe cases, may involve the chest and shoulders, but generally not the lower limbs. Terminally, petechial haemorrhages may be observed in the conjunctivae and under the ventral surface of the tongue. The animal finally becomes restless and may show signs of colic before death from cardiac failure. Difficulty in swallowing due to paralysis of the oesophagus is also seen. The mortality rate is about 50% and death usually occurs within 4–8 days after the onset of the febrile reaction. In recovering cases, swelling gradually subsides within a period of 3–8 days. This clinical form of AHS is usually associated with infection by virus strains of low virulence or is encountered in immune animals infected by heterologous virus strains, or may be a function of biological variation in the infected animal.

The acute or mixed form represents a mixture of the pulmonary and cardiac forms. Although seldom diagnosed clinically, it is the most common form and is seen at post-mortem examination in most fatal cases of AHS in horses and mules. The incubation period varies from 5 to 7 days, and the disease may manifest itself in the following ways:

- Initial pulmonary signs of a relatively mild degree are followed by marked oedematous swellings of the head and neck, with death resulting from heart failure.
- Oedematous swelling, typical of the subacute form, is followed by the sudden onset of dyspnoea and other clinical signs typical of the peracute pulmonary form.
The mortality rate in the mixed form is >80% and death usually follows within 3–6 days after the onset of the febrile reaction.

Horse sickness fever is the mildest form and is frequently overlooked in natural outbreaks. The incubation period varies from 5 to 14 days, and is followed by a febrile reaction (39–40°C) of the remittent type, with morning remissions and afternoon exacerbations, lasting for 5–8 days. Apart from the febrile reaction, other clinical signs are rare. The conjunctivae may be slightly congested, the pulse rate may be increased, and a certain degree of anorexia and depression may be present. This form of the disease is usually observed in partially immune animals or in resistant species, such as the donkey and zebra.

There is no evidence that humans can become infected with any field strain of AHSV, either through contact with naturally or experimentally infected animals or by virus manipulation in laboratories. However, certain neurotropic vaccine strains that may cause encephalitis and retinitis in humans following transnasal infections have been described (24). Experimental and natural transmission of AHS to dogs has been reported through ingestion of infected horse meat (3). However, there is only limited and unsubstantiated evidence that dogs become infected by insect bites (30).

1. Identification of the agent

Several techniques are already available for AHS viral identification ranging from the rapid enzyme-linked immunosorbent assay (ELISA), using either polyclonal antibodies (PAbs) or monoclonal antibodies (MAbs), to the polymerase chain reaction (PCR) test, including a new reverse-transcription (RT) PCR for discrimination of the nine AHSV serotypes (27), or cell culture and inoculation of newborn mice. If possible more than one test should be performed to diagnose an outbreak of AHS, especially the index case. The initial test can be a quick test such as ELISA or PCR, followed by virus isolation in tissue culture. Virus neutralisation (VN) for serotype identification should be performed as early in the outbreak as possible so that the correct vaccine can be selected. Subsequently, the ELISA may be very useful in laboratory diagnosis.

At present, there are no international standards for viruses or diagnostic reagents, and there is no standard methodology for the determination of AHSV. However, a panel of viruses has been evaluated, and comparative studies between different ELISAs for AHSV antigen determination have been carried out in different laboratories. The results have demonstrated a high level of correlation for antigen detection (26) using the indirect sandwich ELISAs for antigen studies (11, 16).

A very important aspect of the diagnosis is the selection of samples and their transportation to the laboratory.

a) Samples for virus isolation

Unclotted whole blood collected during the early febrile stage of the disease from sick animals, as well as small pieces (2–4 g) of spleen, lung and lymph nodes from animals that have died, are the samples of choice for diagnosis. Samples should be kept at 4°C during transportation and storage.

b) Cell culture

Direct isolation of AHSV on baby hamster kidney (BHK-21), monkey stable (MS) and African green monkey kidney (Vero) cell lines has been used successfully. Blood samples collected in heparin can be used undiluted. After 60 minutes of adsorption, the cell cultures are washed and maintenance medium is added. Alternatively and more usually, the blood is washed, lysed and diluted 1/10. This procedure removes unwanted antibody, which could neutralise free virus, and promotes release of virus associated with the red blood cell membranes. When tissue samples, such as spleen, lung etc., are used, a 10% tissue suspension is prepared in phosphate buffered saline (PBS) or cell culture medium, containing antibiotics.

A cytopathic effect (CPE) may appear between 2 and 8 days post-infection. Three blind passages should be performed before considering the samples to be negative.

c) Newborn mice

This method of isolation of AHSV involves the intracerebral inoculation of two families of 1–3-day-old mice. In positive cases, animals develop nervous signs between 3 and 15 days post-inoculation. The brains from sick animals must be collected, homogenised and re-inoculated intracerebrally into at least six 1–3-day-old mice. This second passage should present a shortened incubation period (2–5 days) and 100% infectivity.

d) Sandwich enzyme-linked immunosorbent assay

At least two sandwich ELISAs have been developed and field tested for detection of AHSV antigen from both field samples and laboratory-infected tissue cultures (11, 16).
One technique (11) uses PAbs to the AHSV and the other (16) uses MAbs against one of the major proteins that is more conserved among serotypes – protein VP7. Both methods have been demonstrated to be adequate for the diagnosis of AHS, due to their high sensitivity and specificity and the availability of results in only 2–4 hours (26). The use of a chicken IgY in a double-antibody sandwich ELISA for detecting all AHSV serotypes has been described (5).

Reagents for the ELISA may be obtained from the OIE Reference Laboratories for African horse sickness (see Table given in Part 3 of this Manual).

- The following is an example of a monoclonal antibody enzyme-linked immunosorbent assay
  
  i) **Solid phase:** Coat ELISA plates (e.g. high adsorption capacity Nunc Maxisorb) with a mixture of MAb 5G5 and 3D2 diluted in PBS, pH 7.2 (10 µg/ml each). Incubate overnight at 4°C.
  
  ii) Wash the plates five times with distilled water containing 0.05% (v/v) Tween 20 (washing solution). Gently tap the plates on to absorbent material to remove any residual wash.
  
  iii) Block the plates with PBS + 1% bovine serum albumin (BSA), pH 7.2, 200 µl/well, for 1 hour at 37°C.
  
  iv) Remove the blocking solution and gently tap the plates on to absorbent material.
  
  v) **Test samples:** Add the samples to be tested (twofold dilutions starting with undiluted spleen homogenates, or AHSV cell culture supernatant) diluted in PBS + 1% BSA, pH 7.2, 100 µl/well. Incubate for 1 hour at 37°C. (Spleen homogenate: homogenise approximately 2 cm³ [1 g] of spleen with 3 ml of MEM [minimal essential medium] culture medium. Centrifuge at 600 g for 10 minutes and save the supernatant.)
  
  vi) Wash the plates as described in step ii.
  
  vii) **Conjugate:** Dispense 100 µl/well of biotin-labelled 5G5 MAb diluted 1/500 in PBS + 1% BSA, pH 7.2. Incubate for 1 hour at 37°C. Wash the plates as described in step ii. Add 100 µl/well of avidin/peroxidase at optimal dilution in PBS + 1% BSA. Incubate for 45 minutes at room temperature.
  
  viii) Wash the plates as described in step ii.
  
  ix) **Substrate:** Add 200 µl/well of substrate solution (10 ml of 80.6 mM DMAB [dimethyl aminobenzaldehyde] + 10 ml of 1.56 mM MBTH [3-methyl-2-benzo-thiazolinone hydrazone hydrochloride] + 5 µl H₂O₂). Colour development is stopped by adding 50 µl of 3 N H₂SO₄ after approximately 5–10 minutes (before the negative control begins to be coloured).
  
  x) Read the plates at 600 nm (or 620 nm).
  
  xi) **Interpretation of the results:** Calculate the cut-off value as follows: C ± 0.06 = cut-off (where C is the absorbance value obtained with the negative control). Test samples giving absorbance values lower than the cut-off are regarded as negative. Test samples giving absorbance values greater than the cut-off + 0.20 are regarded as positive. Test samples giving intermediate absorbance values are doubtful and a second technique must be employed to confirm the result.

**e) Polymerase chain reaction**

A PCR assay for the specific detection of AHSV RNA has been developed. Primers correspond to the 5' end (nucleotides 1–21) and 3' end (nucleotides 1160–1179) of RNA segment 8 (20, 29, 35).

- **Test procedure**

Extraction of nucleic acids from spleen samples is carried out as follows: 1 g of tissue sample is homogenised in 1 ml of denaturing solution (4 M guanidium thiocyanate, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol, 0.5% sarcosyl). After centrifugation, 1 µg of yeast RNA, 0.1 ml of 2 M sodium acetate pH 4, 1 ml of phenol and 0.2 ml of chloroform/isoamyl alcohol mixture (49/1) are added to the supernatant. The suspension is vigorously shaken and cooled on ice for 15 minutes. After centrifugation, the RNA present in the aqueous phase is phenol extracted, ethanol precipitated and resuspended in sterile water. The methods for cDNA synthesis and PCR amplification are performed using, in all cases, 37°C as renaturing temperature. The sequences of the PCR primers used are 5'-GTT-AAA-ATT-CGG-TTA-GTG-3', which corresponds to the messenger RNA polarity and 5'-GTA-AGT-GTA-TTC-GGT-ATT-G-3', which is complementary to the messenger RNA polarity. The PCR procedure itself involves 40 cycles (94°C for 1 minute, 55°C for 1.5 minutes, 72°C for 2.5 minutes and 70°C for 7 minutes) and then the PCR tubes are kept at 4°C. Analysis of the PCR products is carried out by electrophoresis in 1.2% (w/v) agarose gels containing ethidium bromide. AHS-positive samples will resolve in a 1179 base-pair band.

A new RT-PCR for discrimination of the nine AHSV serotypes has been described (27). Nine pairs of primers were designed for each specific serotype. The results obtained show a perfect agreement between the RT-PCR and the VN test.
Typing of nine AHS serotypes has also been carried out with probes developed from a set of cloned full-length VP2 genes and can be an alternative to amplification of genome segment 2 (15).

2. Serological tests

OIE International Reference sera are available from the OIE Reference Laboratory in Valdeolmos, Madrid, Spain (see Part 3 of this *Terrestrial Manual*). These sera were developed to standardise the ELISA, which is an OIE prescribed test. In addition, a panel of reference antisera has been evaluated and comparative studies between different ELISAs using MAbs and PAs, and involving several laboratories have been carried out. The results have demonstrated a high level of correlation using indirect or competitive ELISAs for antibody detection (10, 18, 26). More recently, a panel of sera has been generated and is now being used for the annual quality assurance of three antibody detection ELISAs as used by the national laboratories in Europe (8, 10, 18).

Indirect and competitive ELISAs using either soluble AHSV antigen or a recombinant protein VP7 (18), and a competitive ELISA using soluble AHSV antigen (10), have proved to be good methods for the detection of anti-AHSV group-reactive antibodies, especially for large-scale investigations (26). Both of these tests have been recognised by the European Commission (8). The competitive ELISA can also be used for testing wildlife as species-specific anti-globulin is not required with this method. An immunoblotting test has been adapted for anti-AHS antibody determination (18). It is especially suitable for small numbers of sera. Indirect ELISA is also available that uses the AHSV serotype 4 nonstructural protein NS3 as antigen, and can be used for the differentiation of animals infected or vaccinated with the live vaccine from those vaccinated with the inactivated vaccine made with purified virions (19). The complement fixation (CF) test has been widely used, but some sera are anti-complementary.

a) Indirect enzyme-linked immunosorbent assay (a prescribed test for international trade)

The recombinant VP7 protein has been used as antigen for AHSV antibody determination with a high degree of sensitivity and specificity (18, 33). Other advantages of this antigen are its stability and its lack of infectivity.

- Test procedure
  1. **Solid phase:** Coat ELISA plates (e.g. high adsorption capacity Nunc Maxisorb) with recombinant AHSV-4 VP7 diluted in carbonate/bicarbonate buffer, pH 9.6. Incubate overnight at 4°C.
  2. Wash the plates five times with distilled water containing 0.01% (v/v) Tween 20 (washing solution). Gently tap the plates on to absorbent material to remove any residual wash.
  3. Block the plates with PBS, pH 7.2 + 5% (w/v) skimmed milk, 200 µl/well, for 1 hour at 37°C.
  4. Remove the blocking solution and gently tap the plates on to absorbent material.
  5. **Test samples:** Serum samples to be tested, and positive and negative control sera, are diluted 1/25 in PBS + 5% (w/v) skimmed milk + 0.05% (v/v) Tween 20, 100 µl/well. Incubate for 1 hour at 37°C. For titration, add twofold dilution series from 1/25 (100 µl/well), one serum per plate column, and do the same with positive and negative controls. Incubate for 1 hour at 37°C.
  6. Wash the plates as described in step ii.
  7. **Conjugate:** Dispense 100 µl/well of horseradish peroxidase conjugated anti-horse gamma-globulin diluted in PBS + 5% milk + 0.05% Tween 20, pH 7.2. Incubate for 1 hour at 37°C.
  8. Wash the plates as described in step ii.
  9. **Substrate:** Add 200 µl/well of substrate solution (10 ml DMAB + 10 ml of MBTH + 5 µl H2O2). Colour development is stopped by adding 50 µl of 3 N H2SO4 after approximately 5–10 minutes (before the negative control begins to be coloured). Other substrates such as ABTS (2,2’-azino-di-[3-ethylbenzothiazoline]-6-sulphonic acid), TMB (tetramethyl benzidine), or OPD (orthophenyldiamine) can also be used.
  10. Read the plates at 600 nm (or 620 nm).
  11. **Interpretation of results:** Calculate the cut-off value by adding 0.6 to the value of the negative control. (0.06 is the standard deviation derived with a group of 30 negative sera) Test samples giving absorbance values lower than the cut-off are regarded as negative. Test samples giving absorbance values greater than the cut-off + 0.15 are regarded as positive. Test samples giving intermediate absorbance values are doubtful and a second technique must be employed to confirm the result.

b) Immunoblotting

The binding of antibodies to viral proteins separated by electrophoresis and transferred to nitrocellulose paper has been used for the determination of anti-AHSV antibodies (18).
Chapter 2.1.11. – African horse sickness

• **Test procedure**

Semipurified proteins of AHSV are separated by SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis) in 15% (w/v) acrylamide-N,N'-diallyltartrar-diamide (DATD) gels. Separated proteins are transferred to a nitrocellulose membrane filter at a constant current of 280 mA for 6 hours at 4°C. Immunoblotting of a nitrocellulose membrane cut into strips is carried out using the sera at 1/20 dilution, peroxidase-conjugated rabbit-anti-horse immunoglobulin at 1/500 dilution and an incubation time of 1 hour at 37°C. The bands recognised by the sera are developed by the 4-chloro-naphthol technique.

**Interpretation of results:** The comparison between the band pattern of a positive control and a negative control serum permits the identification of the specific viral bands. The appearance of two or more of these specific bands in a problem serum allows it to be classified as positive anti-AHS serum.

c) **NS3 enzyme-linked immunosorbent assay**

An indirect ELISA to distinguish between infected horses and horses vaccinated with the inactivated purified AHSV serotype 4 vaccine, using a recombinant NS3 protein as antigen, has been adapted and evaluated. The results obtained (19) indicate that recombinant NS3 can indeed differentiate between infected and vaccinated animals implying that this recombinant could be an important diagnostic reagent that could allow the transportation of vaccinated horses. To ensure the reliability of results it is essential that the selected AHSV-inactivated vaccine be a purified vaccine. This is to exclude with certainty any trace of NS3 that, if present, would stimulate the production of anti-NS3 antibodies in vaccinated horses, thus mimicking the response to a natural infection. This ELISA will also be useful for distinguishing between infected horses and horses vaccinated with a subunit vaccine in which NS3 protein is not present.

d) **Complement fixation (a prescribed test for international trade)**

The CF test has been used extensively, but due to the anti-complementary effect of some sera, as well as the good results obtained with the ELISA, its use is decreasing. The CF test is frequently used for the demonstration of group-specific antibodies against AHSV. A sucrose/acetone mouse-brain extract is commonly used as antigen.

• **Reagents**

i) Veronal buffered saline containing 1% gelatin (VBSG).

ii) Serum samples, free from erythrocytes, must be heat inactivated: horse serum at 56°C, zebra serum at 60°C and donkey serum at 62°C, for 30 minutes.

iii) The antigen is a sucrose/acetone extract of AHSV-infected mouse brain. The control antigen is uninfected mouse brain, extracted in the same way. In the absence of an international standard serum, the antigen should be titrated against a locally prepared positive control serum. In the test, four to eight units are used.

iv) The complement is a normal guinea-pig serum.

v) The haemolysin is a hyperimmune rabbit serum against sheep red blood cells (SRBCs).

vi) The SRBCs are obtained by aseptic puncture of the jugular vein and preserved in Alsever’s solution or sodium citrate.

vii) The haemolytic system (HS) is prepared by diluting the haemolysin to contain two haemolytic doses and using this to sensitise washed SRBCs. The SRBCs are standardised to a 3% concentration.

viii) **Control sera:** A positive control serum is obtained locally and validated. Serum from a healthy antibody-negative horse is used as the negative control serum.

• **Test procedure**

i) Sera, complement and antigen are reacted in 96-well round-bottom microtitre plates, or in tubes if the macro-technique is used, at 4°C for 18 hours.

ii) Sensitised SRBCs (3%) are added to all wells on the microtitre plate.

iii) The test plate is incubated for 30 minutes at 37°C.

iv) Plates are then centrifuged at 200 g, and the wells are scored for the presence of haemolysis.

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1 20.5 g dextrose (114 mM), 7.9 g sodium citrate 2H₂O (27 mM), 4.2 g NaCl (71 mM), H₂O to 1 litre. Adjust to pH with 1 M citric acid.
v) The following controls are used: (a) serum and complement; (b) serum and SRBCs; (c) CF antigen and control antigen each with 4 CH₅₀ (50% complement haemolytic units), 2 CH₅₀, and 1 CH₅₀ of complement; (d) CF antigen and SRBCs; (e) control antigen and SRBCs; (f) complement dilutions of 4 CH₅₀, 2 CH₅₀, and 1 CH₅₀, and (g) SRBCs.

vi) Results are read using 50% haemolysis as the end point. The inverse of the highest dilution of serum specifically fixing complement with the CF antigen is called the titre.

vii) A titre of 1/10 or more is positive, under 1/10 is negative.

e) Virus neutralisation

VN is the method of choice for serotyping. The test may be performed as follows (12, 13).

i) Stock virus is diluted to yield 30–100 TCID₅₀ (50% tissue culture infective dose) per 25 µl, and 25 µl is added to each of four microtitre wells containing 25 µl serum dilutions. For screening, a final serum dilution of 1/10 is used. Doubling dilutions are used for titrations.

ii) Serum/virus mixtures are incubated for 60 minutes at 37°C prior to the addition of 0.1 ml of Vero cell suspension (200,000 cells/ml) to each test well.

iii) A back titration of virus stock is prepared for each test using four wells per tenfold dilution, 25 µl per well. Test plates are incubated at 37°C, 5% CO₂, 95% humidity for 4–5 days, until the back titration indicates that the stock virus contains 30–100 TCID₅₀.

iv) The plates are then fixed and stained in a solution of 0.15% (w/v) crystal violet in 2% (v/v) glutaraldehyde and rinsed. Alternatively, they may be fixed with 70% ethanol and stained with 1% basic fuschsin.

v) The 50% end-point titre of the serum is calculated by the Spearman–Kärber method and expressed as the negative log₂⁰.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Polyvalent or monovalent live attenuated AHS vaccines based on the selection in Vero cell culture of genetically stable macroplaques are in general use (7). An inactivated monovalent (serotype 4) AHSV vaccine based on virus purification and inactivation with formalin has been produced commercially, but is not available at the present time (4, 14). Requirements for both attenuated and inactivated vaccines are summarised below.

Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 are intended to be general in nature and may be supplemented by national and regional requirements.

C1. Attenuated African horse sickness vaccine

1. Seed management

a) Characteristics of the seed

The seed virus is prepared by selection in Vero cells of genetically stable large plaques from low passage levels of AHSV. The plaque mutants are then further multiplied by three passages in Vero cells. A large quantity of this antigen is lyophilised and stored at –20°C as seed stock antigen.

b) Method of culture

The seed virus is grown in roller cultures of Vero cells.

c) Validation as a vaccine

The seed virus must be shown to be free of contaminating viruses, bacteria and mycoplasmas by the appropriate techniques. The serotype identity of the seed virus is confirmed.

2. Method of manufacture

At the onset of a production run, working antigens are produced from the seed stock antigen in either BHK-21 or Vero cell cultures. The working antigens are tested for sterility, purity and identity and should contain at least 1 × 10⁶ plaque-forming units (PFU)/ml of infectious virus.
Roller bottle cultures of Vero or BHK-21 cells are grown using gamma-irradiated bovine serum in the growth medium. Once the cultures are confluent, the medium is poured off and the cells are seeded with the working antigens. After 1 hour, maintenance medium is added to the cultures. Incubation is continued at 37°C for 2–3 days. When the CPE is advanced, both cells and supernatant medium are harvested. The products from the same serotype are pooled and stored at 4°C.

3. In-process control

The pooled harvests of the individual serotypes are tested for sterility and assayed for infectivity by plaque titration on Vero cell cultures. The minimum acceptable titre is $1 \times 10^6$ PFU/ml.

Finally, two quadrivalent vaccines are constituted by mixing equal volumes of serotypes 1, 3, 4, 5 and 2, 6, 7, 8 respectively. Subsequently, AHSV serotype 5 was withdrawn from this vaccine. A monovalent type can also be prepared. After addition of suitable stabiliser, the vaccine is distributed in 1.0 ml volumes into glass vials and freeze-dried.

4. Batch control

a) Sterility

Following lyophilisation, five bottles of vaccine are selected at random and tested for sterility by internationally accepted methods. Tests for sterility and freedom from contamination of biological products are given in Chapter I.1.5.

b) Safety

 Innocuity of a vaccine is determined by the inoculation of reconstituted vaccine into mice (0.25 ml intraperitoneally), guinea-pig (1.0 ml intraperitoneally), and a horse (5.0 ml subcutaneously). All the animals are observed daily for 14 days. The rectal temperature of the horse is taken twice daily for 14 days and should never exceed 39°C.

c) Potency

Potency is largely based on virus concentration in the vaccine.

The minimum immunising dose for each serotype is about $1 \times 10^3$ PFU/dose. The infectivity titre of the final product is assayed by plaque titration in Vero cell cultures and should contain at least $1 \times 10^5$ PFU/dose. The horse used for safety testing is also used for determining the immunogenicity of a vaccine.

Serum samples are collected on the day of vaccination and 21 days later, and are tested for neutralising antibodies against each serotype by the plaque-reduction test using twofold serum dilutions and about 100 PFU of virus. The horse should develop a neutralising antibody titre of at least 20 against at least three of the four serotypes in the quadrivalent vaccine.

d) Duration of immunity

Duration of immunity is not assessed with every batch of vaccine, but immunity is known to persist for at least 4 years. However, in the light of possible interference between the individual serotypes in each quadrivalent vaccine, annual revaccination is advocated in enzootic regions. Vaccination with monovalent vaccine stimulates a practically lifelong immunity.

e) Stability

In the lyophilised state, the vaccine is known to retain its potency for many years when stored at 4–8°C. However, an expiry date of 2 years is normally given.

C2. Inactivated African horse sickness vaccine

1. Seed management

a) Characteristics of the seed

The seed virus is the attenuated vaccine strain (AHSV serotype 4) used widely in the field in Africa and southern Europe (seed stocks are available from the OIE Reference Laboratory [Onderstepoort]). The virus was passaged ten times in the brains of newborn mice for attenuation, and then passaged a further ten times in roller tube cultures of BHK-21 cells. This material was plaque-purified three times in Vero cell cultures by selection of a large plaque (4–6 mm) at terminal dilutions. The final plaque material was
passaged once in the brain of newborn mice and four times in Vero cell cultures. This material was lyophilised and constitutes the master seed virus.

b) Method of culture
The attenuated virus is propagated by passage in BHK-21 cells in roller bottles.

c) Validation as a vaccine
The seed virus must be shown to be free of contaminating viruses, bacteria and mycoplasmas by the appropriate techniques. The serotype identification is confirmed by a double-sandwich ELISA using MAbs.

2. Method of manufacture

Virus is harvested when characteristic CPE is fully developed. Cultivation thereafter is carried out in cell suspensions with serum-free Stoker’s medium (MEM) at 37°C. Virus growth is checked by cell viability (CPE), and when optimum conditions are reached, the fermentor culture temperature is lowered to 4°C. The virus suspension is aseptically harvested.

The filtered suspension of attenuated virus is inactivated with formaldehyde at a final concentration of 1/1,000. The suspension is transferred to another tank and kept at 4°C under mild agitation for at least 10 days to ensure that all virus is inactivated. Inactivation controls are also performed.

After inactivation, the antigen suspension is concentrated by ultrafiltration, then purified by selective precipitation by a complex of ethylene oxide and bivalent cations according to a patented process.

3. In-process control

Control of virus identity by double-sandwich ELISA, titration of virus before inactivation in Vero cell lines, sucrose gradient analysis to estimate the viral particle concentration, and sterility tests are performed.

4. Batch control

a) Sterility
Tests for sterility and freedom from contamination of biological products are given in Chapter I.1.5.

b) Safety
AHSV-susceptible horses are inoculated intramuscularly or subcutaneously with a single or double dose of vaccine. Temperatures are recorded daily for 14 days, and horses are observed for signs of abnormality. Five guinea-pigs receive a horse vaccine dose by the intramuscular route. Sera collected at day 21 are tested for antibodies by VN.

c) Potency
The potency control is performed by challenge of vaccinated horses. Some animals are vaccinated with one dose, and others with two doses, of vaccine. The challenge is made by the intravenous route at 77 days after the first vaccine inoculation. Where two vaccinations are used, the second dose is given 21 days after the first dose.

Serum samples are collected on the day of vaccination and 21 days later, and are tested for neutralising antibodies and virus isolation.

d) Duration of immunity
No challenges have been performed on horses, only serological studies have been carried out. If the level of antibodies after two injections (recommended field protocol) and at 12 months post-vaccination is equivalent to the levels conferred by a single vaccination at 28 days post-vaccination, the horses are considered to be protected. With this standard, the duration of immunity is 1 year, starting 7–10 days after the first injection. One booster every year is sufficient to confer a protective level in the following years. The protocol for vaccination recommended by the manufacturer is two vaccinations (given 21 days apart) and one booster given at 365 days after the first vaccination.

e) Stability
Due to the fact that the inactivated vaccine is a relatively new product, not much data are available on the duration of its stability. Results have been obtained that demonstrate vaccine stability in excess of 3 years.
C3. African horse sickness subunit vaccine

AHSV serotype 4 outer capsid protein VP2 and VP5 plus inner capsid protein VP7, derived from single and dual recombinant baculovirus expression vectors were used in different combination to immunise horses (21). The crude cell extracts containing the three structural proteins was sufficient to obtain a complete protective immune response in horses challenged with AHS virulence virus (10^6 TCID50). Viraemia was not detectable in vaccinated horse (21). Further analysis of partially protective crude lysate revealed that only soluble VP2 was capable of inducing neutralising antibodies. A definition of neutralising sites of the VP2 virus protein of AHSV serotypes 3, 4 and 9 has been determined (2, 22, 31) and very recently, the full protection of horses immunised with a soluble recombinant VP2 protein (AHSV serotype 5) administrated with saponin to a lethal AHSV challenge has been reported (28). Although further experiments need to be performed to estimate the duration of the immunity induced by these proteins, the data indicate the effectiveness of this candidate vaccine.

REFERENCES


* *

**NB:** There are OIE Reference Laboratories for African horse sickness (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.1.12.

AFRICAN SWINE FEVER

SUMMARY

African swine fever (ASF) is an infectious disease of domestic and wild pigs that affects animals of all breeds and ages, and which is caused by a virus that produces a range of syndromes. Acute disease is characterised by high fever, haemorrhages in the reticuloendothelial system, and a high mortality rate. Infectious virus can survive for several months in fresh and salted dried-meat products.

ASF virus is the only member of the Asfarviridae family.

Laboratory diagnostic procedures for ASF fall into two groups: the first contains the tests for virus isolation and the detection of virus antigens and genomic DNA, while the second contains the tests for antibody detection. The selection of the tests to be carried out depends on the disease situation in the area or country.

In countries free from ASF but suspecting its presence, the laboratory diagnosis must be directed towards isolation of the virus by simultaneously carrying out the inoculation of pig leukocyte or bone marrow cultures, the detection of antigen in smears or cryostat sections of tissues by fluorescent antibody test (FAT) and, where possible, the detection of genomic DNA by the polymerase chain reaction (PCR). The detection of antibodies in tissue fluids by the indirect fluorescent antibody test should also be carried out at the same time.

Identification of the agent: Tissues submitted from suspected cases of disease in the field should be examined for specific antigen by the FAT on smears or cryostat sections, and for the presence of virus by inoculation of primary pig leukocyte cultures, which are examined daily for haemadsorption and cytopathic effects. The cells from negative cultures are examined for antigen by FAT and subinoculation into fresh leukocyte cultures.

The PCR can be used to detect virus genome in tissues and is especially useful if these are unsuitable for virus isolation and antigen detection.

In doubtful cases, the material is passaged and the procedures described above are repeated.

Serological tests: Where the disease is endemic, or where a primary outbreak is caused by a strain of low virulence, the investigation of new outbreaks should include the detection, using the enzyme-linked immunosorbent assay, of specific antibodies in serum or extracts of the tissues submitted.

Requirements for vaccines and diagnostic biologicals: At present, there is no vaccine for ASF.

A. INTRODUCTION

African swine fever virus (ASFV) was originally classified as a member of the family Iridoviridae, but the structure of the genome and the replication strategy of the virus have been shown to have many features in common with members of the Poxviridae (14). The proposal that ASFV be placed in a family separate from the Iridoviridae was accepted at the Sixth Meeting of the International Committee on the Taxonomy of Viruses (ICTV) in Sendai (Japan) in 1984. This virus is currently classified as the only member of a family called Asfarviridae.

ASF viruses produce a range of syndromes varying from peracute to chronic disease, and apparently healthy virus carriers. The more virulent strains produce peracute or acute haemorrhagic disease characterised by high fever, loss of appetite, haemorrhages in the skin and internal organs, and death in 2–10 days. Mortality rates may be as high as 100%. Less virulent strains produce mild clinical signs – slight fever, reduced appetite and
depression – which can be readily confused with many other conditions in pigs and may not lead to suspicion of ASF. In some countries, avirulent, nonhemadsorbing strains produce mainly subclinical nonhemorrhagic infection and seroconversion, but some animals may develop discrete lesions in the lungs or on the skin in areas over bony protrusions and other areas subject to trauma.

ASF cannot be differentiated from classical swine fever (hog cholera) by either clinical or post-mortem examination, and both diseases should be considered in the differential diagnosis of any acute febrile hemorrhagic syndrome of pigs. Bacterial septicaemias may also be confused with ASF and classical swine fever. Laboratory tests are essential to distinguish between these diseases.

In countries free from ASF but suspecting its presence, the laboratory diagnosis must be directed towards isolation of the virus by simultaneously carrying out the inoculation of pig leukocyte or bone marrow cultures, and detection of antigen in smears or cryostat sections of tissues by the fluorescent antibody test (FAT). However, the detection of antibodies in serum or tissue fluids by the enzyme-linked immunosorbent assay (ELISA), immunoblotting or indirect fluorescent antibody (IFA) test should also be carried out at the same time in order to avoid a delay in detecting infection by an unexpected virus of low virulence. Serology can be an invaluable tool for helping to confirm an outbreak as antibody can often be detected in animals that die of acute disease.

An additional technique now available is the polymerase chain reaction (PCR), which can be used to detect the virus genome in blood or tissues and is particularly useful if samples submitted are unsuitable for virus isolation and antigen detection because they have undergone putrefaction.

### B. diagnostic techniques

#### 1. Identification of the agent

Where ASF is suspected, the following samples should be sent to the laboratory: blood in anticoagulant (heparin or ethylene diamine tetra-acetic acid [EDTA]), spleen, tonsil, kidney, lymph nodes. These should be kept as cold as possible, without freezing, during transit. After the samples arrive at the laboratory, they should be stored at −70°C if processing is going to be delayed. As maintaining a cold chain is not always possible, samples can be submitted in glycerosaline; this may slightly decrease the likelihood of virus identification, but it may facilitate the submission of samples to the laboratory so that an outbreak can be confirmed.

- **Sample preparation for hemadsorption and pig inoculation**
  - i) Prepare suspensions of tissues by grinding small pieces with a pestle and mortar containing sterile sand, then add 5–10 ml of a buffered salt solution or tissue culture medium containing antibiotics.
  - ii) Clarify the suspensions by centrifugation at 1000 \( \text{g} \) for 5 minutes.

Use the supernatant for hemadsorption (Section B.1.a. below) and pig inoculation (Section B.1.d. below), although pig inoculation is not recommended.

- **a) Hemadsorption test**

  The hemadsorption (HAD) test (6) is definitive for ASF and depends on the fact that pig erythrocytes will adhere to the surface of pig monocyte or macrophage cells infected with ASFV, and that most virus isolates produce this phenomenon of hemadsorption. A very small number of ‘nonhemadsorbing’ viruses have been isolated, most of which are avirulent, but some do produce typical acute ASF. The test is carried out by inoculating blood or tissue suspensions from suspect pigs into primary leukocyte cultures (Procedure 1 below) or by preparing leukocyte cultures from the blood of pigs inoculated at the laboratory or from the blood of suspect pigs collected in the field (Procedure 2 below). Up to 300 cultures can be prepared from each 100 ml of defibrinated or heparinised blood collected. It is essential to carry out all procedures in such a way as to prevent contamination of the cultures.

  - **Procedure 1: Hemadsorption test in primary leukocyte cultures**
    - i) Collect the required volume of fresh pig blood in heparin (100 International Units [IU]/ml blood).
    - ii) Centrifuge at 700 \( \text{g} \) for 30 minutes, remove the buffy coat cells and wash in medium.
    - iii) Resuspend the cells at a concentration of 10⁷ cells/ml in tissue culture medium containing 10–30% pig serum and antibiotics. In order to prevent nonspecific hemadsorption, the medium should contain serum or plasma from the same pig from which the leukocytes were obtained. If a large volume of samples is to be tested, the homologues serum can be replaced by serum that has been identified by pre-screening as capable of preventing the nonspecific auto-rosette formation.
iv) Dispense the cell suspension in aliquots of 1.5 ml in 160 × 16 mm tubes and incubate in a sloping position (5–10° from the horizontal) at 37°C.

Note: For routine diagnosis, only 2–4-day-old cultures are sufficiently sensitive.

v) Inoculate three tubes of cells by adding 0.2 ml of prepared samples of tissue per tube. It is advisable to inoculate ten-fold and hundred-fold dilutions into cultures, and this is especially important when the field material submitted is in poor condition.

vi) Inoculate positive control cultures with haemadsorbing virus. Uninoculated negative controls are essential to monitor the possibility of nonspecific haemadsorption.

vii) After 3 days, add 0.2 ml of a fresh preparation of 1% pig erythrocytes in buffered saline to each tube.

viii) Examine the cultures daily for 7–10 days under a microscope for cytopathic effect (CPE) and haemadsorption.

ix) Reading the results: Haemadsorption consists of the attachment of large numbers of pig erythrocytes to the surface of infected cells. A CPE consisting of a reduction in the number of adherent cells in the absence of haemadsorption may be due to the cytotoxicity of the inoculum, Aujeszky's disease virus or nonhaemadsorbing ASFV, which can be detected by the FAT on the cell sediment or by use of PCR (see below). If no change is observed, or if the results of the immunofluorescence and PCR tests are negative, subinoculate the supernatant into fresh leukocyte cultures.

• Procedure 2: Haemadsorption ‘autorosette’ test with peripheral blood leukocytes from infected pigs

This procedure is quicker than the preparation and inoculation of primary pig leukocyte cultures (described in Procedure 1 above) and will give more rapid results in positive cases. It can be performed in laboratories that are not equipped for routine virological examinations; the minimum requirements are slides and coverslips, a microscope and sterile medium, tubes or bottles and pipettes. Blood from suspect pigs in the field, or those inoculated in the laboratory, is collected in heparin and leukocyte cultures are prepared for direct examination for haemadsorption. However, the results of the test are difficult to evaluate and it is now being replaced by the PCR.

i) Collect 20 ml of whole blood in a syringe containing 2000 IU heparin in 2 ml of saline, mix and transfer to a glass tube or narrow bottle.

ii) Place the tube/bottle vertically in an incubator or water bath at 37°C, and allow the cells to settle. Sedimentation is improved by the addition of 2 ml of a plasma volume expander, such as 'Dextravan 150' which is a solution of Dextran 150 in 0.9% NaCl for injection (Fisons, United Kingdom).

iii) Incubate the cultures for 6–8 hours at 37°C, and then examine the cultures at 2–3-hour intervals by transferring small aliquots of the white-cell-rich supernatant, together with some erythrocytes, on to a glass slide and identify haemadsorbing cells under a microscope.

b) Antigen detection by fluorescent antibody test

The FAT (2) can be used as an additional method to detect antigen in tissues of suspect pigs in the field or those inoculated at the laboratory. By itself, it is not enough for ASF diagnosis. It can also be used to detect ASFV antigen in leukocyte cultures in which no HAD is observed and can thus identify nonhaemadsorbing strains of virus. It also distinguishes between the CPE produced by ASFV and that produced by other viruses, such as Aujeszky's disease virus or a cytotoxic inoculum.

• Test procedure

i) Prepare cryostat sections or impression smears of test tissues, or spreads of cell sediment from inoculated leukocyte cultures on slides, air dry and fix with acetone for 10 minutes at room temperature.

ii) Stain with fluorescein isothiocyanate (FITC)-conjugated anti-ASFV immunoglobulin at the recommended or pretitrated dilution for 1 hour at 37°C in a humid chamber.

iii) Fix and stain positive and negative control preparations similarly.

iv) Wash in phosphate buffered saline (PBS), mount stained tissues in PBS/glycerol, and examine under an ultraviolet light microscope with suitable barrier and exciter filters.

v) Reading the results: Tissues are positive if specific granular cytoplasmic fluorescence is observed in paracortical tissue of lymphoid organs or in fixed macrophages in other organs.

c) Detection of virus genome by the polymerase chain reaction

PCR techniques have been developed, using primers from a highly conserved region of the genome, to detect and identify a wide range of isolates belonging to all the known virus genotypes, including both
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nonhaemadsorbing viruses and isolates of low virulence. The PCR techniques are particularly useful for identifying virus DNA in pig tissues that are unsuitable for virus isolation or antigen detection because they have undergone putrefaction, or when there is good reason to believe that virus may have been inactivated before samples are received in the laboratory. Two PCR procedures are described and consist of a sample preparation procedure followed by the test procedure.

- **PCR method**

This procedure and procedure 2 serve as a general guideline and a starting point for the PCR protocol. Optimal reaction conditions (incubation times and temperatures, models and suppliers of equipment, concentrations of assay reagents such as the primers and dNTPs) may vary so the described conditions should be evaluated first. Details of PCR validation and procedures to help ensure test validity are given in Chapter I.1.4 of this *Terrestrial Manual*. Alterations can then easily be made to any aspect of the protocol to achieve better performance.

- **Sample preparation**

  i) Prepare suspensions of tissue by grinding up small pieces of tissue with a pestle and mortar containing sterile sand, and make a 1/10 dilution by adding 5–10 ml of PBS containing 1% ox serum and antibiotics.

  ii) Centrifuge at 500 g for 5 minutes.

  iii) Extraction for control samples: 1/10 tissue homogenates (same tissue as the samples to be analysed): (a) a negative control: use 500 µl of a homogenate of ASFV-negative tissue; (b) a positive control: use 500 µl of a homogenate of ASFV-positive tissue.

  iv) Transfer 500 µl to a screw-capped Eppendorf tube and boil for 10 minutes.

  v) Centrifuge at 13,000 g in a microfuge for 5 minutes.

The tissue supernatant is used in the PCR test.

The sample preparation procedure given above is simple and inexpensive, but may produce false-negative results due to the presence of PCR inhibitors. An alternative extraction procedure using the NucleoSpin Virus Kit (Macherey Nagel) is described below. This kit includes the reagents, RAV1, RAV3, and NucleoSpin filter columns.

**Working procedure for fluid samples: plasma, serum, cell culture medium.**

(Note that for organ and tissue samples, first prepare a 1/10 homogenate of the material in PBS, then centrifuge to clarify at 12,000 g for 5 minutes. Use the supernatant fluid.)

Extraction for control samples: 1/10 tissue homogenates (same tissue as samples to be analysed): (a) a negative control: use 150 µl of a homogenate of ASFV-negative tissue; (b) a positive control: use 150 µl of a homogenate of ASFV-positive tissue.

  i) Add 600 µl of RAV1 (carrier RNA included) to 150 µl of sample. Pipette up and down several times and vortex well. Incubate for 5–10 minutes at room temperature.

  ii) Optional: If the resulting solution is turbid, centrifuge the mixture for 1 minute to clarify. Transfer the supernatant to a new tube.

  iii) Add 600 µl ethanol to the clear solution and mix by vortexing.

  iv) Load 700 µl of the sample on to a NucleoSpin column, placed in a 2 ml centrifuge tube.

  v) Centrifuge for 60 seconds at 6000 g at room temperature. Discard the flowthrough.

  vi) Load the remaining sample (about 600 µl) on to the same NucleoSpin column and centrifuge as above. Discard the flowthrough.

  vii) Add 500 µl of buffer RAV3 to the NucleoSpin column.

  viii) Centrifuge for 30 seconds at 6000–8000 g. Discard the flowthrough and repeat this washing step.

  ix) Discard the flowthrough, then place the NucleoSpin column in a fresh 2 ml tube and centrifuge for 5 minutes at maximum speed to completely remove buffer RAV3.

  x) Elution of nucleic acids: Place the NucleoSpin column in a sterile 1.5 ml centrifuge tube, add 50 µl of elution buffer (5 mM Tris/HCl, pH 8.5, preheated to 70°C), and incubate for 2 minutes.

  xi) Centrifuge for 60 seconds at 8000 g.

  xii) Keep the 50 µl of eluted DNA at –20°C until use.
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- **Stock solutions**
  
i) Taq DNA polymerase and PCR amplification buffer (10×) are commercially available.
  
ii) Stock 1.25 mM dNTP: Prepare 50 mM stock solutions of each of the following nucleotides: dATP, dCTP, dGTP and dTTP. Add 10 µl of each of these stock solutions to 360 µl sterile distilled water.
  
iii) Primers at a concentration of 20 pmol/µl: Primer 1 sequence 5’-ATGGA-TACCG-AGGGA-ATAGC-3’ (positive strand); Primer 2 sequence 5’-CTTAC-GATG-AAAAT-GATAC-3’ (negative strand).
  
iv) **Loading buffer:** 0.25% Orange G in an aqueous solution of 30% glycerol.
  
v) **TAE buffer (50× for agarose gel):** Tris base (242 g); glacial acetic acid (57.1 ml); 0.5 M EDTA, pH 8.0 (100 ml); distilled water (to 1 litre).
  
vi) **Marker DNA:** 100 base-pair ladder is commercially available.

- **Procedure 1**
  
i) Add the following reagents to the required number of 0.75 ml polypropyl Eppendorf tubes:

  - Sterile distilled water (24.5 µl); (10× conc.) PCR amplification buffer (5 µl); magnesium chloride 25 mM (4 µl); 1.25 mM stock dNTP solution (8 µl); primer 1, 20 µM (1 µl); primer 2 (1 µl); tissue supernatant (10 µl); Taq DNA polymerase 5 U/µl (0.25 µl).

  - Control tubes contain no tissue supernatant.

  - **Negative control (no DNA):** Add 10 µl of distilled water.

  - **Positive control:** Add 2 µl of ASFV DNA and 8 µl of distilled water.

  - Overlay the mixture with 60 µl of mineral oil.

  - Place all the tubes in an automated DNA thermal cycler and run the following programme:

    - One cycle at 94°C for 5 minutes.
    - Thirty-five cycles at 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute.
    - One cycle at 72°C for 10 minutes.
    - Hold at 4°C.

  - At the end of the programme, carefully remove 20 µl of each reaction mixture from below the mineral oil, transfer to a clean tube and add 2 µl of loading buffer.

  - Load all the samples in a 2% agarose gel in TAE buffer containing ethidium bromide at a final concentration of 0.5 µg/ml.

  - **Add marker DNA** to one lane on each side of the gel.

  - Run the gel at a constant voltage of 150 volts for 2 hours.

  - **Reading the results:** Examine the gel over a UV light source. In a positive sample, a discrete band will be present that should co-migrate with the PCR product of the positive control. Calculate the size of the PCR products in the test samples and the positive control by reference to the standard markers. The PCR product of the positive control has a size of 278 base pairs. No bands should be seen in the negative control.

- **Procedure 2: TaqMan® PCR protocol (5)**

- **Sample preparation**

  The method described below is the one published in reference 5. A number of other DNA extraction kits are commercially available for the preparation of template suitable for PCR depending on the sample submitted for analysis and may be appropriate for use in Reference Laboratories.

  The QiAamp® Viral RNA Mini Kit (QIAGEN) procedure (spin protocol, January 1999) is described below. This kit can be used for blood from suspected swine fever animals. In these cases, detection of ASFV can be performed in parallel to classical swine fever virus (see Chapter 2.1.13. for CSFV molecular detection methods).

  i) Pipette 560 µl of the supplied buffer AVL into a 1.5 ml microcentrifuge tube.

  ii) Add 140 µl of test or control sample and mix by pulse-vortexing for about 15 seconds. Negative ASF control samples consisting of spleen homogenates from uninfected pigs and uninfected porcine bone marrow (PBM) and peripheral blood mononuclear (PBL) cells should be processed alongside the test...
samples. Additional extraction negative controls can also be prepared for each test sample and uninfected negative control by running parallel extractions of nuclease-free water (all controls should subsequently be assayed by the PCR procedure along with the test samples).

iii) Incubate at room temperature for at least 10 minutes.

iv) Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

v) Add 560 µl ethanol to the sample, pulse-vortex for approximately 15 seconds and briefly centrifuge to remove drops from the inside of the lid.

vi) Add 630 µl of the solution from v) to a QIAamp spin column (in a 2-ml collection tube) without wetting the rim. Close the cap and centrifuge for 1 minute at 6000 g. Place the spin column into a clean 2 ml collection tube and discard the tube containing the filtrate.

vii) Carefully open the QIAamp spin column and repeat step vi.

viii) Carefully open the QIAamp spin column and add 500 µl of Buffer AW1. Close the cap and centrifuge for 1 minute at 6000 g. Place the spin column into a clean 2-ml collection tube and discard the tube containing the filtrate.

ix) Carefully open the QIAamp spin column and add 500 µl of Buffer AW2. Close the cap and centrifuge for 3 minutes at 20,000 g.

x) Place the QIAamp spin column in a new 1.5 ml microcentrifuge tube. Discard the old collection tube containing the filtrate. Carefully open the QIAamp spin column and add 60 µl of Buffer AVE. Close the cap and incubate at room temperature for 1 minute. Centrifuge for 1 minute at 6000 g.

xi) Discard the QIAamp spin column. Store the extracted DNA (60 µl) at –20°C until required for PCR amplification procedure.

Stock solutions
i) Nuclease-free or another appropriate sterile water and TaqMan® PCR reaction master mix (2×).

ii) Primers at a concentration of 50 pmol/µl: Primer 1 sequence 5'-CTGCT-CATGG-TATCA-ATCTT-ATCGA-3' (positive strand); Primer 2 sequence 5'-GATAC-CACAA-GATC(AG)-GCCGT-3' (negative strand).

iii) TaqMan® probe at a concentration of 5 pmol/µl: (5'-[6-carboxy-fluorescein (FAM)]-CCACG-GAGGG-AATAC-CACCC-ACTG-3'[6-carboxy-tetramethyl-rhodamine (TAMRA)]).

PCR amplification by TaqMan® assay (5)

i) In a sterile 1.5 ml microcentrifuge tube prepare the PCR reaction mixture described below for each sample. Prepare the reaction mixture in bulk for the number of samples to be assayed but allowing for one extra sample.

Nuclease-free or sterile water (7.5 µl); (2× conc.) TaqMan® PCR reaction master mix (12.5 µl); primer 1, 50 pmol (0.5 µl); primer 2, 50 pmol (0.5 µl); TaqMan® probe, 5 pmol (1 µl).

ii) Add 22 µl PCR reaction mix to a well of a MicroAmp® optical reaction plate for each sample to be assayed.

iii) Add 3 µl of extracted sample template or blank extraction control and securely cover each well with a cap.

iv) Spin the plate for 1 minute in a suitable centrifuge to mix the contents of each well.

v) Place the plate in a TaqMan® Sequence Detection System for PCR amplification and run the following programme:

One cycle at 50°C for 2 minutes.

One cycle at 95°C for 10 minutes.

Forty cycles at 95°C for 15 seconds, 58°C for 1 minute.

Note: If a TaqMan® thermal cycler is not available, an ordinary thermal cycler can be used and the PCR products analysed by end-point fluorescence readers or alternatively by electrophoresis on a 1.5% agarose gel.

vi) Reading the results: Assign a threshold cycle (C_T) value to each PCR reaction from a scan of all amplification plots (a plot of the fluorescence signal versus cycle number). Negative test samples, uninfected negative or extraction blank controls should have a C_T value >40.0. Positive test samples and controls should have a C_T value < 40.0 (strongly positive samples have a C_T value <30.0).
d) Pig inoculation

In the past, pig inoculation has been used to differentiate between classical and African swine fever, as these diseases produce indistinguishable clinical signs. Samples were inoculated into two groups of pigs, one of which had been vaccinated against classical swine fever (hog cholera), the other remaining unvaccinated. This test is now unlikely to be necessary because there are alternative laboratory tests that give reliable results for both ASF and classical swine fever. The pig inoculation test is slow, expensive and difficult to perform and results in acute distress for the animals involved raising serious animal welfare concerns. It can no longer be recommended.

2. Serological tests

Antibodies persist in recovered pigs for long periods after infection, sometimes for life, and a number of tests are available for detecting these antibodies, although only a few of them have been developed for routine use in diagnostic laboratories (1, 3, 8, 9, 12). The most commonly used is the ELISA (13, 15), which is suitable for examining either serum or fluid from the tissues. Confirmatory testing of ELISA-positive samples should be carried out in critical cases using an alternative test, such as the IFA test (11), immunoperoxidase staining or immunoblotting (3, 10). Antibody is usually not produced in pigs infected with virulent ASFV. High levels of antibody are produced in pigs infected with low or moderately virulent ASF viruses, but these are not neutralising antibodies.

Where ASF is endemic, confirmation of suspected cases of disease is best done using a standard serological test (ELISA), combined with an alternative serological test (IFA) or an antigen-detection test (FAT). In some countries, over 95% of positive cases have been identified using a combination of IFA tests and FAT (12).

It should be noted that when pigs have been infected with avirulent isolates or those of low virulence, serological tests may be the only way of detecting infected animals.

Both the counter immunoelectrophoresis test and ELISA can be used for the large-scale screening of sera, although the ELISA is more sensitive for detecting individual positive sera and has been used extensively as part of eradication programmes.

The method used depends on the staff and facilities available.

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

The ELISA (1, 9) is a direct test that can detect antibodies to ASFV in pigs that have been infected by viruses of low or moderate virulence.

• Antigen preparation

The ELISA antigen is prepared from infected cells grown in the presence of pig serum (4).

i) Infect MS (monkey stable) cells at multiplicity of infection of 10 with adapted virus, and incubate in medium containing 2% pig serum.

ii) Harvest the cells at 36–48 hours post-infection, when the CPE is extensive. Wash in PBS, sediment at 650 g for 5 minutes, wash the cell pellet in 0.34 M sucrose in 5 mM Tris/HCl, pH 8.0, and centrifuge to pellet cells.

Carry out steps (iii) to (v) on ice:

iii) Resuspend the cell pellet in 67 mM sucrose in 5 mM Tris/HCl, pH 8.0 (1.8 ml per 175 cm² flask), and leave for 10 minutes with agitation after 5 minutes.

iv) Add nonionic detergent Nonidet P-40 to a final concentration of 1% (w/v), and leave for 10 minutes (with agitation after 5 minutes) to lyse the cells.

v) Add sucrose to a final concentration of 64% (w/w) in 0.4 M Tris/HCl, pH 8.0, and centrifuge at 1000 g for 10 minutes to pellet nuclei.

vi) Collect the supernatant and add EDTA (2 mM final concentration), beta-mercaptoethanol (50 mM final concentration) and NaCl (0.5 M final concentration) in 0.25 mM Tris/HCl, pH 8.0, and incubate for 15 minutes at 25°C.

vii) Centrifuge at 100,000 g for 1 hour at 4°C over a layer of 20% (w/w) sucrose in 50 mM Tris/HCl, pH 8.0.

Remove the band immediately above the sucrose layer and use as the ELISA antigen. Store at –20°C.
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- **Test procedure (13)**
  
  i) Coat ELISA microtitre plate(s) with antigen by adding 100 µl of the recommended or pretitrated dilution of antigen in 0.05 M carbonate/bicarbonate buffer, pH 9.6, to each well.
  
  ii) Incubate at 4°C for 16 hours (overnight) and then wash five times with 0.05% Tween 20 in PBS, pH 7.2.
  
  iii) Dilute the test sera and positive and negative control sera 1/30 in 0.05% Tween 20 in PBS, pH 7.2, and add 100 µl of each diluted serum to duplicate wells of the antigen-coated plate(s).

  If four pairs of each positive and negative control serum are added to wells in different parts of the plate, 40 sera can be tested in duplicate on one plate, as shown on the plate plan below.

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  iv) Incubate plates at 37°C for 1 hour (optionally on a plate shaker), and then wash five times with 0.05% Tween 20 in PBS.
  
  v) To each well add 100 µl of protein-A/horseradish-peroxidase conjugate at the recommended or pretitrated dilution in 0.05% Tween 20 in PBS.
  
  vi) Incubate the plates at 37°C for 1 hour, and then wash five times with 0.05% Tween 20 in PBS.
  
  vii) Add hydrogen peroxide to the substrate solution (0.04% orthophenylenediamine in phosphate/citrate buffer, pH 5.0) at the rate of 10 µl/25 ml, and add 100 µl of substrate to each well.
  
  viii) Incubate at room temperature for approximately 10 minutes; the time necessary for the colour to develop will depend on both the temperature of the substrate when added to the wells, and the room temperature.
  
  ix) Stop the reaction by adding 100 µl of 1.25 M sulphuric acid to each well.
  
  x) **Reading the results:** Positive sera have a clear yellow colour and can be read by eye, but to ensure that all positive sera are identified, it is necessary to read the absorbance in each well spectrophotometrically, at 492 nm, in an ELISA reader. Any serum is considered to be positive if it has an absorbance value of more than twice the mean absorbance value of the control negative sera on that plate.

b) **Indirect fluorescent antibody test**

This test (11) should be used as a confirmatory test for sera from areas that are free from ASF and are positive in the ELISA, and for sera from endemic areas that give an inconclusive result in the ELISA.

- **Test procedure**
  
  i) Prepare a suspension of ASFV-infected pig kidney or monkey cells at a concentration of 5 × 10^6 cells/ml, spread small drops on glass slides, air dry and fix with acetone at room temperature for 10 minutes. Note that slides can be stored at −20°C until ready for use.
  
  ii) Heat inactivate test sera at 56°C for 30 minutes.
  
  iii) Add appropriate dilutions of test sera and positive and negative control sera in buffered saline to slides of both infected and uninfected control cells, and incubate for 1 hour at 37°C in a humid chamber.
  
  iv) Wash the slides with PBS and then distilled water.
  
  v) Add predetermined or recommended dilutions of anti-pig immunoglobulin/FITC or protein-A/FITC conjugate to all slides, and incubate for 1 hour at 37°C in a humid chamber.
vi) Wash the slides with PBS and then distilled water, mount in PBS/glycerol, and examine under an ultraviolet light microscope with suitable barrier and exciter filters.

vii) **Reading the results:** The control positive serum on infected cells must be positive and all other controls must be negative before the test can be read. Sera are positive if infected cultures show specific fluorescence.

c) **Immunoblotting test**

This test should be used as an alternative to the IFA test to confirm equivocal results with individual sera. The immunoblot test is very specific, but its sensitivity may be less than the IFA.

- **Preparation of antigen strips**
  i) Prepare cytoplasmic soluble virus proteins as described for the preparation of ELISA antigen in Section B.2.a.
  ii) Electrophorese through 17% acryl-amide/N,N'-diallyltartardiamide (DATD) gels with appropriate molecular weight standards.
  iii) Transfer the proteins on to a 14 ‰ 14 cm² nitrocellulose membrane by electrophoresis at a constant current of 5 mA/cm in transfer buffer (20% methanol in 196 mM glycine, 25 mM Tris/HCl, pH 8.3).
  iv) Dry the membrane and label the side on to which the proteins were electrophoresed.
  v) Cut one strip from the edge of the filter and carry out the immunoblotting procedure described below. Identify the region containing proteins of 23–35 kDa by comparison with the molecular weight standards run in parallel, and cut this region into 0.5 cm wide strips. Label each strip on the side on to which the proteins were electrophoresed.

These strips (approximately 4 cm long) constitute the antigen strips used for immunoblotting and contain proteins with which antibodies in both acute and convalescent pig sera will react. These antibodies persist for life in some pigs.

- **Preparation of chloranaphthol substrate solution**

This solution must be prepared immediately before use.

  i) Dissolve 6 mg of 4-chloro-1-naphthol in 2 ml of methanol and add this solution slowly to 10 ml of PBS while it is being stirred.
  ii) Remove the white precipitate that is formed by filtration through Whatman No.1 filter paper (optional).
  iii) Add 4 µl of 30% hydrogen peroxide.

- **Test procedure**

The antigen strips must be kept with the labelled side uppermost during the immunoreaction procedure.

  i) Incubate the antigen strips in blocking buffer (2% nonfat dried milk in PBS) at 37°C for 30 minutes with continuous agitation.
  ii) Prepare 1/40 dilutions of test sera and positive and negative control sera in blocking buffer.
  iii) Incubate the antigen strips in the appropriate serum at 37°C for 45 minutes with continuous agitation. Incubate one antigen strip in positive control serum and one in negative control serum. These two strips are controls. Wash four times in blocking buffer; the final wash should be for 5 minutes with continuous agitation.
  iv) Add protein-A/horseradish-peroxidase conjugate at the recommended or pretitrated dilution in blocking buffer to all antigen strips. Incubate at 37°C for 45 minutes with continuous agitation. Wash four times in blocking buffer; the final wash should be for 5 minutes with continuous agitation.
  v) Prepare the substrate solution, add to the antigen strips, and incubate at room temperature for 5–15 minutes with continuous agitation.
  vi) Stop the reaction with distilled water when the protein bands are suitably dark.
  vii) **Reading the results:** Positive sera react with more than one virus protein in the antigen strip; they must give a similar protein pattern and have the same intensity of colour as the antigen strips stained with positive control serum.
d) Counter immunoelectrophoresis (immunoelectro-osmophoresis) test

This test (7) can be carried out rapidly and specific antibody can be detected in some sera 30 minutes after the test is set up. It requires the use of electrophoresis equipment (electrophoresis chamber, slide frames, gel cutter) and a 500-volt constant-current power supply. Due to its low sensitivity, this test is recommended for screening groups of pigs, but not individual animals.

- Test procedure
  
i) Place the required number of 2.5 × 10 cm glass slides in the slide frame on a level table and cover with the recommended volume of 0.6% agarose in veronal/acetate buffer, pH 8.6 (ionic strength 0.025) containing 0.1% sodium azide, and allow to set.
  
ii) Cut four pairs of wells, 3 mm in diameter, 10 mm apart in the gel on each slide as shown below.

\[
\begin{array}{c|c|c|c|c}
 & S & Ag & S & Ag \\
+ & O & O & O & O \\
\hline
& O & O & O & O \\
\end{array}
\]

\(S = \text{serum}; \ Ag = \text{antigen}; + = \text{positive electrode}; - = \text{negative electrode}\)

iii) Fill the wells with the appropriate reagents, including control positive and negative antigens and sera, using capillary (haematocrit) tubes.

iv) Place the frames in the electrophoresis chamber and run for 30 minutes with a constant voltage of 19 volts/cm.

v) After electrophoresis, examine the slides over an indirect light source for specific lines of precipitation.

vi) Wash the slides in 2% NaCl solution overnight and for 2 hours in several changes of distilled water before drying.

vii) Stain dried slides with 0.075% amido black in equal volumes of methanol, 12% acetic acid and 1.6% sodium acetate, containing 0.007% glycerol, for 5–10 minutes and destain with three 10-minute washes in an aqueous solution of 45% methanol and 10% glacial acetic acid.

viii) Reading the results: On the stained slides, the lines of precipitation observed between the antigen and unknown serum wells of a positive sample should be similar to that formed between the positive antigen and serum control wells.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

At present there is no vaccine for ASF.

REFERENCES


*  *  *

NB: There are OIE Reference Laboratories for African swine fever (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CLASSICAL SWINE FEVER
(hog cholera)

SUMMARY

Classical swine fever (CSF), also known as hog cholera, is a contagious viral disease of pigs. The causative virus is a member of the genus Pestivirus of the family Flaviviridae, and is closely related to the viruses of bovine viral diarrhea and border disease. There is only one serotype of CSF virus (CSFV).

The disease may run an acute, subacute, chronic, late onset, or inapparent course, depending on a variety of viral and host factors of which the age of the animals, the virulence of the virus and the time of infection (pre- or post-natal) are of greatest importance. Adult pigs usually display less severe signs of disease than young animals and stand a better chance of survival. In pregnant sows, the virus may cross the placental barrier and reach the fetuses. In-utero infection with strains of the virus of moderate or low virulence can result in what is referred to as the 'carrier sow' syndrome followed by prenatal or early post-natal death, the birth of diseased piglets or an apparently 'healthy' but infected litter. An outbreak of CSF has serious consequences for trade in pigs and pig products.

The highly variable clinical picture of CSF often precludes a diagnosis on clinical and pathological grounds alone. Laboratory methods are therefore essential for an unambiguous diagnosis. Detection of virus in whole blood and of antibodies in serum are the methods of choice for diagnosing CSF in live pigs, whereas detection of virus or antigen in organ samples is most suitable when the pig is dead.

Identification of the agent: The direct fluorescent antibody test (FAT) on cryostat sections of organs from affected pigs is used for the detection of CSF antigen. A panel of monoclonal antibodies (MAbs) is used to determine whether the fluorescence is due to CSF or non-CSF Pestivirus antigens. The isolation of CSFV should be attempted in the pig kidney (PK-15) cell line, or other suitable cell lines. The cultures are examined for virus growth by immunofluorescence or immunoperoxidase staining; positive isolates are further characterised by the use of MAbs and by partial genetic sequencing. Polymerase chain reaction protocols for the identification of CSFV nucleic acid are being used in several laboratories. Isolation and characterisation of suspected pathogenic strains of the virus should be conducted in a virus-secure laboratory.

Serological tests: Detection of virus-specific antibodies is particularly useful in herds suspected of being infected at least 30 days previously with CSFV. Serological methods are also valuable for monitoring and for prevalence studies, and are essential if a country wishes to be internationally recognised as being free from the disease in the absence of vaccination.

As CSFV cross-reactive antibodies against ruminant Pestivirus are occasionally observed in breeding pigs, screening tests have to be followed by confirmatory tests that are CSFV-specific. Certain ELISAs are relatively CSFV-specific, but the definitive method for differentiation is the comparative neutralisation test, which compares the level of antibody to different Pestivirus species.

Requirements for vaccines and diagnostic biologicals: Vaccines against CSF are based on live virus that has been attenuated by passage through cell cultures or through a suitable host species that is not of the family Suidae. The production of these modified live virus (MLV) vaccines is based on a seed-lot system that has been validated with respect to virus identity, sterility, purity, safety, nontransmissibility, stability and immunogenicity. If CSFV is used in the production of
vaccine or in challenge studies, the facility should meet the OIE requirements for Containment Group 4 pathogens.

Effective inactivated, conventional whole virus vaccines are not available. In recent years ‘marker vaccines’ have been developed, which in contrast to MLV vaccines induce antibodies that can be distinguished from antibodies induced by field virus using an accompanying diagnostic test. The presently registered ‘marker vaccines’ are based on the major envelope glycoprotein (E2-subunit) of CSFV, and are produced in insect cells using recombinant DNA technology.

A. INTRODUCTION

The viruses that cause classical swine fever (CSF), bovine viral diarrhoea (BVD) and border disease (BD) are members of the family Flaviviridae, genus Pestivirus, and are closely related, both antigenically and structurally. Clinical signs and lesions seen at post-mortem in pigs affected with CSF are highly variable due to both viral and host factors. Furthermore, congenital infections with ruminant pestiviruses in pigs can give rise to a clinical disease that is indistinguishable from CSF (22, 24, 25).

Spread of disease in all age groups, accompanied by pyrexia, huddling, inappetance, dullness, weakness, conjunctivitis, constipation followed by diarrhoea, and an unsteady gait are the prevailing signs. Several days after the onset of clinical signs, the ears, abdomen and inner thighs may show a purple discoloration. Animals with acute disease die within 1–2 weeks. Sudden death in the absence of clinical illness is not symptomatic of CSF.

Under certain circumstances related to the animals’ age and condition, as well as to the virus strain involved, subacute or chronic clinical illness may develop, which can be protracted for 2–4 weeks or even months. Chronic illness leads to a stunting of growth, anorexia, intermittent pyrexia and diarrhoea. Congenital persistent infections may go undetected for months and may be confined to only a few piglets in the herd. The clinical signs are nonspecific: wasting in the absence of pyrexia. Chronic, persistent infections always lead to the death of the animal. Herd mortality rates may be slightly above the expected level. CSF affects the immune system, a main characteristic being generalised leukopenia, which can often be detected before the onset of fever. Immunosuppression may lead to concurrent infections.

In acute cases, gross pathological lesions are often inconspicuous or absent. In typical cases, the lymph nodes are swollen and marbled red, and haemorrhages occur on the epicardium, and in the kidneys, urinary bladder, the skin and subcutis. In subacute and chronic cases, necrotic or ‘button’ ulcers may be observed in the mucosa of the gastrointestinal tract, epiglottis and larynx, in addition to the above lesions.

Histopathological findings are not pathognomonic. Lesions may include parenchymatous degeneration of lymphatic tissue, cellular proliferation of vascular interstitial tissue, and a nonsuppurative meningoencephalomyelitis, with or without vascular cuffing.

B. DIAGNOSTIC TECHNIQUES

The variability of the clinical signs and post-mortem lesions do not provide firm evidence for unequivocal diagnosis. Other viral diseases, such as African swine fever, post-weaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome, as well as septicaemic conditions such as salmonellosis, pasteurellosis, actinobacillosis and Haemophilus suis infections may be confused with acute CSF. In fact, these bacteria often cause concurrent infections, and isolating these pathogens may obscure the real cause of disease, the CSF virus (CSFV).

A tentative diagnosis based on clinical signs and post-mortem lesions must therefore be confirmed by laboratory investigations. This is all the more necessary in view of the serious consequences of an outbreak of CSF for trade in pigs and pig products.

Laboratory methods for diagnosis of CSF are aimed at detection of the virus, viral nucleic acid or viral antigens, or detection of specific antibodies. For a correct interpretation of the test results the inspecting veterinarian should pay particular attention to the simultaneous and clustered occurrence of two or more of the prevailing signs of disease listed above. Random sampling is unsuitable for CSF diagnosis. As pyrexia is one of the first signs of CSF and is accompanied by viraemia (6), virus detection in whole blood in ethylene diamine tetra-acetic
acid (EDTA) or tissues collected from a few febrile animals is the method of choice for detecting infected herds at an early stage. Additionally, whole blood samples for virus detection can be collected from a larger group of pigs.

CSF is subject to official control and the virus has a high risk of spread from the laboratory: consequently, a risk analysis should be carried out to determine the level of 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 Appendix I.1.6.1. of Chapter I.1.6. of this Terrestrial Manual. Countries lacking access to such a specialised national or regional laboratory should send specimens to an OIE Reference Laboratory.

Antibodies develop in the third week of illness and persist in the surviving animal for life. Samples for antibody detection are collected in ordinary (nonheparinised) tubes from convalescent pigs and from contact herds when ≥30 days have elapsed since the suspected contact with a confirmed outbreak took place.

1. Identification of the agent

a) Immunological methods

• Fluorescent antibody test

The fluorescent antibody test (FAT) is a rapid test that can be used to detect CSFV antigen in cryostat sections of tonsils, spleen, kidney, lymph nodes or distal portions of the ileum. Tissues should be collected from several animals (3) and transported without preservatives under cool conditions, but not frozen. Cryostat sections are stained directly with anti-CSF immunoglobulin conjugated to fluorescein isothiocyanate (FITC) or indirectly using a secondary FITC conjugate and examined by fluorescence microscopy. During the first stage of the infection, tonsillar tissue is the most suitable, as this is the first to become affected by the virus irrespective of the route of infection (18). In subacute and chronic cases, the ileum is frequently positive and occasionally may be the only tissue to display fluorescence. A negative FAT result does not completely rule out CSF infection. When suspicion of CSF continues, further samples should be obtained or attempts made at virus isolation in cell culture (e.g. pig kidney [PK-15]) or another cell line of pig origin that is as sensitive and known to be free from Pestivirus contamination.

• Test procedure

Include positive and negative control sections in each series of organ samples to be examined.

i) Cut out a piece of tonsil, spleen, kidney and ileum of approximately 1 × 1 × 0.5 cm, and mount it with a cryo-embedding compound or distilled water on a cryostat table.

ii) Freeze the piece of organ on to the cryostat table.

iii) Cut sections not more than 4 µm thick and mount these on to 10 × 32 mm grease-free cover-slips with one corner cut-off. All sections are mounted with this corner in the same position (e.g. top right).

iv) After drying, fix the mounted sections for 10 minutes at room temperature in acetone (analytical grade) or air-dry for 20 minutes at 37°C.

v) Immerse the sections briefly in phosphate buffered saline (PBS), remove excess fluid with tissue paper and place them (cut off corner top right) on a frame in an incubation chamber humidified with a small volume of water placed in the bottom of the chamber.

vi) Dispense the anti-CSF immunoglobulin at working dilution on to the entire section and incubate in the closed chamber for 30 minutes at 37°C. If a secondary FITC conjugate is required, wash the section five times for 2 minutes each in PBS at room temperature, then add the FITC conjugate at working dilution and incubate as previously described.

vii) Wash the sections five times for 2 minutes each in PBS at room temperature.

viii) Remove the remaining PBS by touching the cover-slip against tissue paper and mount the cover-slip (with the section between cover-slip and slide) with mounting buffer on to a microscope slide.

ix) Remove excess mounting fluid with tissue paper and examine the sections for fluorescence using a UV microscope. A CSF-positive section shows brilliant green fluorescing cells. In the tonsils, fluorescence in the epithelial lining of the crypts is particularly evident. In kidney sections, fluorescence is most abundant in the proximal and distal tubules of the renal cortex and the collecting ducts in the medulla. In the ileum, fluorescence is most prominent in the epithelial cells of the Lieberkühl glands, whereas in the spleen reactivity is more diffuse, with concentrations of lymphoid cells in the periarterial lymphoid sheath (PALS).
The FAT involves the use of an anti-CSF immunoglobulin prepared from a polyclonal antibody to CSFV that will not distinguish between the antigens of different pestiviruses. Conjugates used for the FAT on cryostat sections or inoculated cell cultures should be prepared from anti-CSFV gamma-globulins raised in specific pathogen free pigs. The working dilution of the conjugates (at least 1/30) should combine a maximum brilliance with a minimum of background.

Strains of modified live virus (MLV) vaccine multiply mainly in the regional lymph nodes and in the crypt epithelium of the tonsils. Pigs vaccinated with MLV strains may yield a positive FAT for 2 weeks after vaccination (15, 19). Rabbit inoculation is used to differentiate between lapinised and field strains of CSFV. In contrast to field strains, lapinised strains given intravenously cause a febrile reaction and induce an immune response in rabbits.

Pigs infected with ruminant pestiviruses can give false-positive FAT reactions. Congenital infections with ruminant pestiviruses can cause clinical signs and pathological lesions indistinguishable from those in chronic CSF (22, 24, 25). Infections by CSFV or ruminant pestiviruses can be differentiated by testing sera from the dam and litter mates, or from other contacts of an FAT-positive piglet, for neutralising antibodies to each virus. Another method of differentiating these viruses is by the inoculation of seronegative piglets with a suspension of suspect material, followed 5 weeks later by virus neutralisation (VN) tests on their sera for the respective antibodies. However, VN tests may take several days, and animal inoculation methods take several weeks.

- **Immunoperoxidase procedure for differentiation of pestiviruses by monoclonal antibodies**

  The use of a panel of three monoclonal antibodies (MAbs), either horseradish peroxidase (HRPO) or FITC-conjugated, or used in conjunction with an anti-mouse conjugate and specifically detecting all field strains of CSFV, vaccine strains of CSFV and ruminant pestiviruses, respectively, would allow an unambiguous differentiation between field and vaccine strains of CSFV on the one hand, and between CSFV and other pestiviruses on the other (10, 26, 28). A prerequisite is that the MAb against CSFV recognises all field strains and that the anti-vaccine MAb recognises all vaccine strains used in the country. No single MAb selectively reacts with all ruminant pestiviruses (10). The use of an MAb to differentiate a CSF vaccine strain can be omitted in nonvaccination areas. A polyclonal anti-CSF immunoglobulin conjugated to HRPO serves as a positive control. Caution should be exercised when using evidence of a single Mab as sole confirmation of an isolate as CSF.

- **Test procedure**

  i) Cut eight or more cryostat sections (4 µm) of the FAT-positive tonsil, or another positive organ if the tonsil is not available.

  ii) Fix the sections on to flying cover-slips for 10 minutes in acetone (analytical grade) and allow to air dry.

  iii) Prepare working dilutions of the respective MAb-peroxidase conjugates in PBS + 0.01% Tween 80 + 5% horse serum, pH 7.6. (FITC–MAb can also be used, as well as unconjugated MAb provided that a secondary conjugate is used.)

  iv) After rinsing with PBS, overlay two sections with the working dilution of the respective monoclonal conjugates, and two sections with the working dilution of the polyclonal conjugate (controls).

  v) Incubate for 1 hour at 37°C in a humid chamber.

  vi) Wash the sections six times for 10 seconds each in PBS.

  vii) Stain the sections with freshly prepared chromogen–substrate solution* for 5–15 minutes at room temperature.

  viii) Rinse the sections in 0.05 M sodium acetate, pH 5.0, in distilled water and mount them on microscope slides.

  *

  **Chromogen–substrate solution**

  A. Stock solution of chromogen: 0.4% 3-amino-9-ethyl carbazole; N,N-dimethyl-formamide (1 ml). Caution TOXIC compound.

  B. 0.05 M sodium acetate, pH 5.0; 19 ml (sterile filtered through a membrane).

  C. Stock solution of substrate (30% hydrogen peroxide).

  Keep stock solutions A and C at 4°C in the dark and solution B at room temperature. Stock solution A can be kept at 4°C for at least 6 months and solution C for 1 year. Immediately before use, dilute 1 ml of solution A in 19 ml of solution B. Then add 10 µl of stock solution C. Mix well and stain the sections.
ix) Examine sections with a light microscope. Dark red staining of the cytoplasm of the epithelial cells lining the tonsillar crypts indicates recognition of the virus isolate by the respective conjugate, and is considered to be positive.

x) Interpretation of the test:

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<th>Interpretation</th>
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<td>CSF vaccine strain</td>
<td>BVD/BD strain</td>
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*The existence of novel strains of CSF should always be considered and any isolate from cases where CSF is still suspected should be sent to an OIE Reference Laboratory.

- **Antigen-capture assay**

For rapid diagnosis of CSF in live pigs, antigen-capture enzyme-linked immunosorbent assays (ELISAs) have been developed for screening herds suspected to have been infected recently. The ELISAs are of the double-antibody sandwich type, using monoclonal and/or polyclonal antibodies against a variety of viral proteins in either serum, the blood leukocyte fraction or anticoagulated whole blood (clarified tissue homogenate may be added here as it is a suitable material for ELISA) (7). The technique is relatively simple to perform, does not require tissue culture facilities, is suitable for automation and can provide results within half a day. The disadvantage of being less sensitive than virus isolation, especially in adult pigs and mild or subclinical cases, may be compensated by testing all pigs of the suspect herd showing pyrexia. However, the lowered specificity of these tests should also be taken into consideration.

b) **Isolation of virus**

Isolation of virus in cell cultures is a more sensitive but slower method for diagnosis of CSF than immunofluorescence on frozen sections. Isolation is best performed in rapidly dividing PK-15 cells seeded on to cover-slips simultaneously with a 2% suspension of the tonsil in growth medium. Other pig cell lines may be used, but should be demonstrably at least as sensitive as PK-15 cells for isolation of CSFV. The cultures are examined for fluorescent foci by FAT after 24–72 hours.

The tonsil is the most suitable organ for virus isolation from pigs that died or were killed for diagnostic purposes. Alternatively, spleen, kidney or lymph nodes can be used.

A detailed procedure for virus isolation is as follows:

1. Prepare a 100-fold strength glutamine–antibiotic stock solution: dissolve glutamine (2.92 g) in 50 ml distilled water (solution A) and sterilise by filtration. Dissolve each of the following antibiotics in 5–10 ml sterile distilled water: penicillin (10⁶ International Units [IU]); streptomycin (1 g); mycostatin (5 × 10⁵ U); polymixin B (15 × 10⁴ U); and kanamycin (1 g). Pool these solutions (solution B). Mix aseptically solutions A and B, make up to 100 ml with sterile distilled water, and store in 5 ml aliquots at −20°C.
2. Cut 1–2 g of tissue into small pieces and, using a mortar and pestle, grind in a small amount of cell culture medium with sterile sand into a homogeneous paste. Alternatively, use an appropriate crushing machine at 4°C.
3. Make a 20% (w/v) suspension by adding Hanks' balanced salts solution (BSS) or Hanks' minimal essential medium (MEM); 1 ml of the glutamine–antibiotic stock is added for each 10 ml of suspension. This mixture is held at room temperature for 1 hour.
4. Centrifuge at 1000 g for 15 minutes.
5. A PK-15 monolayer is trypsinised, the cell suspension is centrifuged at 160 g for 10 minutes, and resuspended to contain 2 × 10⁶ cells/ml in growth medium (Eagle's MEM with Earle's salts; 5% fetal bovine serum free from ruminant pestiviruses and pestivirus antibodies ; and 0.2 ml of the glutamine–antibiotic stock solution per 10 ml cell suspension).
6. Mix nine parts of cell suspension (from step v) and one part of supernatant fluid (from step iv) and inoculate 1.0–1.5 ml into 6–8 Leighton tubes with cover-slips or other appropriate cell culture flasks.
Chapter 2.1.13. – Classical swine fever (hog cholera)

Three tubes are inoculated with 1.0–1.5ml of cell suspension alone as controls. After completion of the sample inoculations, three tubes are inoculated with CSFV as positive controls. Careful precautions must be taken to avoid cross-contamination with this known positive virus suspension. Negative cultures should also be prepared.

vii) At 1, 2 and 3 days after inoculation, two cultures, together with a positive and negative control culture are washed twice for 5 minutes each in Hanks’ BSS, Hanks’ MEM or PBS, fixed with cold acetone (analytical grade) for 10 minutes, and stained with a direct anti-CSFV conjugate at its appropriate working dilution or indirectly, as described in Section B.1.a.

If the 2% tonsil suspension proves to be toxic for the cells, then the test should be repeated using a higher dilution or another organ.

viii) After washing in PBS three times for 5 minutes each, the cover-slip cultures are mounted in 90% carbonate/bicarbonate buffered glycerol, pH>8.0, and examined for fluorescent foci.

Instead of Leighton tubes, 6-well plates with cover-slips can be used. Alternatively, cultures growing on flat-bottomed microtitre plates or M24-plates can also be used for virus isolation. In such case, plates are fixed and stained as described later for the neutralising peroxidase-linked assay (NPLA).

Whole blood (heparin or EDTA treated) from clinically diseased pigs is a suitable sample for early CSF diagnosis. The leukocyte fraction or other components may be used, but for reasons of sensitivity and simplicity whole blood is preferred (9). The procedure is as follows:

i) Freeeze a sample of whole blood at –20°C and thaw in a waterbath at 37°C.

ii) Inoculate 300 µl haemolysed blood on to a PK-15 monolayer grown to approximately 75% confluence* in an M24-plate, and allow adsorption for 1 hour at 37°C.

iii) Remove inoculum, wash the monolayer once with Hanks’ BSS or Hanks’ MEM, and add growth medium.

iv) After a further incubation period of 3–4 days, the plates are washed, fixed and stained, as described later for the NPLA, using in each step a volume of 300 µl to compensate for the larger cell surface.

Note: this method is less sensitive than conventional virus isolation for the detection of acute CSF.

• Reverse-transcription polymerase chain reaction

Many methods for reverse-transcription polymerase chain reaction (RT-PCR) have been described and are still being developed. An internationally accepted alternative to the antigen-capture ELISA and the virus isolation method is a single tube reverse-transcription nested polymerase chain reaction (RT-nPCR) (14). This method is rapid and more sensitive than antigen-capture ELISAs or virus isolation or the RT-PCR, making it particularly suited to preclinical diagnosis. It has the additional advantage of reducing the risk of carry-over contamination because tubes are not opened between the successive RT, first PCR and nested PCR reaction steps (17). Real time PCR methods are now under development. No internationally standardised RT-PCR method is yet available. Examples of a suitable protocol may be obtained from the literature or from the OIE Reference Laboratories for CSF (see Table given in Part 3 of this Terrestrial Manual). Due to its speed and sensitivity, RT-PCR is a useful approach to screening suspect cases of disease and is now accepted by the European Union (EU) (1), although it is recommended that, due to the ease with which false positives can occur, any positive results from primary outbreaks should always be confirmed by other tests. The test can be applied to individual or pooled blood samples as well as solid organs and has been used successfully to control outbreaks.

The molecular epidemiology of CSF is based on the comparison of genetic differences between virus isolates. RT-PCR amplification of CSFV RNA followed by nucleotide sequencing is the simplest way of obtaining the sequence data to make these comparisons. A number of different regions of the CSFV genome may be targeted for molecular epidemiological studies (16). Two regions have been extensively studied and provide large sets of sequence data with which new isolates can be compared. One of these regions lies within the 5’-noncoding region (5’NCR) of the genome (150 nucleotides) and the other lies within the E2 major glycoprotein gene (190 nucleotides). In brief, the method used is to extract virus RNA from infected PK-15 cell cultures, perform RT-PCR to amplify one or both targets within the 5’NCR or the E2 gene, and then determine the nucleotide sequence of the products and compare with stored sequence information held in the databases. A database of these sequences is available from the OIE Reference

* Simultaneous inoculation, though slightly more sensitive, is less suitable as the anticoagulant may interfere with the adhesion of cells on to the surface.
Laboratory for CSF (Hanover, Germany). CSFV isolates from primary outbreaks should be sent to an OIE Reference Laboratory for investigation of molecular epidemiology. An import permit should be obtained prior to despatch.

2. **Serological tests**

Detection of virus-specific antibodies is particularly useful on premises suspected of having infections with CSF strains of low virulence. Due to the immunosuppressive effect of CSFV, antibodies cannot be detected with certainty until 21 days post-infection. Serological investigations aimed at detecting residual foci of infection, especially in breeding herds, may also be useful in a terminal phase of CSF eradication.

As the incidence of infection with ruminant pestiviruses may be high in breeding stock, only tests that will discriminate between CSF and BVD/BD antibodies are useful. VN and the ELISA using MAbs satisfy the requirements for sensitivity, but positive results should be confirmed by comparative VN testing.

Neutralisation tests are performed in cell cultures using a constant-virus/varying-serum method. As CSFV is noncytopathic, any non-neutralised virus must be detected, after multiplication, by an indicator system. The fluorescent antibody virus neutralisation (FAVN) test (13) and the NPLA (20) are the most commonly used techniques. Both tests can be carried out in microtitre plates. The peroxidase system has the advantage that the results can be read by the naked eye.

**a) Fluorescent antibody virus neutralisation test (a prescribed test for international trade)**

i) Seed a suspension of PK-15 cells at a concentration of $2 \times 10^5$ cells/ml into, e.g. 5 cm Petri dishes with cover-slips spread over the bottom, or into Leighton tubes with a cover-slip or into flat-bottomed microtitre plates.

ii) Incubate the cultures for 1–2 days at 37°C in a CO$_2$ cabinet until they reach 70–80% confluency. An ordinary incubator may be used for stoppered Leighton tubes.

iii) Inactivate the sera for 30 minutes at 56°C. For international trade purposes, it is best to test with an initial serum dilution of 1/5 (1/10 final dilution).

iv) Incubate equal volumes of diluted serum and virus suspension containing 200 TCID$_{50}$ (50% tissue culture infective dose) per 0.1 ml for 1–2 hours at 37°C. Thus a constant amount of CSFV of 100 TCID$_{50}$ is used for each reaction well.

v) Remove the cover-slips from Petri dishes or Leighton tubes, wash briefly in serum-free medium, overlay the cell sheet with the serum/virus mixture (from step iv) and incubate for 1 hour at 37°C in a humid atmosphere.

vi) Place the cover-slip in a clean Leighton tube and incubate the cultures in maintenance medium for 2 more days.

vii) Remove the cover-slips from the Leighton tubes, wash the monolayers twice for 5 minutes each in PBS, pH 7.2, fix in pure acetone for 10 minutes and stain with the working dilution of the conjugate for 30 minutes at 37°C before washing.

viii) Mount the cover-slips on grease-free microscope slides with 90% carbonate/bicarbonate buffered glycerol, pH>8.0, and examine for fluorescence.

When the FAVN test is performed in microtitre plates, the procedure for the NPLA (see below) can be followed up to step viii. The plates are then stained with the working dilution of the conjugate for 30 minutes at 37°C and examined for fluorescence. Note: When detecting fluorescence, microplates are best examined from above, using a long focal-length objective.

**b) Neutralising peroxidase-linked assay (a prescribed test for international trade)**

The NPLA is carried out in flat-bottomed microtitre plates. Sera are first inactivated for 30 minutes at 56°C. For international trade purposes, it is best to test with an initial serum dilution of 1/5 (1/10 final dilution). For surveillance schemes within a country, a screening dilution of 1/10 may suffice. Appropriate controls to ensure specificity and sensitivity of reactions are incorporated into each test.

- **Test procedure**

i) Dispense dilutions of serum in growth medium (Eagle’s MEM, 5% fetal bovine serum and antibiotics) in 50 µl volumes into duplicate wells of a microtitre plate. The fetal bovine serum must be free from both BVDV and antibodies to it. A third well may be included for each sample. This well contains serum and not virus and is used as a serum control (for cytotoxicity and/or nonspecific staining).
ii) Add 50 µl of virus suspension to the wells, diluted in growth medium to contain approximately 100 TCID₉₀/50 µl, and mix the contents on a microplate shaker for 20 seconds.

iii) Incubate the plates in a CO₂ incubator for 1 hour at 37°C.

iv) Add to all wells 50 µl of growth medium containing 2 x 10⁵ cells/ml.

v) Allow the cells to grow in 5% CO₂ to become confluent, usually within 3–4 days.

vi) Discard the growth medium and rinse the plates once in 0.15 M NaCl.

vii) Drain the plates by blotting on a towel.

viii) The cell monolayers may be fixed in one of several ways:

   • The plates are incubated for 45 minutes at 37°C, and then for at least a further 45 minutes at −20°C. The plates are removed from the freezer, the wells are filled with 100 µl 4% paraformaldehyde in PBS and reincubated for 5–10 minutes at room temperature. The paraformaldehyde is discarded and the plates are rinsed with 0.15 M NaCl; or

   • The plates are incubated at 70–80°C for 1–2 hours; or

   • The plates are fixed in 20% acetone in PBS for 10 minutes followed by thorough drying at 25–30°C for 4 hours. (This can be done quickly with the aid of a hair-dryer – after 3–5 minutes complete dryness is obtained as observed by the whitish colour of the cell monolayer.)

ix) Add to each well 50 µl of a hyperimmune porcine CSF antiserum, diluted in 0.5 M NaCl containing 1% Tween 80 + 0.1% sodium azide, pH 7.6. Incubate at 37°C for 15 minutes. The working dilution of the antiserum should be determined by prior titration: i.e. a serum with an NPLA titre of 1/30,000 could be used at 1/100.

x) Wash the plates five times in 0.15 M NaCl containing 1% Tween 80, pH 7.6.

xi) Add to each well 50 µl of an anti-porcine IgG-HRPO conjugate, diluted to its working concentration in 0.5 M NaCl with 1% Tween 80, pH 7.6, and then incubate for 10 minutes at 37°C.

xii) Wash the plates five times in 0.15 M NaCl containing 1% Tween 80, pH 7.6.

xiii) Add 50 µl of chromogen–substrate solution to each well and stain for 15–30 minutes at room temperature. This solution is described in Section B.1.a. ‘Immunoperoxidase procedure for differentiation of pestiviruses by monoclonal antibodies’.

xiv) The test is read visually. Infected cell sheets are completely or partially stained reddish brown. In doubtful cases, the monolayer should be examined by low-power microscopy. The cytoplasm of infected cells is stained dark red.

xv) The following controls are included in the test: cell control, positive serum and back titration of test virus. The back-titration should confirm that virus has been used at a concentration of between 30 and 300 TCID₉₀/50µl.

Occasionally, sera from pigs infected with BVDV react in the FAVN or NPLA at low dilution as if they were infected with CSFV. The extent of cross-reactivity depends on the strain of BVDV involved and the interval between infection and time of sampling (27). The usually high antibody levels reached after exposure to CSF infection, including strains of low virulence, allow the use of comparatively high initial dilutions in NPLA tests for CSF antibody, thus avoiding most, but not all, cross-reactions (20, 21). In case of continued doubt, comparative tests using a strain of CSFV, a strain of BVDV and a strain of BDV, that are representative for the country or region, have proved useful. Comparative neutralisation tests are end-point titrations in which the same series of twofold dilutions of the suspected serum sample is tested in duplicate against 100 TCID₉₀ of each selected virus strain. The comparative tests are performed according to the protocols described for the FAVN or NPLA; the cell lines used must be suitable for BVDV and BDV. Neutralisation titres are expressed as the reciprocal of the highest serum dilution that prevents virus growth in 50% of two replicate wells. A four-fold difference or more between end-points of two titrations should be considered decisive for an infection by the virus species yielding the highest titre.

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

Competitive, blocking and indirect techniques may be used on any suitable support. The tests used should minimise cross-reactions with BVDV and other pestiviruses. However, the test system must ensure identification of all CSF infections, and at all stages of the immune response to infection.

Antigen: The antigen should be derived from or correspond to viral proteins of one of the recommended CSFV strains. Cells used to prepare antigen must be free from any other Pestivirus infection.
Antisera: Polyclonal antisera for competitive or blocking assays should be raised in pigs or rabbits by infection with one of the recommended CSFV strains or with the lapinised C strain. MAbs should be directed against or correspond to an immunodominant viral protein of CSFV. Indirect assays should use an anti-porcine immunoglobulin reagent that detects both IgG and IgM.

The sensitivity of the ELISA should be high enough to score positive any serum from convalescent animals, i.e. at least 21 days post–inoculation that reacts in the neutralisation test. The ELISA may only be used with serum or plasma samples derived from individual pigs. If the ELISA procedure used is not CSF-specific, then positive samples should be further examined by differential tests to distinguish between CSF and other pestiviruses.

The complex-trapping blocking ELISA (4) is a one-step method and is suitable for use in automated ELISA systems e.g. robots. The sera are tested undiluted. The test is fast and easy to perform, and detects antibodies against low virulence strains of CSFV at an early stage after infection. As the MAbs are specific for CSFV, the complex-trapping blocking ELISA will only rarely detect antibodies against BVDV, although BD antibodies can be more problematic. Positive sera are retested for confirmation by the NPLA.

More information on the commercial kits can be obtained from the OIE Reference Laboratories.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Effective inactivated whole virus vaccines against CSF are not available.

C1. Modified live virus vaccines

MLV vaccines are produced from CSFV strains that have been attenuated by passage either in cell cultures or in a suitable host species not belonging to the family Suidae. Production is carried out in cell cultures, or in non-Suidae animals, based on a seed-lot system. This must be validated with respect to identity, sterility, purity, safety, nontransmissibility, stability and immunogenicity.

Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 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 CSFV 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 Appendix I.1.6.1. of Chapter I.1.6. of this Terrestrial Manual.

To produce a seed lot and a final vaccine of high quality, the optimal conditions for virus yield must be determined. For vaccines produced in cell cultures, growth curve experiments must be done to study the effect of composition of the medium, regulation of pH and atmospheric CO₂ content, starting concentration of seeded cells, ratio between cell sheet surface and medium volume, phase of the cell growth at the time of viral infection, stationary or rolling conditions during viral replication, etc. For vaccines produced in animals, their age, breed, weight, the size of inoculum (number of animal ID₅₀ [50% infectious dose]), pathogenesis of the infection, and the clinical signs are factors that must be investigated to determine the peak of virus growth and the tissues to be harvested.

Regardless of the production method, the substrate should be harvested under aseptic conditions and be subjected to a freeze–thaw cycle to release cell-associated virus. Coarse cell or tissue elements are removed by filtration or low-speed centrifugation. A stabiliser is added, such as lactose at a final concentration of 5%. The vaccine is homogenised before lyophilisation to ensure a uniform batch.

The vaccine virus in the final product should not differ by more than five passages from the material used for validating the seed lot. The commercial vaccine should be produced in batches in lyophilised form as a homogeneous product.

1. Seed management

a) Characteristics of the seed

To validate a seed lot for an MLV CSF vaccine, samples of the seed lot must first pass pilot experiments. Except for tests to confirm identity, sterility, purity and stability of attenuation, pilot experiments may also be
performed with representative samples of the final commercial product. These samples must originate from the same seed lot as tested above.

Except where otherwise specified, all pigs used in pilot tests are 6–8 weeks of age, healthy, free from antibodies against CSFV and BVDV, of the same breed and origin, grouped at random if necessary, and kept under the same conditions. Pregnant sows must be of equal parity.

The seed virus must be sterile and induce specific neutralising antibodies against a virulent strain of CSFV in pigs.

b) Method of culture

Production is performed in cell cultures or in a suitable host of a species not of the family Suidae.

c) Validation as a vaccine

i) Purity

Vaccine must be virologically pure.

Each of three seronegative pigs is inoculated intramuscularly with an amount of seed-lot virus equivalent to tenfold the amount of virus contained in one dose of vaccine. This is repeated 3 weeks later using the same dose and route of administration. Serum samples are collected 2 weeks after the last inoculation, and tested by the most sensitive method for freedom from antibodies to the viruses of African swine fever, Aujeszky’s disease, BVD, foot and mouth disease (all types), transmissible gastroenteritis, swine vesicular disease, porcine reproductive and respiratory syndrome, and porcine influenza (types H1N1 and H3N2); and for porcine adenoviruses, porcine enteroviruses (types 1 and 2), porcine parvovirus and circovirus.

ii) Safety

For tests of safety, each of ten seronegative pigs is inoculated intramuscularly with ten vaccine doses. Ten other pigs serve as controls. All pigs are observed for 3 weeks thereafter. Body temperatures are recorded and blood samples are collected daily, with an anticoagulant, for the first week. Body weights are recorded at inoculation and 2 weeks later. No animal should die or show signs of illness caused by the vaccine (seed-lot) virus. A daily group average body temperature should not reach 40.5°C or more throughout the trial period. The average weight gain should not fall significantly ($p < 0.05$) below that of the controls. Leukopenia (white blood cell [WBC] count $< 7 \times 10^6$ cells/ml) may be disregarded if it is only in one pig for 1 day.

Each of ten pigs is immunosuppressed by daily injections, each of 2 mg prednisolone/kg body weight, for 5 consecutive days. On day 3, each animal is inoculated with the equivalent of one dose of vaccine, and kept under observation for 3 weeks thereafter. No animal should die or become ill due to the vaccine virus.

Each of ten sows, 25–35 days pregnant, is inoculated intramuscularly with the equivalent of one dose of vaccine. A further ten animals of the same parity and gestation serve as controls. The vaccination should not interfere with normal gestation to term, and the number of live piglets born from the test group should not be significantly fewer ($p < 0.05$) than that for the control pigs.

For field trials, a minimum of 200 pigs is used, farrowed and reared by at least 20 dams, and seronegative for CSF and BVD. The litters are equally distributed over at least two farms. Half of the piglets in each litter are inoculated intramuscularly at 7–14 days of age with the equivalent of one dose of vaccine. The un inoculated littermates are controls. All piglets are weighed at inoculation and 2 weeks later, and are kept under observation for 3 weeks. A mortality rate that exceeds 5% due to causes other than vaccination invalidates the trial. No animal should die or show signs of disease due to the vaccine virus. The average weight gain of the inoculated pigs in the litters should not be more than 20% below that of the controls during the 2 weeks post-inoculation.

iii) Nontransmissibility

To confirm nontransmissibility, 24 seronegative pigs are divided into four groups of equal size. Five pigs in each group are inoculated intramuscularly with the equivalent of one dose of vaccine. The remaining pigs represent in-contact controls. All pigs are challenged 6 weeks later with at least $10^5$ PID$_{50}$ (50% pig infectious dose) of a virulent strain of CSFV. All in-contact animals should be serologically negative at the time of challenge, and then die within 3 weeks. All vaccinated pigs should remain healthy and survive.

iv) Stability of attenuation

To confirm stability of virus attenuation, two pigs are each inoculated intramuscularly with an amount of seed-lot virus equivalent to 100 doses of vaccine, and then killed 6–7 days later. The tonsils of both
pigs are pooled and made into a 10% suspension in PBS, pH 7.2. This is used to inoculate two further pigs intramuscularly with 2 ml, and these are then killed 6–7 days later. This protocol is repeated five times. During these passages, the tonsillar tissue may be stored at 4°C, if storage is to be under 24 hours, or at –70°C for longer periods. At the same time, the presence of CSF antigen is confirmed at each passage by the direct FAT in cryostat sections of the tonsils, or by virus isolation in a suitable substrate. If CSFV or antigen cannot be demonstrated after a certain passage, a second series of passages is performed to show infection, commencing with the last two pigs of the previous series.

Five pigs are inoculated intramuscularly with the sixth pig passage of the seed-lot virus, equivalent to one vaccine dose or, if this passage has not been reached, the highest passage of the two series where virus or viral antigen was detected. Five further pigs are similarly inoculated with one dose of the seed-lot virus, equivalent to one vaccine dose. All pigs are weighed at the time of inoculation and again 2 weeks later. Blood is collected daily into anticoagulant during the first week, and all pigs are kept under observation for 3 weeks. No animal should die or become ill from the vaccine virus. The average weight gain of the two groups during the first 2 weeks should not differ significantly ($p < 0.05$). Leukopenia (WBC count of $<7 \times 10^6/ml$) is permitted, at the most, in one pig of either group for 1 day.

**v) Immunogenicity**

To demonstrate adequate immunogenicity, ten pigs are each inoculated with an amount of virus equivalent to one dose of vaccine, and two other pigs are housed separately as unoinculated controls. All pigs are challenged 7 days later with $10^5$ PID$_{50}$ of a virulent strain of CSFV. Only the controls should die.

In a test for duration of immunity, ten pigs are each inoculated with one dose, and two others are maintained separately as controls. Six months later, the sera of the inoculated pigs are tested for CSF antibodies; at least eight pigs should be positive. All pigs are then challenged with at least $10^5$ PID$_{50}$ of a virulent strain of CSFV, and observed for 3 weeks. Only the controls should die.

To test protection of the development of the carrier sow syndrome, 20 pregnant sows at the same stage of gestation are randomly divided into two groups. The sows of one group are vaccinated once or twice with an amount of virus equivalent to one dose of vaccine, and intranasally challenged 4 weeks after the last vaccination with a field strain of low virulence, together with the unvaccinated control sows. All sows are killed 4 weeks after challenge and the fetuses are examined for the presence of CSFV or viral antigen. Vaccination should significantly reduce transplacental transmission of the virus.

Under the storage conditions prescribed by the manufacturer for the final product, a volume of virus equivalent to one dose of vaccine must maintain its immunogenicity at least until the end of the stated shelf life.

2. **Method of manufacture**

Each batch of MLV CSF vaccine must be derived from the same seed lot that has been used for the pilot tests. Also, each batch must be prepared according to the production protocol and under the conditions laid down for the registration of the final product. The properties of each batch and those of the seed lot must be verified as uniform.

3. **In-process control**

The protocol for production will depend on the vaccine strain, the production system (animals or cell cultures), and available facilities. The norms for cell culture vaccines may vary according to the production system, namely, primary cultures, cell lines, monolayers or suspension cultures.

4. **Batch control**

All pigs used in batch control tests must be 6–8 weeks of age and free from antibodies to CSFV and BVDV. They must be uniform in origin, breed, husbandry, and randomly distributed into any groups where necessary.

**a) Identity**

The vaccine must induce specific neutralising antibodies against a virulent strain of CSFV.

**b) Sterility**

Tests for sterility and freedom from contamination of biological material may be found in Chapter I.1.5.
Chapter 2.1.13. — Classical swine fever (hog cholera)

c) Safety

Each of three pigs is inoculated intramuscularly with ten doses of the reconstituted vaccine as a single injection. The pigs are observed for 3 weeks thereafter and body temperatures are taken daily for the first week. No pig should die or show signs of disease attributable to the vaccine, the average daily body temperature must at no time reach 40.5°C or more, and the pigs should grow normally.

d) Purity

The batch must be virologically pure. To test for this, three pigs are each inoculated intramuscularly with ten vaccine doses. Serum samples are collected at the time of inoculation and again 5 weeks later. These are tested for antibodies to BVD (neutralisation for 1 hour at 37°C) and porcine parvovirus (haemagglutination inhibition using four haemagglutinating units). All three pigs must remain disease free. Tests for virological purity need not be carried out when using vaccines produced in rabbits.

e) Potency

Potency is expressed as the number of 50% protective doses (PD$_{50}$) contained in one vaccine dose. One vaccine dose is at least 100 PD$_{50}$.

Two groups of five 6–8-week-old piglets are inoculated intramuscularly with a 1/40 and a 1/160 dilution of the reconstituted vaccine, respectively, using buffered salt solution, pH 7.2. The vaccinated pigs together with two controls are challenged intramuscularly with 10$^5$ PID$_{50}$ of a virulent strain of CSFV 2 weeks later. The pigs are observed for 2 weeks thereafter, during which time the controls should die. From the pigs that survive without showing any signs of CSF, the number of PD$_{50}$ contained in the vaccine is calculated using the usual statistical methods.

This potency test may be replaced by an infectivity assay, provided that the manufacturer can show that there is a distinct and reproducible relationship between the virus content of the vaccine and the protection it will confer on pigs against challenge.

f) Stability

The period of validity of a batch of lyophilised CSF vaccine should not be under 1 year.

C2. Marker vaccines

Despite the existence of safe and effective MLV vaccines against CSF, their use has been discouraged in the EU and some other CSF-free or near free countries, because antibodies provoked by such vaccines cannot be distinguished from antibodies induced by the wild-type virus. A ‘marker vaccine’ does not have this disadvantage: it can elicit a protective immune response that can be distinguished from the immune response induced by field virus. A prerequisite for the distinction between vaccinated and naturally infected animals is the availability of a companion serological test that is highly discriminatory, for tracing residual infections.

The minimum demands for CSF marker vaccines and the companion discriminatory tests have been formulated as follows (5):

a) Vaccine

The vaccine should provide protection against any natural-contact challenge. The efficacy of vaccination should be shown experimentally by studies in which transmission of wild-type virus in vaccinated groups of pigs is studied. The protective effect of vaccination should be achieved within the shortest possible period. A fast and reliable protection should preferably be obtained after one single application. Furthermore, it should be ensured that infection of vaccinated pregnant sows does not lead to transplacental infection and the birth of litters congenitally infected with CSFV. Duration of immunity should be at least 6 months.

Many different marker vaccines for CSF are under development, but so far, two have been registered in the EU. Both are subunit vaccines that employ the E2 glycoprotein of CSFV as an immunogen and have been subject to independent assessment (8, 23). The E2 subunit is produced by insect cells that are infected by genetically modified baculovirus, which contains the E2 gene of CSFV. The vaccines, therefore, do not contain any CSFV, while the baculo (vector) virus is chemically inactivated. The final preparations are adjuvanted with mineral oils to form a double (water/oil/water), or a single (water in oil) emulsion.

b) Companion discriminatory test

The companion discriminatory serological test should be very sensitive because vaccination will reduce the prevalence of the disease. It should be used as a herd test. If a high sensitivity reduces the specificity of the
test, already compromised by the presence of antibodies to other pestiviruses, good and fast confirmatory assays should be available to discriminate positive from false-positive results.

The existing accompanying tests for E2 subunit vaccines are ELISAs that rely on the detection of antibody to the Erns protein (11, 12). Such a test has recently been approved by the European Commission (2), for use in determining whether herds vaccinated with an E2 subunit vaccine may also have been exposed to field virus.

REFERENCES


*   *

**NB:** There are OIE Reference Laboratories for Classical swine fever (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
Highly pathogenic avian influenza (HPAI), also known as fowl plague, is caused by specified influenza A viruses that are members of the family Orthomyxoviridae. There are three influenza types – A, B and C; only influenza A viruses are known to infect birds. Diagnosis is by isolation and characterisation of the virus. 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 tracheal 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 9–11-day-old embryonated fowl eggs. The eggs are incubated at 35–37°C for 4–7 days. The allantoic fluid of any eggs containing dead or dying embryos as they arise 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 or matrix antigens, both of which are common to all influenza A viruses. Isolation in embryos has recently been replaced, under certain circumstances, by reverse-transcription polymerase chain reaction.

For subtyping the virus, the laboratory must have monospecific antisera prepared against the isolated antigens of each of the 15 haemagglutinin (H1–H15) and 9 neuraminidase (N1–N9) subtypes of influenza A viruses that can be used in immunodiffusion tests. Alternatively, the newly isolated virus may be examined by haemagglutination and neuraminidase inhibition tests against a battery of polyclonal antisera to a wide range of strains covering all the subtypes.

As the terms HPAI and ‘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. Whereas all virulent strains isolated to date have been either of the H5 or H7 subtype, most H5 or H7 isolates have been of low virulence. 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 inoculation of a minimum of eight susceptible 4–8-week-old chickens with infectious virus; strains are considered to be highly pathogenic if they cause more than 75% mortality within 10 days. Isolation and characterisation of suspected pathogenic strains of the virus should be conducted in a virus-secure laboratory.

Serological tests: As all influenza A viruses have antigenically similar nucleocapsid and antigenically similar 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 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.

Requirements for vaccines and diagnostic biologicals: In most countries, vaccines specifically designed to contain or prevent HPAI are banned or discouraged by government agencies because they may interfere with stamping-out control policies. During the 1990s the prophylactic use of inactivated oil-emulsion vaccines was employed in Mexico and Pakistan to control widespread outbreaks of HPAI, and a recombinant fowlpox virus vaccine expressing the homologous HA gene was also tested in field trials in Mexico. During the 1999–2001 outbreak 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 between vaccinated birds and birds infected with the field virus.

If HPAI is used in the production of vaccine or in challenge studies, the facility should meet the OIE requirements for Containment Group 4 pathogens.

A. INTRODUCTION

Highly pathogenic avian influenza (HPAI), which is also known as fowl plague, is caused by infection with influenza A viruses of the family Orthomyxoviridae. Influenza A viruses are the only orthomyxoviruses known to 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, but the overwhelming majority of isolates have been of low pathogenicity for chickens and turkeys, the main birds of economic importance to be affected. Influenza A viruses have antigenically related nucleoprotein and antigenically related matrix proteins, but are classified into subtypes on the basis of their haemagglutinin (H) and neuraminidase (N) antigens (40). At present, 15 H subtypes (H1–H15) and 9 neuraminidase subtypes (N1–N9) are recognised. To date, the highly virulent influenza A viruses that produce acute clinical disease in chickens and turkeys have been associated only with the H5 and H7 subtypes (with the exception of two H10 subtypes that would also have fulfilled the OIE and European Union [EU] definitions), although the reasons for this are not clear. Many viruses of H5 and H7 subtype isolated from birds have been of low virulence for poultry (1).

Depending on the age and type of bird and on environmental factors, the highly pathogenic disease may vary from one of sudden death with little or no overt signs to a more characteristic disease with respiratory signs, excessive lacrimation, sinusitis, oedema of the head, cyanosis of the unfeathered skin and diarrhoea. However, none of these signs can be considered pathognomonic. Diagnosis of the disease, therefore, depends on the isolation of the virus and the demonstration of its virulence for an appropriate host. Testing sera from suspect birds using immunological 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 multiple basic amino acids at the cleavage site of the haemagglutinin). These definitions evolve as scientific knowledge of the disease increases.

HPAI is subject to official control 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 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 Appendix I.1.6.1 of Chapter I.1.6 of this Terrestrial Manual. 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

Samples taken from dead birds should include intestinal contents (faeces) or cloacal swabs and oro-nasal swabs. Samples from trachea, lungs, air sacs, intestine, spleen, kidney, brain, liver and heart may also be collected and processed either separately or as a pool.

Samples from live birds should include both tracheal and cloacal swabs, although swabs of the latter site are the most likely to yield virus. As small delicate birds may be harmed by swabbing, the collection of fresh faeces may serve as an adequate alternative.

The samples should be placed in isotonic phosphate buffered saline (PBS), pH 7.0–7.4, containing 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 tracheal 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. 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. For prolonged storage, diagnostic samples and isolates should be kept at –80°C.
The preferred method of growing avian influenza A viruses is by the inoculation of embryonated specific pathogen free (SPF) fowl eggs, or at least 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 at least five embryonated SPF fowl eggs of 9–11 days' incubation. The eggs are incubated at 35–37°C for 4–7 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 and the allantoic fluids should then be tested for haemagglutination (HA) activity (see Section B.3.b.). Detection of HA activity 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 agar gel immunodiffusion (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 resuspended in a 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 (5). 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 in amnioallantoic fluid, but various enzyme-linked immunosorbent assays (ELISAs) are now also available. There is a sensitive and specific ELISA that demonstrates nucleoprotein of type A influenza virus using a monoclonal antibody against type A influenza nucleoprotein (27, 28, 33). This is available as a commercial kit.

Any HA activity of sterile fluids harvested from the inoculated eggs is most likely to be due to an influenza A virus or to an avian paramyxovirus (a few strains of avian reovirus will do this, or nonsterile fluid could contain HA of bacterial origin). There are currently nine recognised serotypes of avian paramyxoviruses. Most laboratories will have antiserum specific for 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 2.1.15.).

Alternatively, the presence of influenza virus can be confirmed by the use of reverse-transcription polymerase chain reaction (RT-PCR) using nucleoprotein-specific conserved primers (2). Also, the presence of subtype H5 or H7 influenza virus can be confirmed by using H5- or H7-specific primers (13, 26, 39).

The method recommended for definitive antigenic subtyping of influenza A viruses by the World Health Organization (WHO) Expert Committee (40) involves the use of highly specific antisera, prepared in an animal giving minimum nonspecific reactions (e.g. goat), directed against the H and N subtypes (25). An alternative technique is the use of polyclonal antisera raised against a battery of intact influenza viruses. Subtype identification by this technique 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 3 of this *Terrestrial Manual*).

2. Assessment of pathogenicity

The term highly pathogenic avian influenza implies the involvement of virulent strains of virus. It is used to describe a disease of chickens with clinical signs such as excessive lacrimation, respiratory distress, sinusitis, oedema of the head and face, cyanosis of the unfeathered skin, and diarrhoea. Sudden death may be the only sign. These signs may vary enormously depending on the host, age of the bird, presence of other organisms and environmental conditions. In addition, viruses that normally cause only a mild or no clinical disease may mimic highly pathogenic avian influenza if exacerbating conditions exist.

At the First International Symposium on Avian Influenza held in 1981 (3), it was resolved to abandon the term ‘fowl plague’ and to define highly pathogenic strains on the basis of their ability to produce not less than 75% mortality within 8 days in at least eight susceptible 4–8-week-old chickens inoculated by the intramuscular, intravenous or caudal air sac route. However, this definition proved unsatisfactory when applied to the viruses responsible for the widespread outbreaks in chickens occurring in 1983 in Pennsylvania and the surrounding
states of the United States of America (USA). The problem was mainly caused by the presence of a virus of
demonstrable low pathogenicity in laboratory tests, but which was shown to be fully pathogenic following a single
point mutation. Further consideration of a definition to include such ‘potentially pathogenic’ viruses was
undertaken by several international groups.

The eventual recommendations made were based on the finding that while there have been numerous isolations
of strains of H5 and H7 subtypes of low pathogenicity, all the highly pathogenic influenza strains isolated to date
have possessed either the H5 or H7 haemagglutinin. Further information concerning the pathogenicity or
potential pathogenicity of H5 and H7 subtypes may be obtained by sequencing the genome, as pathogenicity is
associated with the presence of multiple basic amino acids (arginine or lysine) at the cleavage site of the
haemagglutinin. For example, all H7 subtype viruses of low virulence have had the amino acid motif at the HA0
cleavage site of either -PEIPKGR*GLF- or -PENPKGR*GLF-, whereas examples of amino acids motifs for HPAI
H7 viruses are: -PEIPKKKRR*GLF-, -PETPKRRKR*GLF-, -PEIPKKKREKR*GLF-, -PETPKRRR*GLF-. Amino
acid sequencing of the cleavage sites of H5 and H7 subtype influenza isolates of low virulence for birds should
identify viruses that, like the Pennsylvania virus, have the capacity, following simple mutation, to become highly
pathogenic for poultry.

The OIE subsequently adopted the following criteria for classifying an avian influenza virus as highly pathogenic:

a) Any influenza virus that is lethal for six, seven or eight of eight 4–8-week-old susceptible chickens within
10 days following intravenous inoculation with 0.2 ml of a 1/10 dilution of a bacteria-free, infective allantoic
fluid.

b) The following additional test is required if the isolate kills from one to five chickens but is not of the H5 or H7
subtype: growth of the virus in cell culture with cytopathic effect or plaque formation in the absence of
trypsin. If no growth is observed, the isolate is not considered to be a HPAI isolate.

c) For all H5 and H7 viruses of low pathogenicity and for other influenza viruses, if growth is observed in cell
culture without trypsin, the amino acid sequence of the connecting peptide of the haemagglutinin must be
determined. If the sequence is similar to that observed for other HPAI isolates, the isolate being tested will
be considered to be highly pathogenic.

In the EU, a similar definition was adopted in Directive 92/40/EEC (11), although in this case the intravenous
pathogenicity index (IVPI) test was used as a method of assessing virulence. For the purposes of confirming
disease and implementing the control measures in the Directive, the following definition applies:

‘an infection of poultry caused by an influenza A virus that has an intravenous pathogenicity index in 6-
week-old chickens >1.2 or any infection with influenza A viruses of H5 or H7 subtype for which nucleotide
sequencing has demonstrated the presence of multiple basic amino acids at the cleavage site of the
haemagglutinin.’

The IVPI test is carried out as follows.

i) Fresh infective allantoic fluid with a HA titre >1/16 (>2^4 or >log_2 4 when expressed as the reciprocal) is
diluted 1/10 in sterile isotonic saline.

ii) 0.1 ml of the diluted virus is injected intravenously into each of ten 6-week-old SPF chickens.

iii) Birds are examined at 24-hour intervals for 10 days. At each observation, each bird is scored 0 if
normal, 1 if sick, 2 if severely sick, 3 if dead. (The judgement of sick and severely sick birds is a
subjective clinical assessment. Normally, ‘sick’ birds would show one of the following signs and
‘severely sick’ more than one of the following signs: respiratory involvement, depression, diarrhoea,
cyanosis of the exposed skin or wattles, oedema of the face and/or head, nervous signs. Dead
individuals must be scored as 3 at each of the remaining daily observations after death.)

iv) The intravenous pathogenicity index (IVPI) is the mean score per bird per observation over the 10-day
period. An index of 3.00 means that all birds died within 24 hours, and an index of 0.00 means that no
bird showed any clinical sign during the 10-day observation period.

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. The most commonly used method has been RT-PCR using
oligonucleotide primers complementing areas of the gene either side of the cleavage site coding region, followed

1 For example primary cells such as chick embryo cells or cell lines such as MDCK cells, although most cell cultures
support the growth of HPAI influenza viruses or those of low pathogenicity in the presence of trypsin.
by cycle sequencing (38). Various stages in the procedure can be facilitated using commercially available kits and automatic sequencers.

Now that the presence of multiple basic amino acids at the HA0 cleavage site is well-established as an accurate indicator of virulence or potential virulence for H5 and H7 influenza viruses, it appears inevitable that determination of the cleavage site by sequencing or other methods will become the method of choice for initial assessment of the virulence of these viruses and incorporated into agreed definitions. This will have the advantage of reducing the number of in-vivo tests, although at present the inoculation of birds may still be required to confirm a negative result as the possibility of virus cultures containing mixed populations 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 (H10 N4 and H10 N5), have been reported that would have fulfilled both the OIE and EU definitions for HPAI viruses (36) as they killed 7/10 and 8/10 chickens with IVPI values >1.2 when the birds were inoculated intravenously. However, they produced no deaths or disease signs when inoculated intranasally and these viruses did not have multiple basic amino acids at their haemagglutinin cleavage sites.

3. Serological tests

a) Agar gel immunodiffusion

All influenza A viruses have antigenically similar nucleocapsid and antigenically similar matrix antigens. This fact enables the presence or absence of antibodies to any influenza A virus to be detected by AGID tests. 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. These have generally employed nucleocapsid-enriched preparations made from the chorioallantoic membranes of embryonated fowl eggs (5) 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, and 2–5 mm apart, are cut in 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. These 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 due to 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 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 (0.1 M), pH 7.0–7.2, and red blood cells (RBCs) 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 birds that are regularly monitored and shown to be free from antibodies to avian influenza). 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.
Chapter 2.1.14. — Highly pathogenic avian influenza

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 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

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 after gentle mixing, 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 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 (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 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 (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.

Chicken sera rarely give nonspecific positive 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, 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. Alternatively, RBCs of the avian species under investigation could be used.

The neuraminidase-inhibition test has been used to identify the AI neuraminidase type of isolates and 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 also relies on using a serological test to detect specific anti-N antibodies; the test procedure has been described (9).

Commercial ELISA kits that detect antibody against the nucleocapsid protein are available. 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.

4. Developing techniques for the diagnosis of avian influenza

At present the conventional isolation and virus characterisation techniques for the diagnosis of AI remain the method of choice, for at least the initial diagnosis of AI infections. However, conventional methods tend to be costly, labour intensive and slow. The past 10 years or so has seen enormous developments and improvements in molecular and other diagnostic techniques, many of these have been applied to the diagnosis of AI infections.

a) Antigen detection

The commercially available Directigen® Flu A kit (Becton Dickinson Microbiology Systems), which is an antigen-capture enzyme immunoassay system, has been used for detecting the presence of influenza A viruses in poultry (28), particularly in the USA. The kit uses a monoclonal antibody against the nucleoprotein and should therefore be able to detect any influenza A virus. Although it was developed to detect virus in mammalian infections it has been successfully applied to detecting viruses in poultry and other birds,
although there may be some variation in the sensitivity for different specimens. The main advantage of the test is that it can demonstrate the presence of AI within 15 minutes. The disadvantages are that it may lack sensitivity, it has not been validated for different species of birds, subtype identification is not achieved and the kits are expensive.

b) Direct RNA detection

Although, as demonstrated by the current definitions of HPAI, molecular techniques have been used in the diagnosis of AI for some time, recently there have been developments in their application for detection and characterisation of AI virus directly from clinical specimens from infected birds.

RT-PCR techniques on clinical specimens could, with the correctly defined primers, result in rapid detection and subtype (at least of H5 and H7) identification, plus a cDNA product that could be used for nucleotide sequencing (22, 30, 31). Results obtained by Koch (19) indicated that care should be taken in clinical specimens used as while tracheal samples from infected birds showed high sensitivity and specificity relative to virus isolation, RT-PCR tests on faecal samples lacked sensitivity. The real application of direct RT-PCR tests may be on rapidly identifying subsequent outbreaks once the primary infected premises has been detected and the virus characterised. This technique was used with success during the 2003 HPAI outbreaks in The Netherlands.

Modifications on the use of RT-PCR have been applied to reduce the time for both identification of virus subtype and sequencing. For example Spackman et al. (29) used a ‘real time’ single-step RT-PCR primer/fluorogenic hydrolysis probe system to allow detection of AI viruses and determination of subtype H5 or H7. The authors concluded that the test performed well relative to virus isolation and offered a cheaper and much more rapid alternative.

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 electro-chemiluminescent 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 AI virus subtypes H7 and H5 in clinical samples within 6 hours (10, 18).

It seems highly likely that within a very short time molecular based technology will have developed sufficiently to allow rapid ‘flock-side’ tests for the detection of the presence of AI virus, specific subtype and virulence markers. The extent to which such tests are employed in the diagnosis of AI will depend very much on agreement on and adoption of definitions of statutory infections for control and trade purposes.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

In some countries, vaccines designed to contain or prevent HPAI are specifically banned or discouraged by government agencies because they may interfere with stamping-out control policies. However, most HPAI control regulations reserve the right to use vaccines in emergencies, and this has been done in Mexico and Pakistan.

Since the 1970s in the USA there has been widespread use of inactivated vaccines produced under special licence on a commercial basis (16, 21, 24). These vaccines have been used primarily in turkeys against viruses that are not highly pathogenic but which may cause serious problems, especially in exacerbating circumstances. Significant quantities of vaccine have been used (15, 21). Inactivated vaccine was prepared from the virus of low virulence of H7N3 subtype responsible for a series of outbreaks in turkeys in Utah in 1995 and used, with other measures, to bring the outbreaks under control (17). Following the stamping out of the HPAI H7N1 outbreaks in Italy, low virulence virus of the same subtype re-emerged and vaccination under strict control measures was allowed.

The existence of a large number of virus subtypes, together with the known variation of different strains within a subtype, pose serious problems when selecting strains to produce influenza vaccines. In addition, some isolates do not grow to a sufficiently high titre to produce adequately potent vaccines without costly prior concentration. The vaccines produced have either been autogenous, i.e. prepared from isolates specifically involved in an epizootic, or have been prepared from viruses possessing the same haemagglutinin subtype that yield high concentrations of antigen. In the USA, some standardisation of the latter has been carried out in that the National Veterinary Services Laboratories have propagated and hold influenza viruses of each subtype for use as seed virus in the preparation of inactivated vaccines (4). These vaccines and those for use in Italy (9, 12) against viruses of low pathogenicity, and against HPAI in Mexico (14) and Pakistan (23), have been prepared from infective allantoic fluid inactivated by beta-propiolactone or formalin and emulsified with mineral oil.
During the 1999–2001 outbreak in Italy, the HPAI H7N1 subtype was eradicated by stamping-out, but a low pathogenic AI virus re-emerged. In order to supplement direct control measures, a DIVA strategy was developed based on the used of an inactivated oil emulsion vaccine containing the same H subtype as the field virus, but a different N, in this case H7N3. Vaccinated and naturally infected birds were differentiated using a serological test to detect specific anti-N antibodies (7, 8).

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 (34). The basic principles for producing vaccines, particularly inactivated vaccines, are common to several viruses e.g. Newcastle disease (Chapter 2.1.15.).

Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 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 HPAI virus 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 Appendix I.1.6.1 of Chapter I.1.6 of this Terrestrial Manual.

Live conventional influenza vaccines against any subtype are not recommended.

1. Seed management
   a) Characteristics of the seed
      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.

   b) Method of culture
      A master seed is established, and from this a working seed. If the strain has been cloned, 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).

   c) Validation as a vaccine
      The master seed should be checked after preparation for sterility, safety, potency and absence of specified extraneous agents.

2. Method of manufacture
   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 below –60°C as lyophilised virus does not always multiply to high titre on subsequent first passage.

   The inactivated influenza vaccines prepared from conventional virus are produced in embryonated fowl eggs. The method of production is basically that of propagating the virus aseptically; all procedures are performed under sterile conditions.

   It is usual to dilute the working seed in sterile PBS, pH 7.2, so that about 10^3–10^4 EID_{50} (50% egg-infective dose) per 0.1 ml are inoculated into the allantoic cavity of 9- or 10-day-old embryonated 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 inactivation.

   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.

3. **In-process control**

For inactivated vaccines, the efficacy of the process of inactivation should be tested in embryonated eggs, taking 25 aliquots of 0.2 ml from each batch and passaging each aliquot three times through SPF embryos.

4. **Batch control**

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.

   a) **Sterility**
   Tests for sterility and freedom from contamination of biological materials may be found in Chapter I.1.5.

   b) **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.

   c) **Potency**
   Conventional potency testing involving the use of three diluted doses and challenge with virulent virus (e.g. Chapter 2.1.15.) may be used for vaccines prepared to give protection against HPAI or H5 and H7 subtypes generally. 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. Assessment of haemagglutinin antigen content (37) could allow _in-vitro_ extrapolation to potency for subsequent vaccine batches.

   d) **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.

   e) **Preservatives**
   A preservative may be used for vaccine in multidose containers.

   f) **Precautions (hazards)**
   Care must be taken to avoid self-injection with oil emulsion vaccines.

5. **Tests on the final product**

   a) **Safety**
   See Section C.4.b. above

   b) **Potency**
   See Section C.4.c. above.

6. **Novel vaccines**

Recombinant fowl pox virus vaccines containing H5 HA have been prepared, evaluated (6, 32) and used in field trials in Mexico (15).

A baculovirus-expression system has been used to produce recombinant H5 and H7 antigens for incorporation into vaccines (35).

DNA encoding H5 haemagglutinin has been evaluated as a potential vaccine in poultry (20).
REFERENCES


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**NB:** There are OIE Reference Laboratories for Highly pathogenic avian influenza (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
NEWCASTLE DISEASE

SUMMARY

Newcastle disease (ND) is caused by specified viruses of the avian paramyxovirus type I (APMV-1) serotype of the genus Avulavirus belonging to the family Paramyxoviridae. There are nine serotypes of avian paramyxoviruses designated APMV-I to APMV-9.

Strains of ND virus vary widely in the severity of the disease they may produce in birds. The less pathogenic strains may induce severe 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 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.

Any haemagglutinating agents should be tested for specific inhibition with a monospecific antiserum to ND virus. ND virus (APMV-1) may show some antigenic cross-relationship with some of the other avian paramyxovirus serotypes, particularly APMV-3 and APMV-7.

The pathogenicity of any newly isolated virus can be assessed by determining the mean death time in chicken embryos, the intracerebral pathogenicity index in 1-day-old chicks or by the intravenous pathogenicity index in 6-week-old chickens. In some countries, variations of these standard techniques are used. The pathogenicity of isolates can also be evaluated using molecular techniques, i.e. reverse-transcription polymerase chain reaction and sequencing. Isolation and characterisation of suspected pathogenic strains of the virus should be conducted in a virus-secure laboratory.

Serological tests: The haemagglutination inhibition test is used most widely in ND virus 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 and diagnostic biologicals: Live viruses of low virulence (lentogenic) or of moderate virulence (mesogenic) are used for the vaccination of poultry depending on the disease situation. 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 embryonating 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.

If pathogenic forms of ND virus are used in the production of vaccine or in challenge studies, the facility should meet the OIE requirements for Containment Group 4 pathogens.
A. INTRODUCTION

Newcastle disease (ND) is caused by specified viruses of the avian paramyxovirus type I (APMV-I) serotype of the genus *Avulavirus* belonging to the subfamily Paramyxovirinae, family Paramyxoviridae. The paramyxoviruses isolated from avian species have been classified by serological testing into nine serotypes designated APMV-1 to APMV-9; ND virus has been designated APMV-1 (4).

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 ND virus has been their great variation in pathogenicity for chickens. Strains of ND virus have been grouped into five pathotypes on the basis of the clinical signs seen in infected chickens (13). 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 enteric: a form that usually consists of a subclinical enteric infection.

Pathotype groupings are rarely clear-cut (6) 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.

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), spleen, brain, liver and heart tissues. These may be collected separately or as a pool, although intestinal samples are usually processed separately from other samples.

Samples from live birds should include both tracheal and cloacal swabs, the latter should be visibly coated with faecal material. Small delicate birds may be harmed by swabbing, 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) and tracheal 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. 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 solution to pH 7.0–7.4 following the addition of the antibiotics. 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 culture
The supernatant fluids of faeces or tissue suspensions 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. After inoculation, these are incubated at 35–37°C for 4–7 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 and the allantoic fluids tested for haemagglutination (HA) activity. Fluids that give a negative reaction should be passaged into at least one further batch of eggs.

c) Virus identification
HA activity detected in bacteriologically sterile fluids harvested from inoculated eggs may be due to the presence of any of the 15 haemagglutinin subtypes of influenza A viruses or of the eight other paramyxovirus serotypes. (Nonsterile fluid could contain bacterial HA.) ND virus 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 ND virus is used.

Cross-reactions in HI tests between ND virus and some of the other APMVs, especially APMV-3 and APMV-7 serotype viruses may cause some problems that can be resolved by the use of suitable antigen and antiserum controls.

d) Pathogenicity indices
The extreme variation in virulence of different ND virus isolates and the widespread use of live vaccines means that the identification of an isolate as ND virus 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'). Several potential in-vitro tests for establishing virulence usually related to the molecular basis for pathogenicity (Section B.1.e. below) are being investigated by various groups around the world. At present, a definitive assessment of virus virulence is usually based on one or more of the following in-vivo tests, although the current OIE definition (Section B.1.f. below) allows molecular assessment of virulence:

• Mean death time in eggs
  i) Fresh, sterile, infective allantoic fluid is diluted in sterile saline to give a tenfold dilution series between $10^{-6}$ and $10^{-9}$.
  ii) For each dilution, 0.1 ml is inoculated into the allantoic cavity of each of five 9–10-day-old embryonated SPF fowl eggs, which are then incubated at 37°C.
  iii) The remaining virus dilutions are retained at 4°C and another five eggs are inoculated with 0.1 ml of each dilution 8 hours later and incubated at 37°C.
  iv) Each egg is examined twice daily for 7 days and the times of any embryo deaths are recorded.
  v) The minimal lethal dose is the highest virus dilution that causes all the embryos inoculated with that dilution to die.
  vi) The mean death time (MDT) is the mean time in hours for the minimum lethal dose to kill all the inoculated embryos.
  vii) The MDT has been used to classify ND virus strains into the following groups: velogenic (taking under 60 hours to kill); mesogenic (taking between 60 and 90 hours to kill); and lentogenic (taking more than 90 hours to kill).

• Intracerebral pathogenicity index
  i) Fresh infective allantoic fluid with a HA titre $>2^4 (>1/16)$ 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. (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 strains will give values close to 0.0.

- **Intravenous pathogenicity index**
  
  i) Freshly collected infective allantoic fluid (which should be no older than 24–48 hours and should have tested negative for bacterial contamination) with a HA titre of \( >2^4 \) (\( >1/16 \)) is diluted 1/10 in sterile isotonic saline.
  
  ii) 0.1 ml of the diluted virus is injected intravenously into each of ten 6-week-old SPF chickens.
  
  iii) Birds are examined at 24-hour intervals for 10 days and scored at each observation: 0 if normal, 1 if sick, 2 if paralysed or showing other nervous signs, and 3 if dead. (Dead individuals must be scored as 3 at each of the remaining daily observations after death.)
  
  iv) The intravenous pathogenicity index (IVPI) is the mean score per bird per observation over the 10-day period.

Lentogenic strains and some mesogenic strains will have IVPI values of 0, whereas the indices for virulent strains will approach 3.0.

Some variations have been recommended in these tests. Swabbing of the cloaca and conjunctiva of 8-week-old chickens with undiluted allantoic fluid has been substituted for the IVPI test (23). The intention is to distinguish between viscerotropic velogenic and neurotropic velogenic viruses.

- **Interpretation of pathogenicity indices**

  Interpretation of the pathogenicity indices obtained with a view to imposing trade or movement restrictions, or other policies, is not straightforward. The objective is to control strains significantly more virulent than lentogenic strains, such as Hitchner-B or La Sota. As viruses capable of producing quite severe disease may have IVPI values of 0, the ICPI test is used most often for such assessments. However, as different strains show a range of values from 0.00 to 2.00 in this test, it is clear that any value used for definition must be governed by practicality.

**e) Molecular basis for pathogenicity**

During replication, ND virus 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-translation cleavage is mediated by host-cell proteases. Trypsin is capable of cleaving F0 for all ND virus 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 ND viruses that are pathogenic for chickens have the sequence \(^{112}R/K-R-Q-K/R-R^{116}\) 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 \(^{112}G/E-K/R-Q-G/E-R^{116}\) and L (leucine) at residue 117. Some of the pigeon variant viruses (PPMV-1) examined have the sequence \(^{112}G-R-Q-K-R-F^{117}\), but give high ICPI values. 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.

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 (for a review see ref. 2). 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 ND virus 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 mean that virus isolation and an *in vivo* assessment of virulence will still be required.
Recent analyses of viruses isolated in Ireland in 1990 and during the 1998–2000 outbreaks of ND in Australia have given strong evidence that virulent viruses may arise from progenitor viruses of low virulence (5, 39). Virulent ND virus has also been generated experimentally from low virulence virus by passage in chickens (34).

f) Definition of Newcastle disease

It seems likely that the vast majority of birds are susceptible to infection with ND viruses of both high and low virulence for chickens, although the clinical signs seen in birds infected with ND virus 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, such as chickens, ND viruses show a considerable range of virulence. Generally, variation consists of clusters around the two extremes in tests used to assess virulence, but, for a variety of reasons, some viruses may show intermediate virulence.

The enormous variation in virulence and clinical signs means it is necessary to define carefully 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 (17).

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.’

Monoclonal antibodies

Mouse monoclonal antibodies (MAbs) directed against strains of ND virus have been used in HI tests to allow rapid identification of ND virus without the possible cross-reactions with other APMV serotypes that may occur with polyclonal sera. MAbs have been produced that give reactions in HI tests that are specific for particular strains or variant ND virus isolates (4, 9).

Panels of MAbs have been used to establish antigenic profiles of ND virus isolates based on whether or not they react with the viruses. This has proven to be a valuable method for grouping and differentiating isolates of ND virus, and has been particularly valuable to the understanding of the epidemiology of outbreaks (9).

h) Phylogenetic studies

Development of improved techniques for nucleotide sequencing, the availability of sequence data of more ND 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 (3, 8, 16, 24, 27, 28, 33, 36–38).

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 that retrospective (2).
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 have been several reports of the use of such molecular techniques to detect ND virus in clinical specimens, the advantage being the extremely rapid demonstration of the presence of virus and even its virulence if primers covering the part of the genome coding for the F0 cleavage site are used (12, 20, 21). 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 (20, 21, 25). As with virulence determination, it is important that such techniques alone are not used to record a negative result in investigations of suspected ND.

ND, as defined in Section B.1.f. of this chapter, is subject to official control 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 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 Appendix I.1.6.1 of Chapter I.1.6. of this Terrestrial Manual. Countries lacking access to such a specialised national or regional laboratory should send specimens to an OIE Reference Laboratory.

2. Serological tests

ND virus may be employed as an antigen in a wide variety of serological tests, enabling neutralisation or enzyme-linked immunosorbent assays (ELISA) to be used for diagnosis. At present, the HI test is most widely used. Chicken sera rarely give nonspecific positive reactions in this 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 (0.1 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 ND virus.) 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.

a) Haemagglutination and haemagglutination inhibition tests

- 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 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.
  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
  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.
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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 (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.

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.

HI titres may be used to assess the immune status of a flock. 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 (11), 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 ND virus (7).

There are a variety of commercial ELISA kits available and these are based on several different strategies for the detection of ND virus 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.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

A detailed account of all aspects of ND virus vaccines, including their production and use, has been published (11) and should be referred to for details of the procedures outlined here. Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 are intended to be general in nature and may be supplemented by national and regional requirements.

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 ND virus are expressed. Several of these recombinant viruses have been licensed for use in certain countries.

ND virus strains used in commercial live virus vaccines fall into two groups: lentogenic vaccines, such as Hitchner-B1, La Sota, V4, NDW, I2 and F, and mesogenic vaccines, such as Roakin, Mukteswar and Komarov. Strains from both these groups have been subjected to selection and cloning to fulfill 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 primarily used in countries where ND is endemic this may not necessarily preclude their use.

The vaccine production facility should operate under the appropriate biosecurity 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 Appendix I.1.6.1 of Chapter I.1.6 of this Terrestrial Manual.

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.

Live virus vaccines may be administered to birds by incorporation in the drinking water, delivered as a coarse spray, or by intranasal or conjunctival instillation. Some mesogenic strains are given by wing-web intradermal inoculation. Vaccines have been constructed to give optimum results through application by specific routes. 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 La Sota strain will cause considerably greater problems in young susceptible birds than the Hitchner-B1 strain, although La Sota induces a stronger immune response.

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, 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. A high yield of virus to produce a potent vaccine is important, and the Ulster 2C strain is very suitable for this purpose.

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 3–4 weeks later. It has been demonstrated that inactivated vaccines may also be usefully employed to vaccinate 1-day-old chicks that have a degree of maternal immunity (15), and the best results of all were obtained when 1-day-old maternally immune chicks were given a combination of live and inactivated vaccines, compared with live or inactivated vaccines given alone (14). Vaccination of fully susceptible 1-day-old birds, even with the most mild of live vaccines, may result in respiratory disease, especially if common pathogenic bacteria are present in significant numbers.

Vaccination after 3 weeks of age is normally practised only in breeding hens and hens laying table eggs. This 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.

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 (11). 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 La Sota at 18–21 days of age in the drinking water; live La Sota 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 La Sota 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 (11).

1. **Seed management**

   a) **Characteristics of the seed**

   The first principle to consider when selecting a strain for a live ND virus 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. The use of MAbs has demonstrated considerable variation in the antigenicity of different strains (9). This may indicate a need to tailor vaccines more carefully to relate antigenically to any prevalent field virus.

   A live vaccine based on ND virus strain V4, selected for heat stability, has been introduced to combat the specific problems associated with village chicken rearing in developing countries. The intention is that this vaccine could be coated on food fed to scavenging chickens. To date, trials in different countries have produced mixed results; it may well be that local factors are extremely important in affecting the success of this strategy (35). More recently the thermostable I2 vaccine has been developed specifically for vaccinating village chickens; it is currently recommended that this vaccine be given by eye drop (1).

   Use of live vaccines may be restricted by legislation. For example, Commission Decision 93/152/EEC (18) restricts the use of vaccines in member states of the European Union from 1 January 1995 to those for which the master seed has been tested and shown to have an ICPI of <0.4 if no fewer than $10^7$ mean egg infectious doses (EID$_{50}$) are administered to each bird, or <0.5 if no fewer than $10^8$ EID$_{50}$ are administered to each bird. The OIE Standards Commission has similarly recommended that while in principle vaccines should have an ICPI < 0.7 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 (30).
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. This risk is reflected in Commission Decision 93/152/EEC (18), which restricts the use of viruses used for inactivated vaccine in member states of the European Union from 1 January 1995 to those for which the master seed has been tested and shown to have an ICPI of <0.7 if no fewer than 10^8 EID50 are administered to each bird. Some lentogenic strains grow to very high titres in eggs. Exceptionally high titres can be obtained by the Ulster 2C strain, which has been recommended as a seed for inactivated vaccine (22). However, successful commercial inactivated vaccines are produced when the Hitchner B1, La Sota or F strains are used as seeds.

b) Method of culture

A master seed is established, and from this a working seed. If the strain has been cloned by 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).

c) Validation as a vaccine

Seed viruses of unknown pedigree should be passaged through SPF eggs and cloned before producing the master seed. Some passage through SPF chickens may also be desirable (11). In either case, the master seed should be checked after preparation for sterility, safety, potency and extraneous agents.

2. Method of manufacture

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 (11).

Most ND vaccines are produced in embryonated fowl eggs, and live virus vaccines should be produced in SPF eggs. The method of production is large-scale aseptic propagation of the virus; all procedures are performed under sterile conditions.

It is usual to dilute the working seed in sterile PBS, pH 7.2, so that about 10^3–10^4 EID50/0.1 ml is inoculated into the allantoic cavity of 9- or 10-day-old embryonated 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 Marcol 52, Drakeol 6VR and BayolF, plus one volume of emulsifying agent, such as Arlacel A, Montanide 80 and Montanide 888 (31). 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. Too high viscosity and the vaccine is difficult to inject; too low viscosity and the vaccine is unstable.

3. In-process control

Each batch of live virus vaccine should be tested for viability and potency. 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 remain undetected at the time of harvest.
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 (11).

4. Batch control

Most countries have published specifications for the control of production and testing of ND virus vaccines (e.g. ref. 29), which include the definition of the obligatory tests on vaccines during and after manufacture.

It is necessary to test the infectivity of live virus vaccines to enable adequate levels of virus to be administered. The virus is usually titrated in embryonated fowl eggs to give the EID$_{50}$. This involves making tenfold dilutions of virus; 0.1 ml of each dilution is inoculated into between five and seven 9–10-day-old embryonated fowl eggs. After 5–7 days’ 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 (10).

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter I.1.5.

b) Safety

The use of chickens for the testing of vaccines involves the inoculation of ten or more birds of stated age that originate from an SPF flock. 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 (19).

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 the absence of clinical signs of disease or local lesions.

c) Potency

Various methods for the testing of ND virus vaccines for potency have been proposed. The importance of using a suitable challenge strain for assessment has been stressed (11). A suitable strain is the Herts 33 strain. For live vaccines, the method recommended (19) involves the vaccination of 20 SPF or other fully susceptible birds at the minimum recommended age by the suggested route using the minimum recommended dose. After 14–21 days, each vaccinated bird and ten control birds are challenged intramuscularly with 10$^5$ LD$_{50}$ (50% lethal dose) of ND challenge virus. The vaccine passes the test if at the end of 10 days, 90% of the vaccinated chickens survive with no signs of disease, but all controls die within 6 days.

For inactivated vaccines, 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 10$^5$ LD$_{50}$ of ND challenge virus, 17–21 days later. Chickens are observed for 21 days. The PD$_{50}$ (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 PD$_{50}$ is not less than 50 per dose and if the lower confidence limit is not less than 35 PD$_{50}$ 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.

d) Duration of immunity

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 (11).

e) Stability

When stored under the recommended conditions the final vaccine product should maintain its potency for at least 1 year. Accelerated stability tests such as reduction of infectivity following incubation at 37°C for 7 days (26) 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. Live virus vaccines must be used immediately after reconstitution. Inactivated vaccines must not be frozen.
f) **Preservatives**

For live vaccines preservatives must not be included in the freeze-dried product, but antimicrobial preservatives may be incorporated in the diluent used to reconstitute the vaccine.

g) **Precautions (hazards)**

Live ND virus 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 incision and washing of the site, as for a ‘grease-gun’ injury.

5. **Tests on the final product**

a) **Safety**

See Section C.4.b. above

b) **Potency**

See Section C.4.c. above.

**REFERENCES**


Chapter 2.1.15. – Newcastle disease


*   *

NB: There are OIE Reference Laboratories for Newcastle disease (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
SECTION 2.2.

MULTIPLE SPECIES DISEASES IN LIST B

CHAPTER 2.2.1.

ANTHRAX

SUMMARY

Anthrax is primarily a disease of herbivorous animals, although all mammals, including humans, and at least 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 Bacillus anthracis. The disease has world-wide distribution and is a zoonosis. Anthrax is of importance to the livestock industry, certain populations of wildlife, and humans, especially those who are occupationally exposed.

The disease is mediated 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. Chronic disease may show localised swelling, fever and enlarged lymph nodes; death may occur if the airway becomes obstructed. Post-mortem examination of recently dead animals (which must be performed with great care to avoid infection of the operator or contamination of the environment) may show any number of lesions, none of which is pathognomonic or entirely consistent. Lesions most commonly seen are those of a generalised septicaemia often accompanied by an enlarged spleen having a 'blackberry jam' consistency and poorly clotted blood. Haemorrhage from the nose, mouth and/or anus at death is not a common sign.

Identification of the agent: Visualisation of the encapsulated bacilli, usually in large numbers, in a blood smear stained with polychrome methylene blue (M'Fadyean reaction) is fully diagnostic. Bacillus anthracis is readily isolated in relatively high numbers from blood or tissues of a recently dead animal that died of anthrax, and in pure culture, on any nutrient agar incubated aerobically at 37°C. Its characteristic appearance on blood agar makes this the medium of choice. As the carcass decomposes, especially in a warm climate, the putrefactive bacteria out compete and eventually eliminate the infective organism within the carcass. Confirmation of anthrax in these cases may depend on isolation from soil contaminated by the terminal discharges.

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 grey, nonhaemolytic with a rough, ground-glass appearance and has a very tacky, butyrous consistency. 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' appearance.

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. The bacilli in infected tissue are encapsulated, but this characteristic is lost when the bacterium is grown aerobically in vitro. The capsule can be induced by incubating in defibrinated horse blood for at least 5 hours, or by
culturing the isolate on nutrient agar containing 0.7% sodium bicarbonate with incubation at 37°C in the presence of CO₂.

Additional useful laboratory tests are the absence of motility, susceptibility to the specific diagnostic ‘gamma’ bacteriophage and sensitivity to penicillin. Caution is advised when using these tests, as it has been shown that some genotypes are resistant to the gamma phage and penicillin. Primers are now available that can be used to show the presence of the toxin and capsule genes by polymerase chain reaction as confirmation of virulence, replacing animal inoculation. A thermoprecipitin test described by Ascoli in 1911 is still used in some countries to supply retrospective evidence of anthrax in decomposed carcasses or animal products.

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

**Requirements for vaccines and diagnostic biologicals:** 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 some Eastern European countries, an equivalent type of vaccine is used (strain 55). The Pasteur vaccine is no longer used in Italy. A new vaccine, Carbosap, has been developed that retains both plasmids and exhibits very low virulence. A list of producers is given in the World Health Organization anthrax guidelines (14).

There are no standardised requirements for diagnostic biologicals. Diagnostic gamma phage may be obtained from, for example, the United States Centres for Disease Control and Prevention1 or various central veterinary or anthrax reference laboratories.

### A. INTRODUCTION

Anthrax is primarily a disease of herbivorous animals, although all mammals, including humans, and at least 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 *Bacillus anthracis*, the only obligate pathogen within the genus *Bacillus*. Most of the other species of *Bacillus* are common ubiquitous environmental saprophytes, although a number, notably *B. cereus*, *B. licheniformis* and *B. subtilis*, are occasionally associated with food poisoning in humans and with other clinical manifestations in both humans and animals.

Animal anthrax occurs in at least three different forms: peracute or apoplectic form, acute form, and subacute to chronic form. Ruminants are most likely to manifest the peracute and acute forms, horses the acute form, and dogs, cats, and pigs a subacute to chronic or localised condition. In the peracute disease, signs preceding death often go unobserved. The clinical history usually describes the animal to be in good health a few hours before death. If the animal is observed shortly before death, fever up to 42°C (107°F), muscle tremors, dyspnoea, and mucosal congestion are the most common signs. Shortly afterwards, the animal will often have terminal convulsions, collapse and then die. Following death, unclotted blood may sometimes be seen to exude from the anus, vulva, nostrils, and/or mouth. Incomplete rigor mortis is also common.

The acute form may occur in cattle and clinical signs, such as depression, anorexia, fever, rapid respiration, increased heart rate, congested mucous membranes, or oedematous swellings, may be observed up to 48 hours before death. The acute form is usually seen in horses and varies with the site of exposure. Enteritis and colic are accompanied by high fever and depression. Death usually occurs within 48–96 hours. Spores introduced subcutaneously, for example by biting insects, result in a hot, oedematous swelling at the site that spreads to the throat, thorax, abdomen, prepuce or mammary gland. Dyspnoea due to throat swelling with resulting compression of the trachea may also be apparent. The course of the disease is usually 1–3 days, with some animals surviving for 1 week or more.

The subacute to chronic form of anthrax occurs in domestic and wild pigs, dogs and cats. The infectious bacteria are usually ingested when the host feeds on a contaminated source. The organism tends to localise in the regional lymph nodes of the pharyngeal area, where severe swelling may occur, resulting in death by occlusion of the airway. In cases where this does not occur, a fatal bacteraemia may develop, although recovery after a few days of illness is not uncommon. An intestinal form with severe acute gastroenteritis is also seen in carnivores and omnivores.

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1 The Division of Bacterial and Mycotic Diseases, Centers for Disease Control, 1600 Clifton Road, Atlanta, Georgia 30333, United States of America.
Suspicion of anthrax will depend on signs, such as sudden death with or without haemorrhaging from the orifices and incomplete rigor mortis, or on the history of the site, herd, etc. If anthrax is suspected, a thin smear is made on a microscope slide from a small drop of blood. This may be obtained by making a small cut in an ear vein or with a syringe from any available vein (care must be taken to avoid contaminating the environment or the operator). The blood smear is air-dried, fixed by immersion in 95–100% alcohol for 1 minute and stained with polychrome methylene blue (further details below). The presence of the encapsulated bacilli resembling railway ‘box-cars’ in pairs or short chains, usually in large numbers, is definitive for anthrax. Smears made from swabs of the blood emerging from any of the orifices will also reveal the capsulated bacilli, but may be contaminated with other organisms or artefacts. Samples of the blood can also be taken for culture.

To prevent environmental contamination, it is usual not to perform a necropsy on the carcass of suspected or confirmed cases. If the carcass is opened by necropsy or scavengers, the vegetative form of *B. anthracis* is released from the acidic environment of decay and produces spores that create foci of contamination. In some countries post-mortem is forbidden. Post-mortem findings are well documented, however. There are no consistent pathognomonic lesions and considerable similarities are seen to other infectious and toxic causes of acute death. Poorly clotted dark blood, enlarged pulpy spleen with a ‘blackberry jam’ consistency, and multiple petechial haemorrhages characteristic of a septicaemia are usual. In horses, post-mortem findings may be similar to those of ruminants, but sometimes may just consist of oedematous lesions confined to the throat and neck with no involvement of internal organs. In omnivores (pigs) and carnivores, findings of septicaemia as described for ruminants may occur, but more often there is extensive oedema and inflammation in the pharyngeal area. If the focus of infection is in the gastrointestinal tract, severe inflammation, sometimes with haemorrhage and necrosis, may be seen in the stomach, intestines, and mesenteric lymph nodes, accompanied by peritonitis and excessive peritoneal fluid.

Natural decomposition of a carcass destroys most of the vegetative organisms through the action of putrefactive bacteria. This can occur within a day or two in hot climates if the carcass is left undisturbed. The capsulated bacilli may not be readily seen in smears of blood samples taken after this has occurred, though the blood may remain culture positive for a day or so more. Some sporulation may have occurred in fluids exuded through natural body openings, and, particularly if a carcass has been opened by scavengers, many spores may be dispersed into the environment. Swabs of concealed fluids or samples of soil contaminated by the fluids are likely to yield *B. anthracis* on culture. Culture of soil samples may be the best way to confirm that death was due to anthrax in well putrefied carcasses.

- **Disposal after sample collection**

Proper disposal of an infected carcass by incineration (although labour intensive and fuel consumptive) is the most desirable method and is often required by law. The contaminated soil, bedding, etc. should be incinerated after use and the bedding incinerated afterwards. Care should be taken not to create dusty aerosols when handling the bedding. Care should also be taken to avoid skin wounds from sharp instruments or animal bites and scratches.

- **Risk factors for handlers of anthrax carcasses**

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, woolen, 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. Laboratory workers should use good laboratory practice when working with specimens from suspected anthrax cases and when culturing *B. anthracis*. A biological safety cabinet is required for the manipulation of broth cultures or spore suspensions. Decontamination of all contaminated materials and surfaces should be accomplished by either autoclaving, incineration, or 10% sodium hypochlorite (bleach). The use of laboratory animals for diagnostic purposes is no longer required, but if they are used, the bedding and cages should be autoclaved after use and the bedding incinerated afterwards. Care should be taken not to create dusty aerosols when handling the bedding.

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

Demonstration of encapsulated *B. 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. anthracis* from old decomposed carcasses, processed specimens (bone meal, hides), or environmental samples (contaminated soil) is also often difficult, requiring demanding and labour-intensive procedures.

a) Fresh specimens

- Capsule visualisation

As described above, virulent encapsulated *B. anthracis* present in tissues and blood and other body fluids from animals that have died from anthrax should be looked for in smears of these specimens that have been dried, fixed in absolute alcohol for 3 minutes and stained with polychrome methylene blue (M’Fadyean’s reaction) or Giemsa stain. 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’ appearance). The Gram stain does not reveal the capsule. The capsule is not present on *B. anthracis* grown aerobically on nutrient agar or in nutrient broths, but can be seen when the virulent bacterium is cultured for a few hours in a few millilitres of blood (defibrinated horse 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 reconstituting enough nutrient agar base powder for 100 ml of agar in 90 ml of water. It is then autoclaved and cooled to 50°C in a water bath; 10 ml of a filter-sterilised (0.22–0.45 μm filter) 7% solution of sodium bicarbonate is added and mixed. The solution is then poured 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 as before.

- Polychrome methylene blue (M’Fadyean’s stain)

Polychrome methylene blue is 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 now 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. A commercial preparation of polychrome methylene blue (M’Fadyean) strain is becoming increasingly hard to obtain.

In making smears for staining, only small drops of blood or tissue fluid are needed and a thin, small smear is best. After fixing in ethanol 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 x10 objective lens under which the short chains appear like short hairs; once found, these can be observed under oil immersion (x1000) 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.

- Culture and identification of *Bacillus anthracis*

*Bacillus anthracis* grows readily on most types of nutrient agar, however, 5–7% horse or sheep blood agar is the diagnostic medium of choice. Inclusion of polymyxin (100.000 units per litre of medium) will suppress contaminant bacteria and aid in isolation of *B. anthracis*. 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. anthracis* colonies are grey-white to grey, 0.3–0.5 mm in diameter, nonhaemolytic, with a ground-glass moist 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’ appearance. Confirmation of *B. anthracis* can 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.

The susceptibility of *B. anthracis* to the gamma bacteriophage was first described by Brown & Cherry in 1955 (3). The phage is available from CDC (see footnote 1), various national central veterinary laboratories, and other anthrax reference laboratories. The procedure for the test is simply to streak a lawn on a blood or nutrient agar plate, or portion of a plate (several tests can be done on one plate) with the suspect organism and place a 10–15 μl drop of the phage suspension on one side of the streaked area and place a 10-unit penicillin disk to the other side. Allow the drop of phage suspension to soak in and incubate the plate at 37°C. A control culture should be included; the Sterne vaccine can be used for this. If the culture is *B. anthracis*, the area under the phage will be devoid of bacterial growth, due to lysis, and a clear zone will be seen around the penicillin disk after overnight incubation. (Note: phage-resistant *B. anthracis* isolates are encountered very occasionally; similarly, there are a few reports in the literature of penicillin-resistance.)
Induction of the capsule is described above.

- Confirmation of virulence with the polymerase chain reaction

Full confirmation of virulence can be carried out using the polymerase chain reaction (PCR). The following instructions are taken from ref. 14. Template DNA for PCR can be prepared from a fresh colony of *B. anthracis* on nutrient agar by resuspension of a loopful of growth in 25 µl sterile deionised (or distilled) water and heating to 95°C for 20 minutes. It is important that a fresh colony be used otherwise there may be some live bacteria in the lysate. Following cooling to approximately 4°C, and brief centrifugation, the supernatant can be used for the PCR reaction.

Suitable primers (2, 6) for confirming the presence of the pX01 and pX02 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 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 from Pharmacia Biotech². 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 minutes followed by 55°C for 0.5 minutes followed by 72°C for 0.5 minutes; 1 × 72°C for 5 minutes; cool to 4°C.

It should be noted that, in use for some years now in an anthrax reference facility, the primers given in the table above have worked well in confirming the presence or absence of pXO1 and/or pXO2 in pure cultures of isolates from animal (including human) or environmental specimens or samples. They are unsuitable, however, for direct detection of *B. anthracis* in such specimens or samples. A choice of alternatives can be found in ref. 7. 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 supplied in ref. 7.

b) Agent identification from old, decomposed specimens, processed materials, and environmental samples, including soil

These specimens more often than not have saprophytic contaminants that outgrow and obscure *B. anthracis* on nonselective agars. The following procedure is suggested:

i) 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 15 minutes.

ii) Tenfold dilutions to 10⁻² or 10⁻³ are then prepared. From each dilution, 10–100 µl are plated on to blood agar and 250–300 µl on to PLET agar (polymyxin, lysozyme, EDTA [ethylene diamine tetra-acetic acid], thallous acetate) (8, 14). All plates are incubated at 37°C.

iii) 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 (8, 14) 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. (NOTE: thallous

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² Uppsala, Sweden, product number 27-9555-01.
acetate is poisonous and should be handled with care.) 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 \textit{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 \textit{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 (Section B.1.a. above), 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 \textit{B. anthracis} present will result in death in 48–72 hours and the organism can be cultured from the blood as described above.

c) Immunological detection and diagnosis

It needs to be borne in mind that \textit{B. anthracis} is antigenically very closely related to \textit{B. cereus}, which is an almost 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 \textit{B. anthracis}. This places considerable constraints on the extent to which immunological methods can be used in routine detection methodology.

- Ascoli test (1)

In 1911, Ascoli (1) 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 \textit{B. anthracis} are shared by other \textit{Bacillus} spp., and is dependent on the probability that only \textit{B. anthracis} would proliferate throughout the animal and deposit sufficient antigen to give a positive reaction. Nowadays, it appears to be used in Eastern Europe only.

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 less than 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 \textit{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 \textit{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.

- Immunofluorescence

While some success has been achieved with immunofluorescence for capsule observation in the research situation (4), it does not lend itself to routine diagnosis.

2. Serological tests

Historically, there has been little need for serological support for the diagnosis of anthrax in animals. Either the animal had anthrax, recognised from the recent history of the herd or site, and was treated accordingly, or it died. Most of the interest in developing serological testing has been for research on humoral responses in humans, and to a lesser extent in animals, for evaluating vaccines and for epidemiological studies involving naturally acquired seroconversion in humans, livestock and wild mammals.
Currently accepted as the best serological procedure is the enzyme-linked immunosorbent assay (ELISA) in microtitre plates coated with the protective antigen (PA) component of the anthrax toxin at 3–5 µg/ml in high pH (9.5) carbonate-coating buffer. The toxin antigens appear to be truly specific for *B. anthracis*, although there is at present no commercial source of these. This means that anthrax serology is currently confined to a few specialist laboratories. Various versions of the ELISA exist and can be found in standard laboratory manuals; any version will do for anthrax serology, although certain sera appear to be more ‘sticky’ than others. A useful tip is to use reconstituted dried milk as the blocking agent and to raise its concentration until control negative sera are giving reliable negative results. For bovine sera, this may be a 10% suspension or higher.

Examples of the successful field application of anthrax serology are given elsewhere (5, 11, 15).

3. **Hypersensitivity test (Anthraxin™)**

In Central and Eastern Europe, a skin test using Anthraxin™, first licensed in the former USSR in 1962, has become widely used for retrospective diagnosis of human and animal anthrax and for vaccine evaluation (12). This is a commercially produced heat-stable protein/polysaccharide/nucleic acid complex, derived from oedematous fluid of animals injected with the vaccine STI or the Zenkowsky strains of *B. anthracis*. The test involves intradermal injection of 0.1 ml of Anthraxin and inspection after 24 hours for erythema and induration at the site lasting for 48 hours after the injection. This delayed-type hypersensitivity is seen as reflecting anthrax cell-mediated immunity and was the test reportedly capable of diagnosing anthrax retrospectively some 31 years after primary infection in up to 72% of cases. It was used with success in a retrospective investigation of a series of cases occurring in a spinning mill in Switzerland where synthetic fibres were combined with goat hair from Pakistan (10). The diagnostic reliability of Anthraxin, like Ascoli test antigen, depends on the nature of anthrax rather than on the specificity of the antigens involved.

C1. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS: VACCINES

The most widely used vaccine for prevention of anthrax in animals was developed by Sterne in 1937 (13). 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 pX02 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 pX02− 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 Appendix V of ref. 14.

The following information concerning preparation of the anthrax vaccine for use in animals is based on refs 9 and 16. Generalised procedures are given; national regulatory authorities should be consulted in relation to Standard Operating Procedures that may pertain locally.

1. **Seed management**

a) **Characteristics of the seed**

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. 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) **Preparation of the master seed**

Seed lots are cultured on solid media formulated to promote sporulation of the organism (see Section C1.2. below). The solid medium formula given in ref. 9 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 them for sterility.

Volumes of 2 ml of vaccine seed from a reference laboratory 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.

c) 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.

d) Safety of the seed lot

Not less than 5 x 10^8 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.

e) Immunogenicity of the seed lot

At least 10 healthy guinea-pigs, 300–500 g in weight should be inoculated with 5 x 10^6 viable spores and observed for 21 days. At least 80% of the animals should 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.

2. Method of production

a) Preparation of vaccine concentrate

Roux bottles with casein digest agar are prepared as for the master seed in Section C1.1.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.

b) Glycerination

Twice the volume of sterile, pure, neutral glycerol should be added to the bulk pool. 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 as before 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.

c) 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 not less than 10 million viable spores per dose for cattle, buffaloes and horses, and not less than 5 million spores per dose for sheep, goats and pigs.
d) **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.

e) **Filling the containers**

Distribution of aliquots of vaccine into single and multidose containers is performed as outlined in World Health Organization Technical Report No. 363 series entitled *General Requirements for Manufacturing Establishments and Control Laboratories* (Requirements for Biological Substances No. 1), 1965, 16–17. 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.

3. **In-process control**

Purity tests consist of microscopic examination of stained smears with culture and motility tests as in Section C1.2.a.

4. **Batch control and tests on the final product**

a) **Sterility**

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 I.1.5.).

b) **Efficacy**

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 nonvaccinated controls are challenged with an appropriate dose of virulent *B. anthracis*. A recommended challenge is 200 LD$_{50}$ of the Pasteur II strain (17JB), which is available from the same source as the Sterne 34F2 vaccine strain. 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) **Dose**

The recommended dose for cattle and horses is a minimum of $10 \times 10^6$ culturable spores; for sheep, goats and pigs, it is $1–5 \times 10^6$ culturable spores. The vaccine should contain these spores in an appropriate volume, e.g. $1 \times 10^7$ml.

d) **Duration of immunity**

Most experts agree that immunity is 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.

e) **Stability**

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.

f) **Preservatives and storage**

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

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. Left over vaccine, empty vials, and equipment used for vaccinating are contaminated with the live spores and should be autoclaved, disinfected, or incinerated. 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.

**5. Tests on the final product**

a) **Safety**

Every batch of vaccine will be tested for safety as described in Section C1.2.d.

b) **Potency**

Every batch of vaccine will be tested for potency, as described in Section C1.4.b.

**C2. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS:**

**DIAGNOSTIC BIOLOGICALS**

- **Propagation of the diagnostic ‘gamma’ bacteriophage**

  1. Prepare a ‘lawn’ of growth of attenuated *B. anthracis* on 150 x 15 mm Mueller–Hinton plates with 5% sheep blood.
  2. Incubate for 4–6 hours at 37°C, if there is sufficient vegetative growth (heavy and visible to the naked eye) then seed the growth with phage. If the growth is thin, then incubate overnight before seeding.
  3. The seeding is done using a sterile transfer pipette. Approximately 2 ml of the stock gamma bacteriophage is dropped over the surface of the vegetative growth. The plate is tilted to allow the phage to cover the whole area of growth. This may be repeated until the whole surface of the vegetative growth has been covered with phage.
  4. Incubate the plates at 37°C overnight.
  5. Place the incubated plates in a 20°C freezer overnight.
  6. The next day remove the plates from the freezer and allow to thaw for 2 hours.
  7. Pour off the brownish red liquid and pre-filter it through Whatman No. 3 filter paper.
  8. Carry out a final sterile filtration into a sterile storage vessel using a 0.22 µ filter.
  9. To confirm the potency of the phage do a serial dilution 1/1, 1/10, through 1/10,000 and test with *B. anthracis* for susceptibility.
  10. Store the phage in a refrigerator at 2–8°C. Do not freeze.

**REFERENCES**


Chapter 2.2.1. — Anthrax


FURTHER READING


Chapter 2.2.1. – Anthrax


* *

NB: There are OIE Reference Laboratories for Anthrax (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
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 virtually all mammals except humans and the tailless apes. It is associated primarily with pigs, the natural host, which remain latently infected following clinical recovery. The disease is controlled by containment of infected herds and by the use of vaccines and 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 virus can also be identified using PCR, but this technique is still quite new.

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.

Since about 1990, it has become possible to distinguish between antibodies resulting from natural infection and those from vaccination by use of gene-deleted vaccines.

Requirements for vaccines and diagnostic biologicals: Vaccines, either modified live virus or inactivated virus antigens, should prevent or at least limit the excretion of virus from the infected pigs. More recently, these conventional vaccines have been supplemented by rDNA-derived gene-deleted or naturally deleted live pseudorabies virus vaccines. The virus used in these new vaccines, sometimes referred to as marker vaccines, lacks a specific glycoprotein (gG, gE, or gC).

A. INTRODUCTION

Aujeszky’s disease, also known as pseudorabies, is caused by an alphaherpesvirus, a member of the family Herpesviridae. The virus infects the central nervous system and other organs, such as the respiratory tract, in virtually all mammals except humans and the tailless apes. It is associated primarily with pigs, the natural host, which remain latently infected following clinical recovery. The disease is controlled by containment of infected herds and by the use of vaccines and removal of latently infected animals.

Whereas isolation of the pseudorabies virus (PRV) will assist in a provisional diagnosis in the case of lethal forms of Aujeszky’s disease or clinical disease in pigs, other techniques and serological tests are required for diagnosis of latent infections. Many affected animals, however, except pigs, do not live long enough to produce any marked serological response.
B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Virus isolation

The diagnosis of Aujeszky’s disease can be confirmed by isolating PRV from the oro-pharyngeal fluid, nasal fluid (swabs) or tonsil biopsies of 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 PRV, samples of brain and tonsil 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 ganglion is the most consistent site for virus isolation, although latent virus is usually difficult to 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 PRV, 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).

PRV 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 PRV 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 PRV genomes in secretions or organ samples. As this technique is still quite new, it is not yet possible to specify a standard procedure. Only some general information can be given.

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). 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 10^6-fold. The primers must be designed to amplify a sequence conserved among PRV strains, for example parts of the gB or gD genes, which code for essential glycoproteins, have been used.

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. More recent techniques involve liquid hybridisation using enzyme-labelled probes, which give a colour reaction after incubation with the appropriate substrate. An improvement of the technique is known as ‘nested PCR’ and uses two sets of primers, one of which is located within the sequence amplified by the first set of primers. By using appropriate hybridisation temperatures in a two-step reaction, both the sensitivity and specificity of the PCR can be improved.

In all cases, the main advantage of PCR when compared with conventional virus isolation techniques is its rapidity, as preliminary identification can be completed within one day with confirmation of the PCR product on the second day. With the most modern equipment the whole process can be completed in 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 I.1.5. Tests for sterility and freedom from contamination of biological materials). This may limit the value of the test for many laboratories, and therefore this technique cannot be fully recommended for routine diagnosis, although methods are available for preventing DNA carry-over contamination (e.g. DUTP-UNG system [d-uracil triphosphate/uracil-N-glycosylase]). Many diagnostic laboratories would restrict the use of PCR to the detection of latent infection.
2. Serological tests

Any serological technique used should be sufficiently sensitive to give a positive result with the OIE International Standard Reference Serum. This serum is obtainable from the OIE Reference Laboratory for Aujeszky’s Disease in France (see Table given in Part 3 of this *Terrestrial Manual* and should be reconstituted before use according to the data sheet instructions. For international trade purposes, the test should be sensitive enough to detect the standard serum diluted 1/2.

Virus neutralisation (VN) has been recognised as the reference method for serology (4, 27), 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 (2, 11, 15, 17). The tests can be performed on meat juice as well as serum.

A latex agglutination test has also been developed and can be used for screening for antibodies. Kits for the test are commercially available.

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 to comply 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 PRV are used; they may be cell lines (e.g. PK-15, SK6), 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 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%). 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, they are suspended in 90 ml of growth medium, and this suspension is distributed into three 75 cm² cell culture bottles.

Virus: A suitable strain of PRV, such as the Kojnok strain, or NIA-3 strain, is stored at a temperature of –70°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 for 1 hour. Then, 30 ml of culture medium is added and the bottle is again incubated at 37°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 –70°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 PRV (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. 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°C for 30 minutes.
  ii) Each undiluted serum is placed in three wells, at 50 µl per well, of a 96-well cell-culture grade microtitre plate.
  iii) 50 µl of virus suspension containing 100 TCID$_{50}$/50 µl (or $2 \times 10^3$ TCID$_{50}$/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 (CO$_2$ 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 CO$_2$), 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 (CO$_2$ optional).
  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 serum neutralisation (target titre 100 TCID$_{50}$/50 µl) is diluted with MEM at 1/10, 1/100 and 1/1000. Of each dilution, 50 µl is placed in at least eight wells, to which 50 µl of medium is added before the wells are incubated for 1 hour at 37°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 two wells.
      - **Positive serum control:** A serum of known PRV 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 TCID$_{50}$/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 ($\times 100$) 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 TCID$_{50}$/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.
  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).
  x) **Interpretation of the results:** This test is capable of detecting the presence or absence of neutralising antibody to PRV. 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
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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 (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, one or more 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, with three wells for each dilution.

i) Complement in the serum samples is destroyed by heating in a water bath at 56°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 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 sera. 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 measuring antibody levels. 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 (6, 20, 21). Alternatively, noncommercial ELISA protocols may be adopted (2, 17) 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 (17).

- **Preparation of antigen**

i) A cell line sensitive to PRV 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 PRV is used, e.g. Kojnock strain. When a confluent cell monolayer has developed (approximately 24 hours after seeding), it is inoculated with 10⁸ TCID₅₀ PRV 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 checkerboard titrations.
  ii) 200 µl of antigen is dispensed into each well of 96-well ELISA-grade plates, coating alternate rows with PRV 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.

- 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 reference 17 and Chapter I.1.3. Principles 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 filter paper disks that have been moistened with a small quantity of blood obtained by puncturing a superficial vein. This technique makes it convenient to collect blood samples from large numbers of pigs (3, 18). 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. The OIE International 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.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Aujeszky’s disease may be controlled by the use of vaccines containing either modified live or inactivated virus antigens. More recently, these conventional vaccines have been supplemented by recombinant DNA-derived gene-deleted or naturally deleted live PRV vaccines. These new 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 described). 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. 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.

Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

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, or rDNA-derived gene-deleted virus. Modified live conventional vaccines and rDNA-engineered vaccines use numerous strains, such as Bartha (8–10, 14, 16, 22, 26), or are derived from Aujeszky's original isolate or from other field isolates, such as the NIA-3 strain.

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.

The MSV 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 culture

Most of the cell lines used to propagate PRV 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. Porcine cells are tested for porcine cytomegalovirus, bovine viral diarrhoea/ classical swine fever virus, the rabies viruses, African swine fever, porcine influenza, porcine respiratory and reproductive syndrome, and vesicular stomatitis. This list may be modified if the country of origin of the cells is free from a certain infection.

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.

c) Validation as a vaccine

i) Purity

The MSV 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.

ii) 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.
iii) **Safety tests**

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.

In general, safety is tested initially under experimental conditions. 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 fraction 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, should be observed after vaccination. These assays have to be performed on at least ten vaccinated pigs, with 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 five passages in piglets are made. If necessary, the organism is propagated *in vitro* between two *in-vivo* passages. 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 processed 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. Four unvaccinated piglets are kept as contact controls. Suitably sensitive tests for the virus are carried out individually on the nasal and oral secretions 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 PRV vaccine strain to spread from a vaccinated pig to unvaccinated ones (diffusibility) must be tested by using the recommended route of administration that presents the greatest risk of spreading. 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 diffusibility of the strain to nontarget species that may be susceptible to the vaccinal 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.

    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 (25). 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 at each recommended route of administration to the pigs for which it is intended.

       Pigs or sows are usually kept under observation and submitted to examinations until any reaction has disappeared. 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 (23). 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 final product pigs.

- **Field testing**

All tests must be carried out on pigs that do not have antibodies against Aujeszky’s disease virus or against a fraction of the virus, except that some tests may be done using maternally immune animals.

Field trials are necessary to assess the safety of an Aujeszky’s disease vaccine in a large number of pigs or sows. In Europe (7), 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, and 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.

iv) **Efficacy tests**

- **Laboratory trials**

All tests must be carried out on pigs that do not have antibodies against Aujeszky’s disease virus or against a fraction of the virus, except that some tests may be done using maternally immune animals.

#### a. Assessment of passive immunity

To test the efficacy of vaccines, it is important to mimic the natural infection conditions (1). PRV 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 PRV strain. It is preferable to use a strain titrated in median lethal doses (LD50). Pigs should be inoculated by the nasal route, 10^2 pig LD50 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).

#### b. 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 (5). The antibody titres have little predictive value for the efficacy of the vaccines. Weight loss compared between the vaccinated and control groups is certainly 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 day 7), has a very good predictive value for the efficacy of the vaccines (13). 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 PRV 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 (12, 19, 24). 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 arbitrary 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 wait 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.

2. Method of manufacture

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.

3. In-process control

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.
4. Batch control

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 to the reproducibility of the quality of the finished product, which has to conform with the quality initially defined in the application for marketing authorisation.

a) Sterility and purity tests

Tests must be carried out for sterility and freedom from contamination (see Chapter I.1.5.)

Each batch of PRV 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.

b) 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.

c) Identity

Where necessary, a specific test for virus identification should be carried out.

d) 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 PRV 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.

e) 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.

f) 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.

g) 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.
5. Tests on the final product

a) Safety

Every batch of vaccine must be tested for safety, as described in Section C.4.d.

b) Potency

Every batch of vaccine must be tested for potency, as described in Section C.4.e.

REFERENCES


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**NB:** There are OIE Reference Laboratories for Aujeszky’s disease (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.2.3.
ECHINOCOCCOSIS/HYDATIDOSIS

SUMMARY

Diagnosis of echinococcosis in dogs or other susceptible carnivores depends on the demonstration of adult cestodes of the Echinococcus genus in their faeces or small intestine. In intermediate hosts, this 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, four species of the genus Echinococcus are regarded as taxonomically valid. These are E. granulosus, E. multilocularis, E. oligarthrus and E. vogeli. The latter two species occur less frequently than the others. These four 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.

Larval forms of Echinococcus can usually be visually detected in organs. Special care has to be taken for a specific diagnosis of echinococcosis 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 Echinococcus 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 is currently used in surveys for E. multilocularis in populations of foxes, dogs 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: Excellent progress is being made in the development of a vaccine against the larval stage of E. granulosus in sheep and cattle.

A. INTRODUCTION

At present, four species of the genus Echinococcus are accepted taxonomically, namely E. granulosus, E. multilocularis, E. oligarthrus, and E. vogeli. These are morphologically distinct both in their adult and larval stages. 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 (21). Further studies are needed to define the full range of genetic diversity (12, 15, 16, 20). Echinococcus granulosus
has a global distribution, *E. multilocularis* occurs in wide areas of the Northern Hemisphere, and *E. oligarthrus* and *E. vogeli* are confined to Central and South America. All four 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 of considerable public health concern in many parts of the world (22).

- **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 may also occur, e.g. wolf/cervid. The adult varies between 2 and 7 mm in length and usually possesses from three to four segments, rarely up to six. 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 on the protoscolex in two rows of varying sizes. The size of the hooks varies between 22 and 45 µm in the first row, and between 18 and 38 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 and Oceania; 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, Sweden and Alaska. The status of dog/camel strains requires further elucidation. This strain has recently been identified in human cases in Argentina, Nepal and Iran (9, 17, 23). To date, all genotypes of *E. granulosus* except the dog/horse strain have been found to infect humans.

- **Echinococcus multilocularis**

  The parasite is transmitted between wild definitive hosts (e.g. *Vulpes vulpes*, *Alopex lagopus*, *Canis latrans*) and small arvicolid rodents (voles and lemmings). Domestic dogs and cats are susceptible, although cats less so than dogs. The adult varies between 1.2 and 3.7 mm in length and usually possesses from four to five segments. 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 rostellarium, the first row of larger hooks vary in size between 27.6 and 34.3 µm and the inner row of smaller hooks between 22.7 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 and this results in infiltration of tissues. Infection with this stage is commonly referred to as alveolar echinococcosis. There is no evidence for distinct strains or genotypes of *E. multilocularis*.

- **Echinococcus oligarthrus**

  The parasite typically uses 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 1.9 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. Preferred sites are internal organs and muscles. To date, only three reports of human disease are on record. The parasite appears not to mature in dogs.

- **Echinococcus vogeli**

  The parasite typically uses the 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 5.6 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 41.64 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*.

Both *E. vogeli* and *E. oligarthrus* are zoonotic agents with approximately 60 human cases caused by the former and only a few caused by the latter species. The infection caused by these two species is commonly referred to as polycystic echinococcosis.

A detailed description of echinococcosis in humans and animals can be found in the WHO/OIE Manual on echinococcosis (22).

**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 cestodes of *Echinococcus* spp. in their faeces or the small intestine or testing for specific coproantigens.

Investigators carrying out these procedures are exposed to 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. Face masks, disposable gloves and an apron must be worn. Chemical disinfection is not reliable, although sodium hypochlorite may destroy a proportion of eggs (3). 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.

1a) **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% formol saline or kept cool at +4°C and deep-frozen at −20°C for subsequent examination.

Larvae can be observed in many organs, but in large animals, such as sheep and cattle, palpation or incision should be done. Pigs, 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. *Ascaris suum* is a cause of ‘white spot’ in sheep livers. 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*. The presence of protoscolecites 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 protoscolecites or larval tissue material that is frozen, refrigerated or preserved in 90% ethanol.

1b) **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. multilocularis* can now be identified and differentiated from other taenilated 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 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 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. 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.

- **Mucosal scrapings**

For necropsy of foxes (or dogs) for *E. multilocularis*, carcasses or intestines should be deep frozen at between –70°C and –80°C for 3–7 days before necropsy. Eggs of *E. multilocularis* are resistant to freezing down to –50°C. Deep mucosal scrapings should be made using microscope slides and adherent material transferred to a square plastic Petri dish. Scrapings are squashed between slides and examined under a stereoscopic light microscope (×120). Five mucosal scrapings from proximal, middle and posterior thirds of the small intestine (total 15) are recommended. *Echinococcus multilocularis* is usually found in the second half of the small intestine.

- **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 pararcarmine (carmine acid [1.0 g], aluminum 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 (35, 50, 70, 85, 95, 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, picolyle, 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 (22).

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).

c) **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, lacrimal, 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. 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 be excluded from treatment. A dose of 4 mg/kg should result in purgation in under 30 minutes. Walking and abdomen massage of recalcitrant cases or enema for constipated dogs may avoid

Dogs that are purged successfully may produce at least two motions; the first will be formed faeces and can be ignored, 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 boiled 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.

d) Coproantigen tests

An alternative to arecoline testing, based on a faecal antigen-detection antibody sandwich enzyme-linked immunosorbent assay (ELISA), has been developed recently 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 (1, 3, 7, 8).

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.

ELISAs\(^1\) 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 (3). When testing for genus-specific *Echinococcus* coproantigens, specificity is around 98% and overall sensitivity approximately 70%; however, when mean worm burdens are >50–100, sensitivity approaches 100% (1, 3, 4, 8). 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 (8, 18). 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* (6, 18). However, for low worm burdens (<50), the sensitivity of the *E. multilocularis* coproantigen ELISA is below that of the mucosal smear method at necropsy (6).

The exact nature of *Echinococcus* antigens released in faeces for coproantigen detection has not been characterised. However, their stability in 5% formal saline after boiling and susceptibility to periodate treatment suggest involvement of carbohydrate antigen(s) (3).

Coproantigens can be detected prior to release of eggs by *Echinococcus* worms, and therefore are not related to egg antigen(s) (8, 18). 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 (8).

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 tract. *Echinococcus* coproantigens are also stable in fox or dog faeces left at 20°C for 1 week. Coproantigen testing has also been successfully used to evaluate the efficacy of deworming wild foxes infected with *E. multilocularis* using praziquantel-laced bait.

• **Coproantigen test procedure (Echinococcus granulosus)** (1, 4)

i) The faecal sample 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 (Immulum #4, Dynex Technologies) is coated with optimal concentration (typically 5 µg per ml) of a protein A purified IgG fraction of rabbit anti-*E. granulosus* proglottid extract (1) 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 per well of faecal sample supernatants is added (in duplicate wells) to 50 µl of neat fetal calf serum. The plate is incubated at room temperature for 1 hour with clingfilm seal covering the plate.

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1 *Echinococcus* coproantigen ELISA is available commercially as a kit from: Genzyme Virotech GmbH, Lowenplatz 5, 65428 Russelsheim, Germany; and Dr Brommeli AG, Liebefeld-Berne, Switzerland.
v) The wells are rinsed as in step iii, but the contents are discarded into a 10% bleach (hypochlorite) solution.

vi) An optimal dilution of an IgG rabbit anti-\textit{E. granulosus} proglottid extract peroxidase conjugate (1) in PBST is prepared and 100 µl per well is added to all wells. The plate is incubated for 1 hour at room temperature.

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.

ix) 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. Absorbance of wells is read at 450 nm.

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 3 of this \textit{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.

e) DNA recognition methods

\textit{Definitive hosts}: Differential diagnosis of \textit{E. granulosus} and \textit{E. multilocularis} infections in definitive hosts may be achieved by specific detection of PCR-amplified DNA from \textit{E. multilocularis} eggs present in faeces (2, 13). Primers from the Ulsn RNA gene of \textit{E. multilocularis} are species specific, exhibiting 100% specificity following zinc chloride flotation purification of eggs from faeces, and gave a sensitivity of 94% for \textit{E. multilocularis} infection in red foxes (13). 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 \textit{E. multilocularis} generally occurs in regions where \textit{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 (5). Further evaluation of \textit{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 \textit{E. granulosus} in different groups.

\textit{Intermediate hosts}: DNA hybridisation methods are not currently used for the detection of \textit{E. granulosus} in livestock intermediate hosts. Molecular methods are, however, important in identification of isolates or strains of \textit{E. granulosus} for epidemiological purposes (15).

For the identification of small or calcified lesions of \textit{E. multilocularis} in intermediate or aberrant hosts, PCR is of great value (14).

2. Serological tests

a) Intermediate hosts

Immunological tests, useful in humans, are less sensitive and specific in livestock and at present cannot replace necropsy (3, 10).

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 has not reached a practical stage as it does not differentiate between current and previous infections.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Intermediate hosts

A vaccine, based on a single polypeptide antigen derived from oncospheres and produced in \textit{Escherichia coli} using recombinant DNA technology has been successfully developed for use against \textit{T. ovis} in sheep. This technology has now been successfully applied to \textit{E. granulosus} (11).
Phase I trials using the recombinant oncosphere antigen vaccine EG95 gave 96–98% protection against experimental challenge of sheep with *E. granulosus*. Protection may last up to 12 months and can be transferred to lambs via colostrum. Phase II trials with natural challenge of vaccinated lambs resulted in similar levels of protection. EG95 vaccine for *E. granulosus* can now be mass produced and has the potential to significantly reduce the time for the attack phase of hydatid control programmes. The vaccine for ovine hydatidosis would be used in parallel with dog-dosing measures and health education programmes.

2. **Definitive hosts**

While considerable research has been undertaken with crude antigens to protect dogs from echinococcosis, no success has been demonstrated so far. Basic research on canine mucosal immunology and *Echinococcus* infection is required for progress.

**REFERENCES**


NB: There are OIE Reference Laboratories for Echinococcosis/Hydatidosis (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
Leptospirosis is a contagious 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 are time-consuming and are tasks for specialised reference laboratories. Isolation followed by typing from renal carriers is 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 can be used for immunohistochemistry and is 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). 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, most 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 primarily as a herd test. To obtain useful information, at least ten animals, or 10% of the herd, whichever is greater, should be tested and the vaccination history of the animals documented. 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.

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 many ELISAs, thus complicating interpretation of the results.

**Requirements for vaccines and diagnostic biologicals:** Vaccines for veterinary use are 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 contagious 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 several species of *Leptospira* (11, 58, 85). There are more than 200 distinct leptospiral serovars recognised and these are arranged in 23 serogroups (46). A recently published textbook (31) reviews *Leptospira* and leptospirosis.

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 systematically; 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 will enhance the chances of detecting the organism (53). 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.

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 leptospirological culture, the sample should be kept at 4°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 media 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) (39) or containing BSA and a combination of Tween 80 and Tween 40 (25). Contamination may be controlled by the addition of a variety of selective agents, e.g. 5-fluorouracil (43), nalidixic acid (44), fosfomycin (54), and a mixture of rifamycin, polymyxin, neomycin, 5-fluorouracil, bacitracin, and actidione (1). However, use of selective agents may reduce the chances of isolation when there are only small numbers of viable leptospires, and many 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 some fastidious leptospiral serovars.

Cultures should be incubated at 29 ± 1°C for at least 16 weeks, and preferably for 26 weeks (25). 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 (9, 26), and various immunohistochemical techniques (4, 28, 63, 65, 88). 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 (6).

Polymerase chain reaction (PCR)-based assays are now used in some diagnostic and most reference laboratories for the detection of leptospires in tissues and body fluids. A variety of primer sets for the conduct of PCR assays have been described (3, 5, 32, 34, 39, 45, 51, 66, 75, 79, 83) with some primers specific for the genus Leptospira and others that are serovar specific. PCR assays can be quite sensitive, but lack of specificity (i.e. false-positive results) can be a problem. 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 (24, 49). 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 (70).

The identification of leptospiral isolates is a task for specialised reference laboratories. For complete identification, a combination of procedures must be 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 is 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 and temperature tolerance (41, 42); PCR-assay-based amplification of 23S rDNA (82); and G+C content of DNA (41).

New leptospiral species have been identified based on DNA–DNA hybridisation analysis (11, 58, 85). 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 genospecies 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 or by genetic analysis of the 16S or 23S ribosomal RNA genes (14, 47, 55, 57, 80, 81).

Strains belonging to Leptospira can be differentiated to the serogroup level by cross-agglutination reactions (23). Further differentiation to the serovar level is traditionally by cross-agglutination absorption, although for most isolates this is now being done using less time-consuming methods: factor analysis (23), monoclonal antibodies (MAbs) (71, 72), restriction endonuclease analysis (38, 48, 73, 74), and various PCR strategies (14, 18, 22, 33, 56, 57, 59, 62, 87, 88). 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 or 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 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. 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 (37, 52).

The strains selected should be grown in Tween 80 BSA or suitable commercial medium at 29 ± 1°C and the culture should be at least 4 days old, but no more than 8 days. The transmittance of the antigen should be 60–70% using a spectrophotometer with a 400 nm filter or at a reading of 25 in a nephelometer. 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. The degree of reaction is interpreted by estimating the percentage of leptospires that are agglutinated. If 100% of the leptospires are agglutinated, the reaction is 4+; 3+ equals approximately 75% agglutination; 2+ equals 50%; and 1+ equals less than 50%. If a screening test is performed, any serum that has a 2+ reaction at a 1/100 dilution is titrated to an end-point using doubling dilutions of serum, starting at a final dilution of 1/100 through to 1/12,800 or higher. The end-point titre is the reciprocal of the highest dilution with a 2+ or greater reaction.

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 prepared using a protocol such as that given by the Subcommittee on the Taxonomy of Leptospirosis (40). Briefly, healthy rabbits weighing 2–4 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 with a density of approximately 2 × 10⁸ leptospires/ml. The culture should be grown in Tween 80 BSA medium or another appropriate medium. Five injections of 1, 2, 4, 6, and 6 ml 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 antisera. If the titres are satisfactory in both rabbits, the sera may be pooled. To preserve potency, it is preferable to freeze-dry the antisera in 2 ml volumes and store it at 4°C. Alternatively, the serum can be stored in 2 ml volumes at −20°C.

Purity of antigens used in the MAT should be checked regularly by culture on blood agar and in thioglycolate broth. Stock cultures of antigens may be stored at −70°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.

In some situations, formalinised or lyophilised cultures may be used in the MAT. The main advantages of the use of nonliving organisms are that the antigens can be standardised and the expense, difficulty, and risk of maintaining live cultures of Leptospira can be avoided. Titres determined using live antigens and killed antigens may not be directly comparable and many diagnosticians feel that maximum sensitivity of the MAT is obtained using live antigens.
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. The test has limitations in diagnosis of chronic infection in individual animals, both in the diagnosis of abortion (27) and in the identification of renal or genital carriers (25). This is particularly true with the host-adapted leptospiral 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% (25). The demonstration of antibodies in fetal blood is diagnostic.

As leptospirosis is a herd problem, the MAT has much greater use as a herd test. To obtain useful information, Cole et al. (17) 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. (37) found that a ten-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 during the acute phase of 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 immunosorbent 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. 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 (35, 36). 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 (2, 16, 21, 50, 60, 67–69, 76, 84). The major identified role of ELISA in livestock species is the use of an IgM ELISA for identification of recent infections (21) 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 (29). 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.

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 (8), 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 I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 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 (15). 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 (7, 64) or low-protein medium (7).

The volume of each master seed culture should be amplified by growth for 2–10 days at 29 ± 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 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 (23) 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 (10). A draft monograph for the efficacy testing of serovar Hardjo vaccines has been prepared and specifies the use of more natural routes of challenge (30).

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 (42), 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 (13, 19, 20, 77). Tests for sterility and freedom from contamination of biological materials may be found in Chapter I.1.5.

   b) **Safety**

   Samples of completed product should be tested for safety. Methods for this have been described elsewhere (12, 19, 78). 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 LD50 (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 ID50 (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 MAbs in a capture ELISA (61). 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 checked in the animal species for which the vaccine is intended using natural routes of challenge (10). 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 4°C, room temperature, and 37°C.
5. Tests on the final project

a) Safety
See Section C.4.b.

b) Potency
See Section C.4.c.

REFERENCES


Chapter 2.2.4. — Leptospirosis


30. European Pharmacopoeia Draft Monograph; Bovine Leptospirosis vaccine (inactivated); PA/PH/Exp. 15V/T (01) 28.


Chapter 2.2.4. – Leptospirosis


Chapter 2.2.4. — Leptospirosis


77. UNITED STATES DEPARTMENT OF AGRICULTURE STANDARD REQUIREMENTS § 113.26.

78. UNITED STATES DEPARTMENT OF AGRICULTURE STANDARD REQUIREMENTS § 113.38.


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NB: There are OIE Reference Laboratories for Leptospirosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
Rabies is a major zoonosis for which diagnostic techniques have been standardised internationally. As there is no gross pathognomonic lesion for rabies, diagnosis can only be made in the laboratory. Laboratory techniques are preferably conducted on central nervous system (CNS) tissue removed from the cranium. 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 done using the fluorescent antibody test (FAT). A drop of purified immunoglobulin previously conjugated with fluorescein isothiocyanate is added to an acetone-fixed brain tissue smear, preferably made from several parts of the brain, including the hippocampus, cerebellum and medulla oblongata. For a large number of samples, as in an epidemiological survey, the immunoenzyme technique can provide rapid results (the rapid rabies enzyme immunodiagnosis [RREID]). FAT provides a reliable diagnosis in 98–100% of cases for all genotypes if a potent conjugate is used, while RREID detects only genotype 1 virus.

Infected neuronal cells have been demonstrated by histological tests and these procedures will reveal aggregates of viral material (the Negri bodies) in the cytoplasm of neurones. However, the sensitivity of histological techniques is much less than that of immunological methods, especially if there has been some autolysis of the specimen. Consequently, histological techniques can no longer be recommended.

As a single negative test on fresh material does not rule out the possibility of infection, inoculation tests, or other tests, should be carried out simultaneously. Newborn or 3–4-week-old mice are inoculated intracerebrally with a pool of several CNS tissues, including the brain stem, and then kept under observation for 28 days. For any mouse that dies between 5 and 28 days, the cause of death should be confirmed by FAT. Alternatively, a monolayer culture of susceptible cells is inoculated with the same material as used for mice. FAT carried out after appropriate incubation will demonstrate the presence or absence of viral antigen. 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 possibly 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 the prescribed tests for international trade. Alternatively, use may be made of a test that is known to correlate with these, notably an enzyme-linked immunosorbent assay using antibody to the G protein or the neutralisation test in mice. Results are expressed in International Units or equivalent units relative to an international standard antiserum.

Requirements for vaccines and diagnostic biologicals: 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 the CNS tissue of newborn animals, in embryonated eggs, 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.

For live virus vaccines, the minimum virus content that will elicit an adequate immune response must be established.

The potency of inactivated virus vaccines is established and controlled by mouse vaccination followed by intracerebral challenge 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 nontarget species must be demonstrated.

A. INTRODUCTION

Rabies is caused by a neurotropic virus of the genus Lyssavirus of the family Rhabdoviridae, and is transmissible to all mammals. As it is transmissible to humans by inoculation or inhalation of infectious virus, all suspected infected material must be handled under the appropriate safety conditions specified by the World Health Organisation (WHO) (37).

Seven distinct genetic lineages can be distinguished within the genus Lyssavirus by cross-protection tests and molecular biological analysis (5, 14, 21), namely the classical rabies virus itself (RABV, genotype 1, serotype 1), Lagos bat virus (LBV, genotype 2, serotype 2), Mokola virus (MOKV, genotype 3, serotype 3), and Duvenhage virus (DUUV, genotype 4, serotype 4). The European bat lyssaviruses (EBLV), subdivided into two biotypes (EBLV1, genotype 5 and EBLV2, genotype 6) and the Australian bat lyssavirus (ABLV, genotype 7), recently isolated in Australia (24), are also members of the Lyssavirus genus, but are not yet classified into serotypes. Viruses of serotypes 2–4, EBLV and ABLV are known as rabies-related viruses. The use of monoclonal antibodies (MAbs) directed against viral nucleocapsid or glycoprotein antigens, and the sequencing of defined genomic areas has made possible the definition of numerous subtypes within each serotype. Lyssaviruses cause a 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. For RABV, DUUV, EBLV and ABLV, conserved antigenic sites on the surface glycoproteins allow cross-neutralisation and cross-protective immunity to be elicited by rabies vaccination. Little or no cross-protection against infection with MOKV or LBV is elicited by rabies vaccination and most anti-rabies virus antisera do not neutralise these lyssaviruses.

Humans working with suspect material must be vaccinated against lyssaviruses or other pathogens that may be present in diagnostic samples. The laboratory must comply with national biocontainment and biosafety regulations to protect staff from contact with pathogens; it should also comply with the guidelines in Chapter I.1.6. Human safety in the veterinary microbiology laboratory.

WHO recommends the preventive immunisation of exposed staff. The immunisation protocol includes three injections, e.g. at days 0, 7, and 28. The serological evaluation of immunisation is made 1–3 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 evidence of infection is rarely useful because of late seroconversion and the high mortality rate of host species, although such data may be used in some epidemiological surveys.

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 (36). The only way to perform 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’.

Several laboratory techniques may be used, and have been detailed and standardised in the fourth edition of the WHO's *Laboratory Techniques in Rabies* (37). The methods vary in their efficiency, specificity and reliability. They are classically applied to brain tissue, but they can also be applied, though less effectively, to other organs (e.g. salivary glands). In the brain, rabies virus is particularly abundant in the thalamus, pons and medulla. The hippocampus (Ammon’s horn), cerebellum and different parts of the cerebrum have been reported to be negative in 3.9–11.1% of the positive brains. The structure of choice is the thalamus as it was positive in all cases. It is recommended that a pool of brain tissues that includes the brain stem should be collected and tested (12). To reach these parts of the brain, it is necessary to remove the entire organ after having opened the skull in a necropsy room. Under some conditions (e.g. in the field or when sampling for large epidemiological studies), a simplified method of sampling through the occipital foramen (11), or through the orbital cavity (26), can be used.

a) Shipment of samples

During the shipment of suspect material for diagnosis (animal heads, brain or other tissue samples), no risk of human contamination should arise: brains must be placed in a leak-proof rigid container (animal heads will be wrapped in absorbent material) as prescribed in the International Air Transport Association (IATA) Dangerous Goods Regulations must be followed. These regulations are summarised in Chapter I.1.1. Sampling methods.

When it is not possible to send refrigerated samples, other preservation techniques may be used. The choice of the preservative is closely linked to the tests to be used for diagnosis:

- Formalin inactivates the virus, thus the isolation tests cannot be used and diagnosis depends on using a modified and less sensitive direct fluorescent antibody test (FAT), immunohistochemistry or histology (33, 37);
- 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/saline should be kept refrigerated. As the virus is not inactivated by glycerol/PBS, all laboratory tests can be used on these samples.

b) Collection of samples

Usually the brain is collected following the opening of the skull in a necropsy room, and the appropriate samples are collected. This step may be hazardous if laboratory technicians are not fully trained, or under field conditions. In such cases, there are two possible methods of collecting some brain samples without opening the skull:

- **Occipital foramen route for brain sampling**
  A 5 mm drinking straw (11) or a 2 ml disposable plastic pipette (16) 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. Bovine spongiform encephalopathy (BSE) should be considered in the differential diagnosis of most cattle that are considered to be ‘rabies suspect’. Sampling of brain specimens for both diseases can be done using the ‘brain scoop or tool’ developed for BSE tissue sampling rather than a straw or pipette. The resulting samples are relatively easily recognised as to the area of brain sampled.

- **Retro-orbital route for brain sampling**
  In this technique (26), a trocar is used to make a hole in the posterior wall of the eye socket, and a plastic pipette 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.

c) Routine laboratory tests

Laboratory diagnosis can be performed by using three kinds of procedure.

- **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. Immunohistochemical tests are the only histological test specific to rabies.

  An unfixed tissue smear may be stained by the Seller’s method, diagnosis is then obtained in under 1 hour. Generally, histological tests, such as Mann’s test, are performed on fixed material after a paraffin-
embedding step, and the result of the test is obtained within 3 days. These techniques have the advantage that the laboratory equipment needed to perform them is inexpensive and any need to keep specimens cold after fixation is avoided. Whichever staining method is used, the evidence of infection is provided by intracytoplasmic acidophilic bodies. These histological methods, especially the Seller's method, can no longer be recommended because they have very low sensitivity and should be abandoned.

- **Immunochromatographic identification of rabies virus antigen**
  - **Fluorescent antibody test**
    The most widely used test for rabies diagnosis is the FAT, which is recommended by both WHO and OIE. This 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 sensitivity of the FAT depends on the specimen (the degree of autolysis and how comprehensively the brain is sampled, see Section B.1) (1, 9), on the type of lyssavirus and on the proficiency of the diagnostic staff. Sensitivity may be lower in samples from vaccinated animals due to localisation of antigen, which is confined to the brainstem. For direct rabies diagnosis, smears prepared from a composite sample of brain tissue, that includes the brain stem, are fixed in high-grade cold acetone and then stained with a drop of specific conjugate. Anti-rabies fluorescent conjugates may be prepared in the laboratory. Those available commercially are either polyclonal conjugates specific to the entire virus or specific to the rabies nucleocapsid protein, or they may be prepared from a mix of different MAbs. In the FAT, the specific aggregates of nucleocapsid protein are identified by their fluorescence. The specificity and sensitivity of these anti-rabies fluorescent conjugates for locally predominant virus variants should be checked before use.

    The FAT may be applied to glycerol-preserved specimens. 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 (6, 7, 32, 33). However, the FAT on formalin-fixed and digested samples is always less reliable and more cumbersome than when performed on fresh tissue.

  - **Immunochromatographic tests**
    The antibody may be conjugated to an enzyme such as peroxidase instead of fluorescein isothiocyanate (FITC). This conjugate may be used for direct diagnosis with the same sensitivity as FAT (22), 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 be used on sections of formalin-fixed tissue for immunohistochemical tests. An enzyme-linked immunosorbent assay (ELISA) that detects rabies antigen is one variation of the immunochromatographic test. This rapid rabies enzyme immunodiagnosis test (RREID) is available commercially (28). The correlation between the FAT and the RREID ranges between 96% and 99% (8, 15). The ‘routine’ version of this test is not sensitive to rabies-related viruses as RREID only detects genotype 1 lyssaviruses.

- **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.

  - **Mouse inoculation test**
    Five-to-ten mice, 3–4 weeks old (12–14 g), or a litter of 2-day-old newborn mice, are inoculated intracerebrally. It is recommended, though not strictly essential, to use specific pathogen free (SPF) mice. The inoculum is the clarified supernatant of a 20% (w/v) homogenate of brain material (cortex, Ammon’s horn, cerebellum, medulla oblongata) in an isotonic buffered solution containing antibiotics. To reduce animal pain, mice should be anaesthetised when inoculated. The young adult mice are observed daily for 28 days, and every dead mouse is examined for rabies using the FAT. For street fox rabies strains, deaths due to rabies generally begin 9 days post-inoculation. 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. This in-vivo test is quite expensive, particularly if SPF mice are used, and should be avoided where possible. It does not give rapid results (compared with in-vitro inoculation tests), but when the test is positive, a large amount of virus can be isolated from a single mouse brain for strain identification purposes. Another advantage of this low-tech test is that it can be easily and practicably be applied in situations where skills and facilities for other tests (e.g. cell culture) are not available.
ii) **Cell culture test**

Neuroblastoma cell lines, e.g. CCL-131 in the American Type Culture Collection (ATCC)\(^1\), is used for routine diagnosis of rabies. The cells are grown in Dulbecco’s modified Eagle’s medium (DMEM) with 5% foetal calf serum (FCS), incubated at 36°C with 5% CO\(_2\). Its sensitivity has been compared with that of baby hamster kidney (BHK-21) cells (29). This cell line is sensitive to street isolates without any adaptation step, but should be checked for susceptibility to locally predominant virus variants before use. Presence of rabies virus in the cells is revealed by the FAT. The result of the test is obtained after at least 18 hours (one replication cycle of virus in the cells); generally incubation continues for 48 hours (10) or in some laboratories up to 4 days.

This test is as sensitive as the mouse inoculation test. Once a cell culture unit exists in the laboratory, this test should replace the mouse inoculation test as it avoids the use of live animals, is less expensive and gives more rapid results.

It is often advisable to carry out more than one type of test on each sample, at least when there has been human exposure.

d) **Other identification tests**

The tests above may be completed in specialised laboratories (such as OIE or WHO Reference Laboratories) using MAbs, nucleic acid probes, or the polymerase chain reaction (PCR), followed by DNA sequencing of genomic areas for typing the virus (16). This enables a distinction to be made between vaccine virus and a field strain of virus, and possibly the geographical origin of the latter.

2. **Serological tests**

Serological tests are rarely used in epidemiological surveys, due to late seroconversion and the low percentage of animals surviving the disease and therefore having post-infection antibodies. Oral immunisation of rabies reservoirs is the method of choice for wildlife rabies control. For follow-up investigations in oral vaccination campaigns, virus neutralisation (VN) tests in cell culture are preferred. However, if poor quality sera are submitted, the VN tests in cell culture are sensitive to cytoxicity, which could lead to false-positive results. For such samples, the use of an indirect ELISA with rabies glycoprotein-coated plates has been shown to be as sensitive and specific as the VN test on cells (19).

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 (18) is the neutralisation *in vitro* of a constant amount of rabies virus ('challenge virus standard' [CVS] strain adapted to cell culture) before inoculating cells susceptible to rabies virus: BHK-21 C13 cells.

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 a standard serum under the same experimental conditions (OIE serum of dog origin or WHO standard for rabies immunoglobulin [human] No. 2, or both). An internal control calibrated against the international control may be used.

This microplate method uses 96-well plates, and is an adaptation of the technique of Smith *et al.* (30), modified by Zalan *et al.* (38) and by Perrin *et al.* (27). Several publications (17, 18) have shown that the FAVN test and the rapid fluorescent focus inhibition test (RFFIT) give equivalent results.

- **Essential equipment**
  - Humidified incubator at 37°C with 5% CO\(_2\); dry incubator at 37°C; biocontainment cabinet; fluorescence microscope suitable for FITC fluorescence equipped with ×10 eye-piece and ×10 objective. 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 Ca\(^{2+}\) and Mg\(^{2+}\), stored at 4°C;
    - Trypsin ethylene diamine tetra-acetic acid (EDTA);
    - High-grade acetone 80% (diluted with deionised water), stored at 4°C;

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\(^1\) American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia 20110-2209, United States of America (USA).
Dulbecco’s modified Eagle’s medium (DMEM) + 10% heat-inactivated FCS;  
FITC anti-rabies conjugate;  
**Cells:** BHK-21 C13 (ATCC CCL-10);  
**Virus:** CVS-11 (ATCC VR 959) strain, which is available from the ATCC or the OIE Reference Laboratory for Rabies, Nancy, France (see Table given in Part 3 of this Terrestrial Manual). Vials are stored at –80°C;  
WHO standard for rabies immunoglobulin (human) No. 2, 30 IU per ampoule2 (reconstituted with 5 ml of sterile deionised or distilled water, stored at –20°C, and diluted to 0.5 IU/ml with deionised water before use), or preferably OIE Standard Serum of dog origin (OIE Reference Laboratory for Rabies, Nancy, France [see Table given in Part 3 of this Terrestrial Manual] stored at –20°C and diluted to 0.5 IU/ml with sterile deionised or distilled water according to the titre of the batch). It is advised that, for internal routine control, laboratories should use a positive serum or a pool of sera of dog origin that has been calibrated against the OIE International Standard Serum;  
Naive serum: This lyophilised pool of ten negative dog sera, is stored at 4°C, and reconstituted with 0.5 ml of sterile deionised or distilled water.

- **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 used 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 infective 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 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 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:

- inoculation of a 24-hour cell layer,  
- tenfold dilutions prepared using 0.9 ml of diluent and 0.1 ml of virus suspension,  
- six 50 µl replicates per dilution,  
- incubation for 72 hours,  
- qualitative reading (i.e. the well is positive or negative),  
- in every titration session, a vial of a control batch of virus is titrated and this titre is integrated in a control card to validate the titration process,  
- calculation according to neoprobit graphic or Spearman–Kärber methods.

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. Tenfold 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.

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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).
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:

- the neoprobit graphic method (2) or
- the Spearman–Kärber formula:

\[
\log_{10} (\text{end-point dilution}) = - \left(x_0 - \frac{d}{2} + d \sum \frac{r_i}{n_i}\right)
\]

- \(x_0 = - (\log_{10} \text{of the lowest dilution with all wells positive})\)
- \(d = \log_{10} \text{of the dilution step, one in this case}\)
- \(n_i = \text{number of replicates, six in this case}\)
- \(r_i = \text{number of positive wells}\)

**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 \(\log_{10}\)

<table>
<thead>
<tr>
<th>Plate 1: Controls</th>
<th>(\text{H})</th>
<th>(\text{G})</th>
<th>(\text{F})</th>
<th>(\text{E})</th>
<th>(\text{D})</th>
<th>(\text{C})</th>
<th>(\text{B})</th>
<th>(\text{A})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Challenge virus standard titration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WHO or OIE standard serum (0.5 IU/ml)</td>
<td>5</td>
<td>50 µl</td>
<td>50 µl</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Internal positive control</td>
<td>5</td>
<td>50 µl</td>
<td>50 µl</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Naive dog serum (negative)</td>
<td>5</td>
<td>50 µl</td>
<td>50 µl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Plate 2: Sera to be tested</th>
<th>(\log (\text{dilution}))</th>
<th>0.48</th>
<th>0.95</th>
<th>1.43</th>
<th>1.91</th>
<th>2.39</th>
<th>2.87</th>
<th>3.35</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>log dilution</td>
<td>0.48</td>
<td>0.95</td>
<td>1.43</td>
<td>1.91</td>
<td>2.39</td>
<td>4.23</td>
<td>0.48</td>
<td>0.95</td>
<td>1.43</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serum 1</th>
<th>Serum 3</th>
<th>Serum 2</th>
<th>Serum 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 50 µl</td>
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<td>50 µl</td>
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<tr>
<td>B 50 µl</td>
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<td>50 µl</td>
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<tr>
<td>C 50 µl</td>
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<td>D 50 µl</td>
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<td>E 50 µl</td>
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<tr>
<td>F 50 µl</td>
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<tr>
<td>G 50 µl</td>
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<td>50 µl</td>
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<tr>
<td>H 50 µl</td>
<td></td>
<td>50 µl</td>
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</tr>
</tbody>
</table>

- **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. Plate No. 2 and others 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; plate 2 and others, 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.

Sera 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. 10−2.36 to dil. 10−2.23). Using a multichannel pipette adjusted to 100 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.

• **Addition of challenge virus standard**

  i) 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.

  ii) One dilution from this tube is prepared in order to obtain 100 TCID 50 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).

  iii) Incubate the microplates at 37°C in a humid incubator with 5% CO2 for 1 hour.

  iv) **Addition of cells:** trypsinise a subconfluent culture of 3-day-old BHK-21 cells. Resuspend the cells to obtain a $4 \times 10^5$ cells/ml suspension in DMEM supplemented with 10% heat-inactivated FCS. Add 50 µl of the cell suspension to each well.

  v) Incubate the microplates for 48 hours at 37°C in a humid incubator with 5% CO2.

• **Fixation and staining**

  i) 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 (without a lid), and are dried at room temperature for at least 1 hour.

  ii) Add 50 µl of the FITC anti-rabies conjugate, at the working dilution, to each well, gently rock the microplates and incubate at 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**

  i) 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.

  ii) Controls are read first. For control cells, titration of CVS, naïve serum, and standard sera (WHO standard and/or OIE Standard Serum), titres are calculated according to the Spearman–Kärber method or the neoprobit graphic method (2).

  iii) Results of titration of CVS (TCID 50), naive serum (D 50 [median dose]) and positive standard (D 50) 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 valid if the values obtained for the three controls in the current test are not statistically different from the mean of all the values obtained in the tests conducted previously according to this technique.

  iv) 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 (2) or with the Spearman–Kärber formula (37). The comparison of the measured titre of the tested sera with that of the positive standard serum of a known neutralising titre allows determination of the neutralising titre of the tested sera in IU/ml.
b) The rapid fluorescent focus inhibition test (RFFIT) for determining rabies virus-neutralising antibody (a prescribed test for international trade)

- **Standard procedure (from WHO Laboratory Techniques in Rabies, 1996; [ref. 37])**
  - **Preparation of seed virus suspension**
    i) Trypsinise one 3-day-old 150 ml flask culture of mouse neuroblastoma (MNA) cells. These cells prefer an acidic medium, supplemented with vitamins (34). 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 (ATCC, 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 (23) 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.

Available on request from the Rabies Laboratory, Division of Viral and Rickettsial Diseases, Centres for Disease Control and Prevention, Atlanta, Georgia, USA.
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, FFD50). The stock virus suspension should contain at least 1 × 10^4 FFD50 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 FFD50.

- **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 Centers for Disease Control and Prevention is the first international standard for rabies immunoglobulin (35), 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 inactive complemnt. 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 (37).
c) **Virus neutralisation in mice**

The principle of this test is the neutralisation *in vitro* of a constant amount of rabies virus (50 LD<sub>50</sub> [50% lethal dose] per 0.03 ml of CVS strain) by varying quantities of the serum to be titrated during an incubation step of 90 minutes at 37°C. The virus/serum mixture (0.03 ml) is inoculated into the brain of 3-week-old mice. The serum titre is the final dilution of serum in the virus/serum mixture that protects 50% of the mice (mortality is 100% in the absence of neutralisation). This titre can be expressed in IU by comparing it with the neutralising dilution of a standard serum under the same experimental conditions.

To perform the test, thaw an ampoule of CVS virus and prepare a suspension containing 100 LD<sub>50</sub>/0.03 ml (taking into account that it will be diluted twofold by the addition of the same volume of test serum before being injected). The amount of virus actually used during the test (permissible limits: 30–300 LD<sub>50</sub>/0.03 ml) is checked by titrating four dilutions of the viral preparation, each of which are inoculated into five mice. The test sera are heated at 56°C for 30 minutes to inactivate any complement.

A standard serum must be included in order to check the titration conditions. The maximum allowable difference between its expected neutralising capacity and that measured during the titration is 10<sup>0.5</sup>. The greatest dilution must not neutralise the virus. The diluent is the same as that used for the viral preparation.

To each serum dilution an equal volume of the viral preparation containing 100 LD<sub>50</sub>/0.03 ml is added. The mixtures are incubated in a 37°C water bath for 90 minutes, and then placed in melting ice to reduce virus inactivation due to temperature. The reaction is stopped by immersion in melting ice. During inoculation, the tubes that are not used immediately are kept at 4°C.

For each dilution, five mice are inoculated intracerebrally with 0.03 ml of the serum/virus mixture. Mortality is recorded during 21 days after inoculation, although deaths occurring during the first 4 days are regarded as nonspecific (due to shock, infection, etc.). The serum titre can be calculated in IU by comparison with an international standard serum.

d) **Enzyme-linked immunosorbent assay**

This indirect ELISA allows a qualitative detection of rabies antibodies in individual dog and cat serum samples following vaccination. In accordance with the WHO recommendations (36), 0.5 IU per ml rabies antibodies is the minimum measurable antibody titre considered to represent a level of immunity that correlates with the ability to protect against rabies infection. Although this indirect ELISA has a lower sensitivity than the FAVN or RFFIT, it can be used as a rapid (~ 4 hours) screening test, that does not require handling of live rabies virus, to determine if vaccinated dogs and cats have sero-converted. Due to the lower sensitivity of the test, negative results should be confirmed by FAVN or RFFIT.

The reaction is composed of three steps:

1. Each serum sample to be tested is placed in a well of a microtitre plate pre-coated with inactivated rabies viral antigens. Antibodies present in the sample bind to the viral antigens on the surface of the plastic.

2. After a wash step, Protein A/peroxidase conjugate is added. It binds to the previously captured immunoglobulins (antibodies), forming a complex: (rabies Ag)–(Ab anti-rabies)–(Protein A/peroxidase).

3. Excess conjugate is eliminated by a wash step. The enzyme linked to the complex is revealed by the addition of a substrate that is transformed into a coloured product. After stopping the reaction, the optical densities are measured.

- **Preparation of antigen**

Rabies virus strain G5,52 Wistar (Pasteur derivative) is grown in low passage NIL2 cells originating from hamster embryo cell culture. The virus harvest is clarified to eliminate cell debris by gel filtration and the virus suspension is inactivated by betapropiolactone. A 4.1 µg/ml of an antigen stock is used for coating plates.

- **Reagents**<sup>4</sup>

Microplate containing six 16-well strips sensitised with rabies antigens. Use in the 4 weeks after opening the sachet, which must be closed after each use;

Conjugate (CJ): Protein A/peroxidase (10 × concentrated). Dilute ten times in the conjugate diluent (CD) and use within 24 hours following dilution;

---

<sup>4</sup> Available from Synbiotics Europe S.A.S., 2 rue Alexander Fleming, 69367 Lyon Cedex 07, France.
Buffered peroxidase substrate (PS); 3,3', 5,5'-Tetramethylbenzidine;

Negative control serum (N), Specific pathogen free sera diluted in Stabilzyme, a commercial stabiliser provide by Surmodics Inc., MN 55344-3523 USA;

Positive control serum (P), hyperimmune sera from vaccinated dogs diluted in Stabilzyme, a commercial stabiliser provided by Surmodics Inc., MN 55344-3523 USA;

Sample diluent (SD), PBS buffer, pH 7.8, including 0.28% (w/v) caseine, 0.055% (v/v) X100 Triton;

0.55% (w/v) PEG, 0.056% (w/v) SDS, 1% (w/v) PVP, 0.42% (w/v) Tetronic and 1% (v/v) heat-inactivated bovine serum;

Wash solution (W), Tris/NaCl buffer, pH 7.5, including 1% Tween 20;

Conjugate diluent (CD), Tris buffer, pH 8;

Stop solution (S), 4 N sulfuric acid solution + 0.02% (w/v) thiomersal.

Diluted reagents should be stored at 5°C ± 3°C. Place all reagents at laboratory temperature for at least 1 hour prior to use.

- **Samples**

The reaction is performed on heat-inactivated (30 minutes at 56°C) individual serum diluted at 1/100. Testing the appropriate set of dilutions of the OIE International Standard Serum containing 6.7 IU/ml is necessary (available from the OIE Reference Laboratory for Rabies, Nancy, France).

Serum samples should be stored at 5°C ± 3°C. For prolonged storage, the serum samples should be frozen at −20°C.

- **Preliminary predilution steps**

Strictly comply with the procedure indicated below. Use negative and positive controls in duplicate for each test run and/or for every plate.

  i) Carefully set up the distribution and identification of controls and samples using the protocol below.

  ii) Prepare the sera to be tested. Dilutions are performed in the sample diluent (SD) as follows: the samples are first prediluted at 1/10 in a blank microplate (10 µl of sample in 90 µl of SD).

  iii) For serum titration, a set of six dilutions of the OIE Standard Serum should be performed either in tubes or a blank microplate, starting with the initial dilution 1/10, then 1/30, 1/100, 1/300, 1/1000 to the final dilution 1/3000. This set of dilutions of the OIE Standard Serum should be included in each test run and/or in microplates with an initial dilution of 1/10, 1/30, 1/100, 1/300, 1/1000 and 1/3000.

The following scheme to prepare the appropriate set of dilutions is recommended:

<table>
<thead>
<tr>
<th>OIE dilution</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/10</td>
<td>10 µl of OIE International Standard Rabies Serum + 90 µl of sample diluent</td>
</tr>
<tr>
<td>1/30</td>
<td>10 µl of OIE International Standard Rabies Serum + 290 µl of sample diluent</td>
</tr>
<tr>
<td>1/100</td>
<td>10 µl of the 1/10 dilution + 90 µl of sample diluent</td>
</tr>
<tr>
<td>1/300</td>
<td>10 µl of the 1/30 dilution + 90 µl of sample diluent</td>
</tr>
<tr>
<td>1/1000</td>
<td>10 µl of the 1/100 dilution + 90 µl of sample diluent</td>
</tr>
<tr>
<td>1/3000</td>
<td>10 µl of the 1/300 dilution + 90 µl of sample diluent</td>
</tr>
</tbody>
</table>

This range of dilution of the OIE Standard Serum should be present in every plate.

- **Test procedure**

  i) **Control distribution:** dispense 90 µl of sample diluent, and add 10 µl of the negative control into wells A1 and A2, and 10 µl of the positive control to wells B1 and B2.

  ii) **Distribution of samples and OIE Standard Serum dilutions:** dispense 90 µl of sample diluent, add 10 µl of either 1/10 sample predilution or each OIE serum dilution from 1/10 to 1/3000 into the test wells and mix thoroughly.

Samples and OIE Standard Serum dilutions should be tested in duplicate. The following distribution plans (reporting final testing dilutions) are recommended:
Strips should always be placed on the frame so that both washer and reader can be used. Cover the wells with adhesive film, cut to the necessary length by the number of strips used. Mix by gently shaking the plate manually or by using a plate agitator.

iii) Incubate microtitre plates for 1 hour ± 5 minutes at 37°C ± 3°C.

iv) Reagent dilution:

- **Wash buffer:** dilute the concentrated washing solution (W) 1/10 in distilled or demineralised water.
- **Conjugate:** dilute the concentrate (CJ) 1/10 in the conjugate diluent (CD); 2 ml is needed for one strip, i.e. 20 µl of CJ in 1.88 ml of CD.

v) Carefully remove the adhesive film and wash four times.

vi) Add 100 µl of diluted conjugate to all the wells and cover with a new piece of adhesive film.

vii) Incubate conjugate for 1 hour ± 5 minutes at 37°C ± 3°C.

viii) Carefully remove the adhesive film and wash four times.

ix) Add 100 µl of buffered peroxidase substrate (PS) per well. Do not cover with adhesive film at this stage. Mix by shaking the plate gently manually or use a plate agitator to ensure correct homogenisation.

x) Incubate for 30 ± 5 minutes at laboratory temperature (20°C ± 5°C), shielded from light.

xi) Add 50 µl of stop solution (S) per well. Mix by gently shaking the plate manually or by using a plate agitator. Make sure that no bubbles occur in the wells. Carefully wipe the bottom of the wells.

xii) Measure the optical density (OD) bichromatically at 450 and 630 nm or monochromatically at 450 nm (in the yellow band).

- **Antibody quantification:** expression and interpretation of results

Titre calculation using the regression curve

i) Calculate the average OD value for each sample tested and each OIE serum dilution.

ii) Calculate the natural logarithm (ln) value for each average OD and the ln value of the rabies Ab concentration for each OIE dilution (from 6.7 to 0.0223 IU/ml, without taking into account the 1/100 testing dilution factor).

iii) Plot the ln (OD) (Y-axis) as a function of the ln (rabies Ab concentration) (X-axis) in order to draw the reference curve for the OIE Standard Serum.

iv) Using all individual results obtained for the OIE Standard Serum dilutions, perform a linear regression between ln rabies Ab concentrations (expressed in ELISA Units/ml) and ln (OD), to establish the corresponding mathematic model:

\[
\text{In rabies Ab concentration (ELISA Units/ml)} = a + b \times \ln \text{OD}
\]

v) For each tested sample, calculate the average OD value and then the rabies antibody concentration of the sample expressed as 'equivalent units per ml' (EU/ml), from the established model:

\[
\text{Sample Rabies Ab concentration (EU/ml)} = e^{(a + b \times \ln \text{OD})}
\]
Test validation

The results of each test run (or for each plate) are valid:

- if the optical density obtained with the positive control (OD \( P \)) is greater than or equal to 0.300, and
- if the optical density obtained with the negative control (OD \( N \)) is less than 0.50 \( \times \) OD \( P \).
- the correlation coefficient between ln \( \text{ODs} \) and ln rabies Ab concentrations for the OIE Standard Serum is greater than 0.95.

Examples

Positive control:

\[
\text{OD well } B_1 = 0.610 \\
\text{OD Well } B_2 = 0.690 \\
\Rightarrow \quad \text{OD } P = 0.650
\]

Negative control:

\[
\text{OD well } A_1 = 0.190 \\
\text{OD well } A_2 = 0.210 \\
\Rightarrow \quad \text{OD } N = 0.200
\]

Sample 1:

\[
\text{OD well } 1 = 1.790 \\
\text{OD well } 2 = 1.750 \\
\Rightarrow \quad \text{OD} = 1.770
\]

Sample 2:

\[
\text{OD well } 1 = 0.350 \\
\text{OD well } 2 = 0.390 \\
\Rightarrow \quad \text{OD} = 0.370
\]

Test validation

\[
\text{OD } P = 0.650 > 0.300 \quad \text{and} \quad \text{OD } N = 0.200 < 0.50 \times 0.650 = 0.325, \text{ therefore the test is valid.}
\]

Results and interpretation (quantitative antibody titration)

If the calculated titre is \( \geq 0.6 \), the animal is considered to have sero-converted following vaccination.
If the calculated titre is \(< 0.6 \), the animal is considered not to have a sufficient antibody level. As the ELISA is intended as a screening test, a confirmatory FAVN test or RFFIT should be carried out on serum samples showing a titre <0.6.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

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; there is no protection provided against Mokola virus (31). 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.

For animals, live vaccines are also effective by the oral route and can be distributed in baits in order to immunise wild (or domestic) animals. Live recombinant vaccine (e.g. vaccinia rabies-glycoprotein recombinant) has also proved to be effective (25).

Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 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 animals, 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 (5), 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 (13). Only in humans has the efficiency of post-exposure treatment 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 (36, 37), the OIE (Chapter I.1.6.) and to national guidelines and regulations.
Chapter 2.2.5. – Rabies

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 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, but, from a practical point of view, it could be easier to obtain 100% mortality of the control animals with a well-known rabies virus strain than with a locally isolated one. In animals vaccinated with inactivated vaccines, the percentage of seroconversion and the mean level of antibody allow a good prognosis for survival to challenge (3).

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 (including recombinant vaccines) used in oral vaccination campaigns, 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 (5).

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. Both animals and eggs should be of SPF origin, and the cell cultures, such as BHK cell lines, should conform to international standards of sterility and innocuity.

a) In animals

The virus is inoculated intracerebrally and the nervous tissue is harvested when the animal has been killed in the terminal stages of rabies. The virus is inactivated by physical methods, such as irradiation with ultraviolet light, or by chemical methods, such as the addition of phenol or beta-propiolactone. Vaccines should be prepared in young animals (mice, lambs, etc.) for high yield of virus and to reduce the myelin content of vaccine and associated adverse events. In certain cases, the virus is not inactivated entirely, as for example in the phenol-treated vaccines of the Fermi type, but such vaccines are no longer recommended.
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 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 (25).

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 animals, 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.

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 I.1.5.

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 90 days for adverse reactions attributable to the vaccine.

c) Potency

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 (20), or with two doses, 1-week apart, according to the NIH test (37). 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 (20, 37).

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 PD\textsubscript{50} (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 at least 10 LD\textsubscript{50}. The challenge dose should be in the range 12–50 LD\textsubscript{50} 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, or the potency demonstrated in the duration of immunity test used to licence the product, 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 (4). 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. After that, it is not tested for every batch (see Section C.4.c. above).

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.

REFERENCES


*  *

NB: There are OIE Reference Laboratories for Rabies (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
SUMMARY

Paratuberculosis (Johne’s disease) is a chronic enteritis of ruminants caused by Mycobacterium avium subsp. paratuberculosis (M. paratuberculosis) (34).

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 response through the use of the gamma interferon assay. 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: The single largest problem in paratuberculosis control is the difficulty of detecting subclinically infected ruminants. The serological tests commonly used for paratuberculosis in cattle are complement fixation (CF), absorbed enzyme-linked immunosorbent assay (ELISA) and gel immunodiffusion. 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 paratuberculosis causes paratuberculosis or Johne’s disease, an
intestinal granulomatous infection. 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 (3, 10). 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 (16) and semen can be infected with the organism (31). 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 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 (33).

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 (9). 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 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 detectable serological response in clinical cases.

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 and M. paratuberculosis infection, both clinically and by the use of specific diagnostic tests. Exposure to environmental saprophytic mycobacteria may also sensitise 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 (14).

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 (2).

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 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. The mesenteric lymph nodes may be 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% formal saline) for subsequent histology. Both haematoyxin-and-eosin-stained sections and Ziehl–Neelsen-stained sections should be examined. The pathognomonic lesions consist of infiltration of the lamina propria, Peyer’s patches and the cortex of the mesenteric lymph nodes with large, pale-staining epithelioid cells and multinucleated Langhans' giant cells, in both of which clumps or singly disposed acid-fast bacilli are usually, but not invariably, found. Langhans' giant cells are not uncommon and contain few organisms.

Lesions in sheep and goats are similar to those observed in cattle. There is often only slight thickening and inflammation of the mucosa, but nodules of caseation and calcification sometimes form in the intestine and in associated lymph nodes. Enlargement of the mesenteric lymph nodes is seen in alpaca. Sometimes the pigmented form of paratuberculosis is observed in sheep.

b) Bacteriology (microscopy)

Ziehl–Neelsen-stained smears of faeces are examined microscopically. A 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 does not indicate a definitive diagnosis. The disadvantage of this test are that only about one-third of cases can be confirmed on microscopic examination of a single faecal sample.

c) Bacteriology (culture)

*Mycobacterium paratuberculosis* infection mainly involves the lower small intestine and adjacent caecum. *Mycobacterium paratuberculosis* organisms are vastly outnumbered by other bacteria in faecal and intestinal tissue specimens.

Primary colonies of *M. paratuberculosis* may be expected to appear any time from 5 to 14 weeks after inoculation. Primary colonies on Herrold’s medium containing mycobactin\(^1\) are very small (1 mm in diameter), colourless, translucent and hemispherical. Their margins are round and even, and their surfaces are smooth and glistening. The colonies become more opaque and increase in size (4 or 5 mm) as incubation continues. The colonial morphology changes with age from smooth to rough, and from hemispherical to mammilate (32).

The uncommon, bright yellow pigmented sheep strain is difficult to grow on artificial media. It has been reported that unpigmented sheep strains grow less well than cattle strains, and no cultures should be discarded as negative without prolonged incubation.

For identification of *M. paratuberculosis*, small inocula of suspect colonies should be subcultured on the same medium with and without mycobactin, to demonstrate mycobactin dependency. (The test is not reliable if large numbers of bacilli are present.)

There are two basic methods in use for the culture of *M. paratuberculosis* from clinical specimens: the method using oxalic acid and NaOH for decontamination and Löwenstein–Jensen 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.

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\(^1\) 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.
• Media

Examples of suitable media are:

i) **Herrold’s egg yolk medium with mycobactin** (19)

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 yolks² 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** (28)

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 Seltz ‘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) **Middlebrook 7H9, 7H10 and 7H11 media** (Difco), and 7H12 Bactec medium enhanced with mycobactin in the same proportion as for Herrold’s medium can also be used. The advantage of this media is that it is transparent, which facilitates the early detection of colonies.

iv) **Löwenstein–Jensen medium** with or without mycobactin (13).

• Sample preparation

Although faecal culture is technically difficult and time-consuming to carry out, it is still a good method for diagnosis of paratuberculosis in live animals. It is the only test that does not produce false-positive results (100% specificity). It will detect infected animals 6 months or more before they develop clinical signs, and during the clinical stage its sensitivity approaches 100%.

• Processing tissue specimens

Chemical preservatives should not be used. The tissues can be frozen ay –20°C.

To avoid contamination, the faeces should be rinsed from portions of intestinal tract before shipment to the laboratory.

i) **Digestion 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.

ii) **Decontamination of inoculum**

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. Other methods of decontamination can be used, such as treatment with 5% oxalic acid.

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2 Use fresh eggs not more than 2 days old from a flock that is not receiving antibiotics. With a brush, scrub the eggs with water containing a detergent. Rinse with water and place the eggs in 70° alcohol for 30 minutes. Dry by inserting between two sterile towels. With sterile rat-tooth forceps, crack one end of the eggshell, making a hole of approximately 10 mm, and remove the egg white with the forceps and gravity. Make the hole larger and break the yolk. Mix the egg yolk by twirling the forceps, and remove the yolk sac. Pour the mixed egg yolk into media.
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 by Merkal & Richards (22) to control effectively fungal overgrowth of inoculated media. 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 15–20 weeks and observed weekly from the sixth week onwards.

- **Processing faecal specimens**
  - No refrigerant or chemical preservative is used. The faecal specimens can be frozen at −70°C.
  - i) **Suspension and decontamination of faeces**
    
    1 g of faeces is transferred to a 50 ml tube containing 20 ml of sterile distilled water. The mixture is shaken for 30 minutes at room temperature. The larger particles are allowed to settle for 30 minutes. The uppermost 5 ml of faeces suspension is transferred to a 50 ml tube containing 20 ml of HPC. The tube is inverted several times to assure uniform distribution and allowed to stand undisturbed for 18 hours at room temperature.
  - ii) **Inoculation of culture media**
    
    0.1 ml of the undisturbed sediment is transferred to each of four slants of Herrold’s medium, three with mycobactin and one without mycobactin. A smear may be made from the sediment and stained by the Ziehl–Neelsen method.
  - iii) **Incubation and observation of slants**
    
    The same as for tissue specimens.

Variations in the above methods have been described (4, 15, 21, 24, 27, 38, 39). The sensitivity of culture may be enhanced using liquid media and with centrifugation rather than sedimentation techniques. The double incubation method assists with decontamination of the inoculum (30).

A more rapid technique for the isolation of *M. paratuberculosis* employs the use of a radiometric-based detection system, the Bactec 460. Growth of mycobacteria is measured by the release of $^{14}$CO$_2$ from palmitate as a consequence of bacterial metabolism. However, as this system is radiometrically based, it is not feasible for use in some laboratories and has been phased out in others. Recently three other fluorescence-based rapid methods were introduced, the Bactec 960 system, the MGIT (Mycobacterial Growth Indicator Tube) system (Becton Dickinson) and the MBBact system (Organon Technica). Severe problems were encountered during initial experiments in which these methods were tested on faecal samples due to overgrowth by other bacteria (spore forms and fungi); however, these methods have been further developed and are now used with some success in many laboratories.

d) **DNA probes**

DNA probes are being developed that offer a means of detecting *M. paratuberculosis* in diagnostic samples and of rapidly identifying bacterial isolates (8, 20). They have been used to distinguish between *M. paratuberculosis* and other mycobacteria, especially those of the *M. avium* group.

McFadden et al. have identified a sequence (17, 18), 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* (5). The use of IS900 as a DNA probe for specific identification of *M. paratuberculosis* in faecal samples from cattle by enzymatic amplification of DNA using the polymerase chain reaction (PCR) has been reported (36). A commercial diagnostic test based on the detection of IS900 sequences following isolation of mycobacteria from faecal samples and enrichment of a DNA fraction from IS900 sequences by
PCR has been developed. The test is available in kit form\textsuperscript{3} suitable for use in laboratories. It has poor diagnostic sensitivity compared with faecal culture. Another kit, PCR Adiavet Paratb, is now available\textsuperscript{3}.

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) (29) 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 \textit{M. paratuberculosis} 316F). \textit{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 H\textsubscript{50} (50% haemolysing dose) as calculated by titration against the antigen.

iv) Sheep erythrocytes, 2.5%, are sensitised with 2 units of H\textsubscript{100} 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) \textit{Reading and interpreting the results}: Plates may be left to settle or centrifuged and read as follows: 4\textsuperscript+ = 100% fixation, 3\textsuperscript+ = 75% fixation, 2\textsuperscript+ = 50% fixation, 1\textsuperscript+ = 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\textsuperscript+ 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 \textit{M. paratuberculosis}. 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 \textit{M. phlei} absorption of sera.

The absorbed ELISA, designed by Yokomizo \textit{et al}. (43, 44) and modified by Milner \textit{et al}. (23), was developed into a commercial kit by Cox \textit{et al}. (6). This kit was evaluated by Ridge \textit{et al}. (25), and was found to have a sensitivity in clinical cases of 88.3%, and in subclinical cases of 48.8%, and a specificity of 99.8% in cattle, and in sheep a sensitivity of 35–54% and a specificity of 98.2–98.5%. However, other workers have found a lower sensitivity and specificity (40).

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 \textit{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 \textit{M. phlei}, which were removed by centrifugation prior to testing.

A microtitre plate format has been developed in which \textit{M. paratuberculosis} antigen is coated on to 96-well plates. Samples are diluted in sample diluent containing \textit{M. phlei} to remove cross-reacting antibodies. On incubation of the diluted sample in the coated well, antibody specific to \textit{M. paratuberculosis} forms a complex with the coated antigens. After washing away unbound materials from the wells, horseradish peroxidase (HPRO)-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.

\textsuperscript{3} IDEXX DNA Probe test kit for the detection of \textit{Mycobacterium paratuberculosis} or the PCR Adiavet Paratb kit.
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An anti-bovine IgG labelled with HRPO is 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. The procedure is the same as for any routine ELISA. Screening tests are usually performed in one well per serum, but are unreliable, and diagnostic tests should be performed in duplicate wells.

c) **Agar gel immunodiffusion test**

The AGID test is useful for the confirmation of the disease in clinically suspect cattle, sheep and goats (26). Several variations of the method are in use.

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.

3. **Tests for 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) (42). The quantitative detection of bovine gamma interferon is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine gamma interferon. The sensitivity and specificity may be comparable with the tuberculin skin test. A commercial diagnostic test based on the detection of gamma interferon has been developed. The method and test materials needed, the interpretation of results, and calculations, are fully described in the instructions accompanying the commercial kit.

b) **Delayed-type hypersensitivity**

The 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 avian PPD tuberculin⁵ or johnin (avian tuberculin and johnin are of comparable sensitivity and specificity) into a clipped or shaven site, usually on the side of the middle third of the neck. 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 (12). A herd test gives only an indication of the number of sensitised animals and may thus be used only as a preliminary test prior to the initiation of a control programme.

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

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⁴ *Mycobacterium paratuberculosis* gamma-interferon test kit, CSL Limited (Veterinary), 45 Poplar Road, Parkville, Victoria 3052, Australia.

⁵ Avian tuberculin can be obtained from VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom, or from Symbiotic Society, 299 av. Jean Jaurès, 69007 Lyon, France.
bacterins. Vaccines may be prepared from one strain of \textit{M. paratuberculosis} 316F or 2E (Weybridge) or \textit{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 (7, 35, 41). Vaccination may cause a reaction at the site of injection. Vaccination may also interfere with eradication programmes based on elimination of infected animals and can interfere with the interpretation of DTH skin tests for bovine tuberculosis.

\textit{Diagnostic products:} Johnin PPD is a preparation of the heat-treated products of growth and lysis of \textit{M. paratuberculosis}. Avian tuberculin PPD is a preparation of heat-treated products of growth and lysis of \textit{M. avium} D4ER or TB 56. Details of avian tuberculin PPD are in Chapter 2.7.8. Avian tuberculosis. These two preparations are used, by intradermal injection, to reveal DTH as a means of identifying animals infected or sensitised with \textit{M. paratuberculosis}.

Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management
   a) Characteristics of the seed
      \textit{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.
      
      \textit{Johnin:} Strains of \textit{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.
   
   b) Method of culture
      \textit{Vaccine:} Seed cultures may be made on potato slants partly immersed in a suitable medium, such as Reid’s synthetic medium$^6$ (37). Cultures may be stored lyophilised. Active cultures are normally incubated at 37°C.
      
      \textit{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
      \textit{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 \textit{M. paratuberculosis} (41). 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 (11).
      
       \textit{Johnin:} Cultures should be checked by staining smears for the presence of contaminating organisms.

\textit{6} 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. (48 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.
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 (7).

**Johnin:** Johnin for skin test diagnosis is a PPD prepared from one or more strains of *M. paratuberculosis* (available from VLA 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, 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 (1). 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 I.1.5. 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 (1).

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 (1).

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 (14).

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 (11). In humans, accidental injection of vaccine has resulted in chronic inflammatory reactions requiring surgical treatment (14).

5. **Tests on the final product**

a) **Safety**

See Section C.4.b.
Chapter 2.2.6. — Paratuberculosis (Johne’s disease)

b) Potency

See Section C.4.c.

REFERENCES


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**NB:** There are OIE Reference Laboratories for Paratuberculosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
Heartwater (also known as cowdriosis) is a 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 and in Madagascar, and also in the Caribbean, threatening the American mainland.

Clinically, the disease is characterised by a sudden high fever, often hydropericardium, lung oedema and, in acute and peracute forms, by nervous signs and a high mortality. Subacute heartwater also occurs, and has a higher recovery rate.

Wild animals could play a role as reservoir; Rusa deer seem to be the only wild ruminant in which heartwater has an economic impact.

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 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 under 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. The colonies are still visible 2 days after death in a brain that has been stored at room temperature and up to 34 days in a brain that has been stored in a refrigerator.

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 Ehrlichia in the brain of the inoculated animal are diagnostic for heartwater.

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 actively infected animals, to a lesser extent in the blood or bone marrow of carrier animals, and in the tick vectors. Apart from diagnosis, PCR is widely used for research on the Ehrlichia genome and for epidemiological studies.

Serological tests: Serological tests include indirect fluorescent antibody tests, enzyme-linked immunosorbent assays (ELISAs) and Western blotting. However, when the whole Ehrlichia is used as antigen, cross-reactions with Ehrlichia spp. occur in all of these tests.

Two recently developed ELISAs that use recombinant major antigenic protein 1 (MAP1) antigens – the MAP1-B ELISA and the MAP1 competitive ELISA – have shown a dramatic improvement in specificity compared with previous tests, making the interpretation of serological results more reliable in regions where Ehrlichia infections occur in ruminants. These tests can help to monitor
requirements for vaccines and diagnostic biologicals: The immunisation against heartwater by the ‘infection and treatment’ method using infected blood or homogenised, pre-fed Amblyomma ticks 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 is being evaluated in the field in endemic areas. In the near future, it could replace the infection and treatment method, which has practical difficulties.

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 (2, 6, 23). It is also known by the synonyms malkopsiiekte (Afrikaans), péricardite exsudative infectieuse (French), hidrocarditis infecçiosa (Portuguese), idropericardite dei ruminanti (Italian), and a variety of names in different African languages (5). Ehrlichia ruminantium is classified in the order Rickettsiales and in the Tribe Ehrlichieae, together with the genera Anaplasma.

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 Tome. The disease is also reported in the Caribbean (Guadeloupe, Marie-Galante and Antigua) (21), 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 seem to be the only wild ruminant in which heartwater has an economic impact.

The average natural incubation period is 2 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 with small fluctuations and drops shortly before death.

Fever is followed by inappetence, sometimes listlessness, diarrhoea, particularly in cattle (3), 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 head 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 a nervous 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 Ehrlichia.

The most common macroscopic lesions are hydropericardium, hydrothorax, pulmonary oedema, intestinal congestion, oedema of the mediastinal and bronchial lymph nodes (3), 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, of clinical nervous signs, and of transudates in the pericardium and thorax at post-mortem examination. Differential clinical diagnosis should be made with bovine cerebral babesiosis and theileriosis, anaplasmosis, botulism, and small ruminant haemonchosis.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Typical colonies of E. ruminantium can be observed in brain smears made after death.

The tedious task of opening the cranium is not necessary. One alternative method (26) is to cut off the head in front of the first cervical vertebra. Then, a curette is introduced 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 simple 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 and appropriate restraint with large and especially with 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 *Ehrlichia* are still present 48 hours after death in a brain that has been stored at room temperature, and for up to 34 days in a brain that has been stored in a refrigerator (4).

A small fragment of grey matter (approximately the size of a match head) is placed on a microscope slide, crushed 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 rickettsias. Experience is required to identify *Ehrlichia* colonies and to differentiate them 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* 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 drug-treated 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 *Ehrlichia* in the brain of the inoculated ruminant are diagnostic for heartwater.

Transmission electron microscopy has been used to demonstrate that the *Ehrlichia* organisms develop inside a vacuole-like structure, which is surrounded by a membrane in the endothelial cell’s cytoplasm (25). Each organism is enclosed by a double membrane. Within the vacuole-like structure, *Ehrlichia* electron-dense forms (elementary bodies), as well as intermediate reticulate forms, are identified.

### a) Isolation of *Ehrlichia ruminantium* using *in-vitro* culture

Isolation of *E. ruminantium* in cell culture is not the first choice of a test to confirm the diagnosis of cowdriosis, as it is a time-consuming laboratory procedure, although numerous cell lines support growth. However, *Ehrlichia* isolation is necessary for typing the strains present in a region for the purpose of vaccination programmes. *Ehrlichia* can be isolated from the blood of reacting animals by cultivation on ruminant endothelial cells. 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**
  - (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:)

  - The blood of the reacting animal is collected in anticoagulant (heparin or sodium citrate) and diluted 1/2 in the culture medium consisting of Glasgow minimal essential medium (MEM) supplemented with 10% inactivated fetal bovine serum, 2.85 mg/ml tryptose phosphate broth, 200 mM L-glutamine, and antibiotics if necessary (penicillin 100 international units/ml, streptomycin 100 µg/ml).
  - 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.
  - After incubation, the blood is poured off and the monolayer is gently washed three times with prewarmed culture medium. Fresh culture medium is added and the flask is incubated at 37°C. The medium is changed twice weekly.

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• Seed 4 ml of plasma 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. Passage on uninfected cell monolayers is performed when the lysis reaches 80% of the cell layer. The remaining cells are stained with eosin/methylene blue 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 a *Ehrlichia*-specific antiserum.

b) **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 *Ehrlichia*, by inoculating blood or tick homogenate into a susceptible animal. Blood from individual animals, or pooled blood, is injected slowly at a dose of 10–100 ml intravenously into a susceptible sheep or goat. Another method consists of collecting and homogenising adult *Amblyomma* ticks, and after centrifuging the homogenate, inoculating the resulting supernatant. The latter method is more sensitive because the concentration of *Ehrlichia* is higher in the tick than in the blood. However, the tick infection rate is variable and sometimes as low as 1% (15). In this case, to detect an infection, at least 100 ticks are needed and as many as possible should be used. In both cases, the inoculum with 10% dimethyl sulphoxide 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 bacteria by molecular methods and/or the demonstration of *Ehrlichia* in the brain of the inoculated ruminant are diagnostic for heartwater.

2. **Molecular methods**

a) **Detection of *Ehrlichia ruminantium* using DNA probes**

A genomic DNA fragment specific for *E. ruminantium* has been cloned and used as a nucleic acid probe (29). It recognises all strains of *E. ruminantium* tested so far. This probe, designated pCS20, readily detects infection in clinically ill animals and experimentally infected *Amblyomma* ticks. However, it is not sufficiently sensitive to detect most carrier animals and/or low level infections in ticks. The pCS20 probe proved nonetheless 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 polymerase chain reaction (PCR)-amplified product of the homologous DNA fragment (1).

b) **Detection of *Ehrlichia ruminantium* using PCR and nested PCR**

Two primers – AB128 and AB129 – have been designed from the DNA sequence of the pCS20 probe (15) for use in a PCR. A modification of the PCR method consists in blotting the pCS20 probe on the amplification fragment in an additional step, which results in a tenfold increase in the sensitivity (24). This latter technique (PCR/hybridisation) has been shown to be 350-fold more sensitive than the nucleic acid probe alone. 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 usually remains negative (24). Experimentally, the detection limit of the conventional PCR assay was found to be between 10 and 10² 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 97 to 88% with tick samples containing 10⁷ to 10³ organisms, and dropping to 61% and 28% with samples containing 10³ and 10² organisms, respectively (22). Consequently, the rate of 86% of ticks testing positive when fed on a reacting animal dropped to 21% when fed on carrier animals due to a lower rickettsemia in such animals.

A nested PCR targeting the same pCS20 DNA fragment has also been developed (18). The 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 matrix 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 2 log 10 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.
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 (18). A high genetic diversity of *E. ruminantium* is observed in the field that influences 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.

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, but after that it seems to be absent from the circulation at a detectable level for long periods. In one study in Zimbabwe only between 3.3 and 26.7% of cattle, and 23.3% of goats were found to be positive whereas almost 100% of them had been infected with *Ehrlichia* (13). 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 known. 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 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 microorganisms in the circulation during feeding.

Primers 32F1 and 32R1 designed from the sequence of the MAP1 gene of *E. ruminantium* were successfully used in a PCR to detect the pathogen in blood and bone marrow of carrier sheep and wild African ungulates, but the method has not been widely evaluated and used.

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 (2). 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 Omatjente*, *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, whereas no hybridisation occurred when the PCR-RLB was applied to *Terassoma 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.

- **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 confirm serological results when, for example, seronegative animals originating from an endemic area must be translocated to a heartwater-free area at risk (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, and further research is needed before a standard test with known sensitivity can be designed. The current results obtained with the PCR, the nested PCR or the RLB assay 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 *Ehrlichia*. 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) is now seldom used.

One drawback of all of these tests is the detection of false-positive reactions due to common antigenic determinants between the *E. ruminantium* MAP1 (9) and the presence of similar proteins in several *Ehrlichia* species. Almost all of these tests are no longer used for epidemiology or 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 overcome 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) (28). 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) (10). 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.

So far, the MAP 1-B ELISA has been the most extensively used and will be described in more details.

a) Indirect fluorescent antibody test with infected endothelial cell tissue culture as antigen (CIFA test) (17)

To prepare the antigen, a *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 supernatant. The cells are centrifuged three times with phosphate buffered saline (PBS) at 200 g for 10 minutes. Of the washed cell suspension, 10 µl is 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 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 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) Indirect fluorescent antibody test with infected mouse peritoneal macrophages as antigen (MIFA test) (8)

Mice are injected intraperitoneally with 0.2 ml of Kümml strain stabilate following retrieval from liquid nitrogen storage. Clinical signs – ruffled hair coat and lethargy – appear 12 days later, and several mice may die. The surviving mice are killed. The peritoneal cells containing some macrophages with morula colonies are removed by injecting 2 ml of PBS into the peritoneal cavity and withdrawing the fluid. The pooled peritoneal fluid is centrifuged for 5 minutes at 2000 g and the pellet is resuspended in 0.3 ml of buffer.

A droplet of the cell suspension is placed in every well of an immunofluorescence slide to form a monolayer of cells. The antigen slides are air-dried, wrapped in tissue and tinfoil and stored: at 4°C for 21 days, at –18°C for 6–9 months, or at or under –70°C for over 1 year.

Just before use, the antigen slides are plunged into cold methanol for 1–3 seconds. A thick felt pen is used to separate the wells to avoid confluence of sera between wells.

The IFA procedure is the same as the previous test, but the initial serum dilution is 1/80.
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c) **MAP1-B enzyme-linked immunosorbent assay (28)**

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.

**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% nonfat 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 by adding 50 µl of 2 N H₂SO₄. Absorbance is read at 495 nm. Positive and negative controls are included in each plate.

d) **MAP1 competitive enzyme-linked immunosorbent assay (20)**

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, isoytpe 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 *Ehrlichia* antigens, such as CIFA, ELISAs, and Western blotting, are still used for experimental studies but are no longer used for sero-epidemiological studies. The

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1 Qiagen, Max-Volmer-Straße 4, 40724 Hilden, Germany.
tests have been compared and applied to known positive and negative sera to *E. ruminantium* (7). 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 *Ehrlichia* 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*.

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* (20). 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.

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 (20). 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 (19). 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 even never seroconvert. 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 (14). Similar observations were made in the Caribbean.

Serological tests are useful for the assessment of heartwater infection in vaccinated animals. The tests can also be used to screen animals prior to importation into heartwater-free areas, bearing in mind that antibodies are maintained at detectable levels in naturally infected domestic ruminants for a few months only, and that 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 be regarded as a diagnostic method to be applied at the herd level, not at the individual level.

Molecular methods, such as PCR assay, could potentially help in detecting carrier animals without detectable antibodies, but this approach has still significant drawbacks (see Section B.2. Molecular methods).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Although promising research results have been obtained with attenuated and inactivated organisms, no commercial vaccines are available at present. The only method of immunisation against heartwater remains the "infection and treatment" method using infected blood or homogenised pre-fed infected ticks followed by treatment of reacting animals with tetracycline. This method is still in use in several areas. However, it is likely to be replaced soon by vaccination with preparations of inactivated *E. ruminantium* elementary bodies emulsified in oil adjuvants, following the demonstration that susceptible goats can be protected by inactivated *Ehrlichia* in Freund's adjuvant (16). This vaccine also protected against challenge in sheep (11) using different strains of *E. ruminantium*, and in cattle (27) using the same strain as in goats. A first generation vaccine preparation of inactivated *Ehrlichia* in Montanide ISA 50 oil adjuvant (adjuvant licensed for animal use) was shown to be similarly effective to the Freund's adjuvant preparation on laboratory challenge of immunised goats.

Animals can be immunised with two subcutaneous injections of 250 µg of antigen emulsified (50/50) in Montanide ISA 50 adjuvant in a volume of 2 ml. Further studies on optimisation of vaccine production, quality control and efficiency in the different target species are currently being conducted. In experimental conditions, it has recently been shown on goats that the vaccine dose can be lowered down to 32 µg of antigen without decreasing the effect on protection.

Evaluation of an inactivated vaccine adjuvanted with ISA 50 has demonstrated protection of sheep against natural field challenge in Zimbabwe (12). In addition, large field evaluation trials are currently being done in Africa in several farming systems. 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.

REFERENCES


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NB: There is an OIE Reference Laboratory for Heartwater (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.2.8.

NEW WORLD SCREWWORM
(Cochliomyia hominivorax) AND
OLD WORLD SCREWWORM (Chrysomya bezziana)

SUMMARY

The New World screwworm¹ (NWS), Cochliomyia hominivorax (Coquerel), and the Old World
screwworm² (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 growth 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 reinfection. Preventive
measures include the spraying or dipping of susceptible livestock with organophosphorus
compounds and, more recently, use of avermectins 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.

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, Chrysomya bezziana Villeneuve, are members of two genera of the subfamily Chrysomyinae of the Dipteran family Calliphoridae (blowflies). Both species are obligate parasites of mammals 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 respective niches in their 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 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 (18), 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.

Following two moult, the larvae reach maturity about 5–7 days after egg hatch. They 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. Upon 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 ready to mate within 24 hours, but females mature their ovaries, become responsive towards males and mate only 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, firmly attached to each other and to the oviposition substrate. The number 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 (38). Following the first egg batch, further batches are laid at intervals of 3–4 days (46). 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 (23). Thus, the complete life cycle of NWS may take 2–3 months in cold weather (33), whereas in temperate conditions with an average air temperature of 22°C, it is completed in about 24 days (23), and in tropical conditions averaging 29°C it is completed in about 18 days (46).

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 (21). 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 (6), Mexico (16), Curacao, Puerto Rico, and the Virgin Islands and, in Central America, from Guatemala, Belize, El Salvador, Honduras, Nicaragua and, in 2000, Costa Rica (49). 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. 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 (13, 24). 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. Cases of NWS have been reported recently in Mexico, USA, and even in the United Kingdom (28).

The distribution of OWS is confined to the Old World, as the name suggests, throughout much of Africa (from Ethiopia and sub-Saharan countries to northern South Africa), the Gulf countries, the Indian subcontinent, and South-East Asia (from southern China [People’s Rep. of] through the Malay Peninsula and the Indonesian and Philippine islands to Papua New Guinea) (21, 38, 42, 50). OWS myiasis has also been reported from Algeria (1).
in a local shepherd, but in the absence of other reported cases, particularly animal cases, a continuing presence there seems unlikely. The situation in the Gulf area and surrounding regions is dynamic with recent reports confirmed from Iran (32) and Iraq (2). The climatic requirements of the two screwworm species are very similar and their potential distributions, if unrestrained, would overlap considerably (42).

Organophosphorus insecticides such as dichlofenthion, fenchlorphos, and in particular, coumaphos are recommended for the treatment of wounds infested with OWS and NWS (15, 34, 40). 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 wett able 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 (34). 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% wett able 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, 35). 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 (34) and scrotal strike of castrated calves (39). 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 (48). 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 ref. 16), but more recent studies demonstrated that it can produce a significant reduction in the incidence of navel and scrotal myiasis due to NWS (7, 26). Although results of ivermectin trials show variation, results of doramectin trials are overwhelmingly positive (17). 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 (4, 30, 31). 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 (29). Doramectin also provided complete protection for 21 days and partial protection (56%) at 28 days post-treatment (29). 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 (10). Abamectin (subcutaneous injection, 200 µg/kg) gave good, but not 100%, prevention of post-castration myiasis by NWS (3). Pour-on formulations of moxidectin, eprinomectin and doramectin gave poor protection against OWS myiasis (48) when compared with injectable formulations of doramectin against NWS. There are early indications that fipronil (a phenyl-pyrazole) might be effective as a preventive of post-castration myiasis. Similarly, topical application of an insect growth regulator (IGR), dicyclanil, to castration wounds in cattle gave good protection (>90%) against NWS myiasis (5). IGRs are very specific to insects and, therefore, are less hazardous in the environment than many other groups of insecticides.

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 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 (Figure 1) and then be preserved in 80% ethanol and returned to the laboratory for examination under a dissecting microscope at up to x50 magnification (for further techniques see refs 12, 20, 37, 50). Optimal preservation of larvae, in their natural extended state, is made by killing them by brief immersion (15 seconds) in boiling water before storage in 80% ethanol.

**Second instars larvae:** Second instars larvae have only two spiracular slits in each of the posterior spiracular plates compared with the three slits of third instar larvae (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 (22). 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 meat, 50 g dried bovine blood, and 1.5 ml formalin (44), 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 instars larvae:** Third instars larvae 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 (23, 37). 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. 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 larvae 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 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 refs 12, 14, 17, 20, 21, 37, 50 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 reversed between the two screwworm species.

**Adult:** Adult flies needed for identification purposes are often collected using wind-oriented traps (8) and sticky traps (37) baited with a synthetic odour, swarm lure-4 (27). Alternative sampling systems, using electrocuting grids or sticky surfaces at odour-baited visual targets, have been used for research purposes (18). 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 pollinose 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 4; see also refs 11, 14, 23, 37).

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 squamae (s in Figure 4) 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) (37, 50).
Chapter 2.2.8. – Screwworm (Cochliomyia hominivorax and Chrysomya bezziana)

**Fig. 1.** Identification key for the diagnosis of third instars 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.
Fig. 2. Head and first two thoracic segments of third instars larva of Cochliomyia hominivorax (as = anterior spiracle; inset is the anterior spiracle of Chrysomya bezziana).

Fig. 3. Characteristics of third instars larva 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. [23].)

Fig. 4. 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 (9), analysis of mitochondrial DNA (19, 25, 45), and use of random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) (36). Problems with identification of larvae or adults from cases of myiasis can be referred to the Food and Agriculture Organisation of the United Nations Collaborating Centre on Myiasis-Causing Insects and Their Identification2.

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 (47).

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 (43). The only proven method of eradication of NWS relies on a biological technique, the sterile insect technique, SIT (16, 24), which has also been applied experimentally to OWS (41). In this technique, male flies sterilised in their late pupal stage by gamma 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. references 13, 24). Presently, only one New World sterile screwworm production facility exists, at Tuxtla Gutiérrez in the south of Mexico. Another will be built in Panama3. An experimental facility to produce sterile OWS opened in Malaysia in 19984.

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Chapter 2.2.8. — Screwworm (Cochliomyia hominivorax and Chrysomya bezziana)


Chapter 2.2.8. — Screwworm (Cochliomyia hominivorax and Chrysomya bezziana)


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**CHAPTER 2.2.9.**

**TRICHINELLOSIS**

**SUMMARY**

Trichinella infection of meat from food animals and game is important because of the risk of trichinellosis in humans who eat raw or undercooked meat. The adults of Trichinella species survive less than 2 months and may be found in the small intestine of humans, pigs, rats, bears, walruses and many other flesh-eating mammals, and occasionally in horses. Larvae of most genotypes of Trichinella occur in the voluntary skeletal muscles of their hosts and ingestion of tissue containing these larvae transmits infection to a susceptible individual.

Diagnostic tests for Trichinella infection 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. The sensitivity and specificity of serological methods is strongly related to the antigen type and quality used. A good level of validation has been reached in pigs. However, a serological response in pigs with a light or moderate infections is often not detected for 3 weeks or longer after muscle larvae become infective. In such cases, a false-negative serological result might be obtained. A low rate of false-positive results has been reported for serological tests. For the purposes of individual carcass inspection, only direct methods can be recommended. For surveillance or verification of trichinae-free herds or regions, serological methods are acceptable.

**Identification of the agent:** Two general methods, compression or digestion of muscle tissue, are used for the direct detection of Trichinella infection. Both methods test for the presence of parasites in tissues where infection is heaviest, these being in descending order of incidence in pigs: the diaphragm (crus), tongue, masseter and abdominal muscles, although this partially depends on the degree of infection. In horses, muscles of the tongue and masseter harbour the most worms, followed by the diaphragm and muscles in the neck.

The compression method involves visual inspection of compressed pieces of muscle tissue for the presence of larvae in situ. This method can be performed with a stereomicroscope, but usually a specialised microscope, the trichinoscope, is used, which has an estimated efficiency of detecting as few as three larvae/g of tissue. It has the disadvantage of requiring considerable time for the inspection of multiple samples from each carcass. It is also very difficult to detect the larvae of T. pseudospiralis, T. papuae, and T. zimbabwensis from African crocodiles, which are not surrounded by collagen. For these reasons, compression is not recommended for routine inspection, but is useful for detecting medium to high infections when a few animals require examination and facilities are not available for testing by artificial digestion.

The artificial digestion methods involve the enzymatic digestion of individual or pooled muscle tissue samples, followed by selective screening, filtration, or sedimentation procedures. Samples processed by these methods are inspected microscopically for the presence of larvae. Digestion methods include mechanical homogenisation and stirring, using a 1 g sample for pigs and 10 g for game and horses, and have an efficiency of approximately three larvae/g of tissue examined. These methods are recommended for the individual inspection of carcass of pigs, horses and game carcasses where their meat is intended for human consumption.

**Serological tests:** The enzyme-linked immunosorbent assay (ELISA) is the only suitable method for the ante-mortem detection of Trichinella infection and levels as low as one larva/100 g of tissue have been detected in pigs. The specificity of ELISA for Trichinella infection is directly linked to the type and quality of the antigen employed in the test. Secretory antigens collected by short-term maintenance of T. spiralis muscle larvae in vitro and synthetic carbohydrate antigens currently...
provide the most specific and economical source, although a low rate of false-positive results has been obtained in some studies.

A low rate of false-negative ELISA results may be obtained with recently infected animals that have low-grade infections. For this reason, the use of secretory antigens in an ELISA is recommended for surveillance programmes but is not recommended for individual carcass testing. The digestion of 100 g or more of tissue is recommended as a confirmatory test for serological positive animals.

Requirements for vaccines and diagnostic biologicals: A vaccine for Trichinella infection in food animals is not practical. There are no biological reagents required for direct detection methods. For indirect (serological) methods TSL-1 antigens must be used to ensure test specificity. These antigens may be obtained as secretory products recovered from in-vitro maintenance of muscle larvae or as synthetic carbohydrate antigens produced commercially.

A. INTRODUCTION

Trichinella infection in food animals is important because of the risk of trichinellosis in humans who eat raw or undercooked meat. The adults of Trichinella sp. may be found in the small intestine of humans, pigs, rats, bears, walruses and many other flesh-eating mammals, but may also occur in horses, birds and crocodiles. The parasite has a direct life cycle. The females are ovo-viviparous. The larvae are shed into the lacteals, enter the lymph, reach the venous blood, and though many die out, others survive to settle in voluntary muscles, especially those of the diaphragm and tongue in pigs and tongue and masseters in horses. Larvae encyst in muscle tissue and ingestion of tissue containing these larvae transmits infection to another host.

Eight species of Trichinella are recognised (19, 23, 24). Trichinella spiralis (also called T-1) is distributed in temperate regions world-wide and is commonly associated with domestic pigs. It is highly infective for pigs, mice and rats. Trichinella nativa (T-2) and its subspecies, Trichinella T-6 found in North America, is a cold-climate-adapted species. It has limited infectivity for pigs, but is commonly found in wild canids, bear, and walrus; it is further distinguished by its resistance to freezing. Trichinella britovi (T-3) is found predominantly in wild animals, although it may occasionally be found in pigs or horses. It occurs in temperate regions of Europe and Asia. Trichinella britovi and its related genotypes, Trichinella T-8 from Southern Africa and Trichinella T-9 from Japan, have some intermediate characteristics of other species, including some resistance to freezing, moderate infectivity for swine and slow capsule formation (larvae have been confused for non-encapsulating species in some cases. Trichinella murrelli (T-5) is a North American species found in wildlife and occasionally horses and humans. It has very low infectivity for domestic pigs, but poses a risk to humans who eat game meats. Trichinella nelsoni (T-7) has been isolated sporadically from wildlife in Africa. It is characterised by greater resistance to elevated temperatures as compared with other species of Trichinella. Three species of Trichinella do not form a collegen capsule in muscle. Trichinella pseudospiralis (T-4) is cosmopolitan in distribution and has been recovered from raptorial birds, wild carnivores and omnivores, rats and marsupials in Asia, North America, Europe and the Australian subcontinent. Trichinella papuae (T-10) is a second species that does not form a capsule. To date, it has only been reported in wild pigs and humans from Papua New Guinea. It is resistant to freezing, has low infectivity for pigs, is found in a variety of wild mammals and has been implicated in human disease. Trichinella zimbabwensis (T-11) has been recently discovered in crocodiles from farms in Zimbabwe. The species can infect pigs and rats. All species and genotypes of Trichinella cause disease in humans.

Human trichinellosis is a serious disease that can cause much suffering and may result in death. The worms may have little effect within the intestine, but severe signs and symptoms may result from the migration of the larvae and their presence in voluntary muscle. The disease is transmitted by eating infected meat that has not been sufficiently cooked (or otherwise made safe). Prevention of human infection is accomplished by meat inspection, by processing (cooking, freezing, or curing of meat), and by prevention of exposure of food animals to infected meat including uncooked food waste, rodents and other wildlife (10, 12). Game meats should always be considered a potential source of infection, and therefore game meats should be tested or cooked thoroughly. Trichinella found in game meats (mainly T. nativa, T-6 and to a lesser degree T. britovi) may be resistant to freezing and therefore frozen meat may still pose a public health risk.

B. DIAGNOSTIC TECHNIQUES

Testing methods for the detection of Trichinella infection in pigs and other species may be grouped as those that result in either: (a) the direct demonstration of the parasite in tissue samples or digests; or (b) the indirect demonstration of the parasite by the detection of specific antibodies using serological methods.
1. **Identification of the agent (the prescribed test for international trade)**

The direct demonstration of the parasites is limited to post-mortem inspection of carcasses. The sensitivity of direct testing methods depends on the amount of tissue examined and the site from which the sample was obtained. Current methods for testing by artificial digestion employing a 1-g sample (2, 3) have a sensitivity of approximately three larvae/g of tissue (9, 13); testing of a 5-g sample increases sensitivity to 1 larva/g of tissue. Direct methods will identify infected pigs, horses or other animals as early as 17 days after exposure, coincident with the time that muscle larvae become infective for a new host. Direct methods remain effective as long as muscle larvae remain viable. Where large quantities of tissue (up to 100 g) are available for digestion, the sensitivity of this test is greatly increased. The disadvantages of direct inspection methods, particularly by trichinoscopy, are the time and labour required.

Two methods are generally employed for the direct diagnosis of trichinellosis:

a) **The trichinoscope or compression method**

Trichinoscopy for the inspection of pork has been described elsewhere (2, 3). Samples are taken for examination from the pillars of the diaphragm (crus) and cut into at least 28 pieces, each about 2 × 10 mm in size. Alternative sites for tissue collection include the tongue, masseter and abdominal muscles, but larger samples are required for comparable sensitivity. (The predilection sites of muscle larvae vary with host species and have only been well established in pigs and horses. As a general rule, the tongue is one of the most infected muscles.) The tissues are compressed between glass plates (compressorium; Hauptner Herberholz, Solingen, Germany) until they become translucent. They are then examined for larvae through a specially constructed projection microscope, the trichinoscope, or a conventional stereo-microscope at ×15–40 magnification. Most larvae will appear coiled within an individual muscle fibre, and the muscle cell typically appears oval in shape as a result of the formation of the capsule. In heavy infections, multiple larvae may be seen in a single cell.

Non-encapsulating *Trichinella*, including *T. pseudospiralis*, *T. papuae* and *T. zimbabwensis* from crocodiles may be seen outside muscle cells and typically are not coiled, making the identification of these species by trichinoscopy quite difficult. Because of this limitation, and lower sensitivity as compared with artificial digestion methods, trichinoscopy and similar compression methods are not recommended for the routine examination of food animals and game for meat consumption.

b) **The digestion method**

Muscle tissue can be digested with artificial digestive fluid releasing live trichinae from the muscle. Digestion procedures are recommended within the European Union (EU) (2, 3), and EU Directives may be consulted for more complete details. Many other countries have similar legislation for the post-mortem inspection of pork (8) and horsemeat (4). The International Commission on Trichinellosis (ICT) also provides general guidance on the performance of artificial digestion methods (12).

The widely used ‘magnetic stirrer method for pooled samples’ can be employed in a variety of circumstances with a minimum of equipment. A general outline of the principles of this method is as follows:

- **Sampling**

Muscle samples are taken from the diaphragm pillars or tongue of pigs, or tongue or masseter muscles of horses; as a general rule, the tongue is one of the most infected muscles, other sites generally have lower numbers of larvae. Sample sizes can vary; individual samples of 100 g may be taken from one animal, or multiple samples may be collected from a number of animals to make a 100-g pool. The size of the samples that make up the latter will determine the sensitivity of the method. EU Directives require 1-g samples in 100-g pools for testing pig carcasses. In the United States of America (USA), 5-g samples are required for pigs and in Canada, 10 g per pig is tested. For testing pigs in endemic areas, 5-g samples are recommended by the ICT. For testing horsemeat, the EU requires at least a 5-g sample. For horses originating from endemic areas, a 10-g sample is recommended. Prior to testing, the samples are ground, blended or diced to facilitate digestion.

- **Digestion and recovery**

Each 100 g of tissue is digested in 1–2 litres of artificial digestive fluid containing 1% (w/v) pepsin (1/10,000 National Formulary) and 1% (v/v) hydrochloric acid (0.12 N final). The ground or diced sample is added to the digestion fluid and the mixture is stirred on a magnetic stirrer for 3 hours at 37°C (or for shorter periods of time at higher temperatures (e.g. 30–60 minutes at 44–46°C). At the conclusion of digestion, the digest is allowed to settle for 15–20 minutes and the upper two-thirds of the fluid is decanted. The remaining fluid and deposit are poured through a 355 µm (177–180 µm is also acceptable) mesh screen into a conical settling glass and allowed to settle for a further 15–20 minutes. The maximum possible supernatant fluid is
aspirated without disturbing the sediment: the latter is washed with warm (37°C) tap water and allowed to settle for another 15–20 minutes; if necessary, the washing step is repeated until the supernatant fluid is clear. The washed sediment is transferred to a 50-ml tube, allowed to settle, and aspirated down to a final volume of 10 ml. All 10 ml are poured into a gridded Petri dish and examined for *Trichinella* larvae with a dissecting microscope (×15–40 magnification). Alternatively, following digestion in 3 litres of artificial gastric fluid, the digest suspension may be poured into a 4 litre separatory funnel through a 177–180 µm sieve, the sieve is rinsed thoroughly into the separatory funnel with tap water and the suspension allowed to settle for 30 minutes (5). Then 125 ml is drained into a 500 ml separatory funnel, 375 ml of tap water is added, and the suspension is allowed to settle for an additional 10 minutes. Finally, 22–27 ml of sediment is released into a Petri dish and counted as previously described. This method has fewer steps, requires less time and seldom needs further clarification steps.

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 (for dead larvae). In case of doubt, worms should be viewed at higher magnification and further tissues should be examined. If the counts are high, appropriate dilutions must first be made. When larvae are detected in pooled sample digests, the entire procedure must be repeated on the individual samples comprising the pool in order to identify infections in individual carcasses.

Use of a thermostatically controlled tissue blender (Stomacher) shortens the time required for digestion to about 12–15 minutes. Details of other methods using a Stomacher Lab Blender 3500T (Seward, London, United Kingdom) or double separatory funnels (8) have been described (3) and an alternative protocol for the magnetic stirrer method for pooled samples, approved by the EU (84/319/EEC), can also be recommended (3).

### Quality assurance in digestion testing

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 International Commission on Trichinellosis (12) and elsewhere (5, 7) and should include regular use of proficiency panels (6).

### c) Polymerase chain reaction

Identification of the species/type of *Trichinella* recovered from muscle tissue can be valuable in understanding the epidemiology of the parasite in animals and in assessing the relative risk of human exposure. Specific primers have been developed that allow the differentiation of all known species and genotypes of *Trichinella* by polymerase chain reaction (PCR) (1, 25). Requests for speciation/typing of *Trichinella* isolates can be made through the OIE Reference Laboratories in Rome, Italy and Saskatoon, Canada (see Table given in Part 3 of this Terrestrial Manual).

Limited studies have shown that PCR can be used to detect larvae in the musculature of infected animals (26). However, this technology in its present form is not a practical method for routine testing of food animals.

### 2. Serological tests

The use of enzyme-linked immunosorbent assay (ELISA) to detect the presence of parasite-specific antibodies provides a rapid method that can be performed on blood or serum collected before or after slaughter. Infection levels as low as one larva/100 g of tissue have been detected by ELISA in pigs (11). 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 detection of *Trichinella* infection is the occurrence of a low rate of false-negative results in the case of infected animals. Such results are due to a lag in the kinetics of antibody responses in animals mildly or moderately infected with *T. spiralis* or infected with sylvatic *Trichinella* species. This slow rate of antibody production means that infected animals cannot be detected for several (3–5) weeks following exposure (9, 13). For this reason, serological testing is not recommended for individual carcass testing.

Serological responses in pigs persist for at least 6 months after infection with no decline (9), however, antibody has been reported to decline in horses within a few months following infection, coincident with a decline in muscle larvae (20). Thus, serological evaluation of *Trichinella* in horses may not have any value as horses harbouring hundreds of larvae per gram of muscle tissue can show a negative serology. Little is known of
antibody responses to *Trichinella* infection in game species. The quality of serum samples from game animals is of great importance for avoiding false positive reactions.

Several antigen preparations have been developed that provide a high degree of specificity for *Trichinella* infection in pigs and horses (11, 16, 24). In abattoir testing, the ELISA yielded less than 0.3% false-positive results and was nearly 100% sensitive in detecting infected pigs with more than one larva/g of tissue (21). The *T. spiralis* secretory (ES) antigens used in the ELISA are conserved in all species/types of *Trichinella* (22), and therefore infection may be detected in pigs or other animals harbouring any of the eight species. Serological tests other than ELISA (e.g. indirect immunofluorescence tests) lack specificity and are not suitable for detection of *Trichinella* infection.

- **Enzyme-linked immunosorbent assay**

  Diagnosis of *Trichinella* infection by ELISA can be accomplished by using secreted stichosome antigens collected from *T. spiralis* larvae (11). The antigens recognised in worm secretions consist of a group of structurally related glycoproteins with molecular weights of 45–55 kDa (14, 22). A synthetic carbohydrate antigen has also been used in ELISA, but this antigen is not yet widely available and has been shown to have a lower sensitivity (17).

- **Antigen production**

  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 those antigens that are recognised by *Trichinella*-infected animals. The species of *Trichinella* normally used for antigen preparation is *T. spiralis* or T-1. For purposes of standardisation, it is recommended that this species be used for ELISA testing in food animal species. 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 (18). Parasites to be used for antigen preparation may be maintained by serial passage in mice or rats.

  To prepare antigen for use in the ELISA (16), *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 3 hours 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. Secretory (ES) antigens thus recovered may be stored frozen for short periods at –20°C or for longer at –70°C; they comprise 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.

  The quality of antigen 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 20 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.

- **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 1/10 or 1/100 in wash buffer. Alternative sources of antibodies that may be used in place of sera include whole blood or tissue fluids (15). 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, e.g. a 1/1000 dilution of rabbit anti-swine IgG (0.1 mg/ml) produced by Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA. 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. However, the cut-off value is influenced by the breed of pig.

Commercial adaptations of the ELISA are available in a shorter format, taking less than 1 hour for completion.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

A vaccine for Trichinella infection in food animals is not practical. There are no biologicals required for direct detection methods. For indirect (serological) methods TSL-1 antigens must be used to ensure test specificity. These antigens may be obtained as secretory products recovered from in-vitro maintenance of muscle larvae (the muscle larvae are initially obtained ex vivo) or as synthetic carbohydrate antigens produced commercially.

REFERENCES


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NB: There are OIE Reference Laboratories for Trichinellosis (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.2.10.  

Q FEVER

SUMMARY

Query (Q) fever is a zoonosis that occurs in most countries. Humans acquire infection from animal reservoirs, especially from domestic ruminants. Q fever is a highly infectious disease, which is due to the proliferation of Coxiella burnetii, a small and pleomorphic bacterium measuring 0.3–1.5 µm long × 0.2–0.4 µm wide. As an obligate intracellular bacterium, C. burnetii can be grown only in embryonated eggs or cell cultures or, when necessary, in inoculated laboratory animals. It occurs in two antigenic forms: the pathogenic phase I, found in infected animals or humans, and the avirulent phase II, obtained by repeated passages in embryonated eggs or in cell cultures. Because this microorganism is extremely hazardous, handling viable C. burnetii must be done in facilities that meet the OIE requirements for Containment Group 3 pathogens.

In humans, Q fever occurs in either an acute form (self-limiting febrile episode, pneumonia, hepatitis) or a severe chronic form (endocarditis) following an early infection that may be passed 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 some countries, a vaccine is available for professionally exposed population groups.

The signs of Q fever in cattle include abortion, dead or weak offspring, retained placenta, metritis, and infertility. In small ruminants, Q fever is often associated with sporadic abortions or outbreaks of abortions followed by recovery without complications. Coxiella burnetii infection persists for several years, and is probably life long. Sheep, goats and cows are mainly asymptomatic carriers, but can shed massive numbers of bacteria at parturition, and intermittently in various secretions and excreta. Domestic animals, such as cats, rabbits, birds, etc., are also susceptible to infection and should be considered as possible sources of infection for animals and humans.

Identification of the agent: For laboratory diagnosis, samples can be taken from the placenta, vaginal discharges, and liver, lung or stomach contents of aborted fetuses, and from milk, colostrum and faeces.

The bacteria can be visualised in stained tissue smears using a microscope with an oil-immersion objective lens. Because of its acid–alcohol resistant, the bacteria can be stained by several methods: Stamp, modified Ziehl–Neelsen, Gimenez, Giemsa and modified Koster. This finding is presumptive evidence of Q fever, but coupled with serological tests, clinical findings and other infectious abortive agents, it may be sufficient to establish a diagnosis of the disease at the flock or herd level.

To date, demonstration of the agent by immunohistology using specific antibodies or by polymerase chain reaction (PCR) has proven to be more specific and sensitive than classical staining methods. Nonetheless, no specific antibodies for immunochemistry are available commercially, and PCR can be done in suitably equipped laboratories. PCR is considered a useful test for screening large numbers of samples and various types of samples. Furthermore, samples can be heat inactivated and therefore ensure the safety of laboratory personnel.

Coxiella burnetii can be isolated by inoculation of specimens into conventional cell cultures or embryonated chicken yolk sacs or laboratory animals. Inoculation of laboratory animals (guinea-pig, mouse, hamster) is helpful in cases requiring isolation from tissues contaminated with various microorganisms or in order to obtain phase I Coxiella antigens.

Serological tests: The diagnosis of Q fever often relies on serology. A number of tests can be used, particularly the indirect immunofluorescence test, the enzyme-linked immunosorbent assay,
Abortions in ruminants have usually been investigated to determine if Q fever is present as it may affect the health of humans or other animals. Diagnosis of Q fever, and other abortive diseases, made on the basis of abortion or fetal death (27). The infection is endemic in many areas leading to sporadic cases or explosive epidemics. Its incidence is probably greater than reported. The epidemiology of Q fever suggests that the disease affects mostly humans, cattle, sheep and goats (18, 21). 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 (49). *Coxiella burnetii* has now been placed in the Coxiellaceae family in the order Legionellales of the gamma subdivision of Proteobacteria (17). The complete genome sequencing of *C. burnetii* has now been achieved recently and confirms its systematic position (38). Unlike rickettsiae, *C. burnetii* produces a small, dense, highly resistant spore-like form that is highly stable in the environment, a trait that is important for transmission (22). This ability has been attributed to the existence of *C. burnetii* developmental cycle variants: large-cell variants (LCV), small-cell variants (SCV), and small dense cells (SDC) (10, 39). The SDC and SCV represent the forms of the bacteria likely to survive extracellularly as infectious particles. Another essential characteristic is that *C. burnetii* has two antigenic forms: the pathogenic phase I, isolated from infected animals or humans, and the avirulent phase II, obtained *in ovo or in vitro*. 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. The LPS phase variation is accompanied by a permanent chromosomal deletion that makes impossible cell reversion from phase II to phase I.

Q fever is a zoonosis. In humans, the infection has an acute and a chronic form (29). The acute forms commonly include a self-limiting febrile episode, pneumonia, and granulomatous hepatitis. The main clinical manifestation of chronic Q fever is endocarditis in patients with valvulopathies. In the absence of any appropriate antibiotic treatment, complications of the chronic form may be severe to fatal (6). Moreover, *C. burnetii* infection of pregnant women can provoke placentitis and often leads to premature birth, growth restriction, spontaneous abortion or fetal death (27). The infection is endemic in many areas leading to sporadic cases or explosive epidemics. Its incidence is probably greater than reported. The epidemiology of Q fever suggests that the infection is principally transmitted by inhalation of desiccated aerosol particles, and through contact with infected animals and their reproductive tissues (20, 21). Ingestion has been often suggested, particularly through the consumption of dairy products derived from contaminated raw milk, and even possibly following pasteurisation (9, 29, 44). Q fever is very rarely transmissible from person to person; whereas exposure during childbirth, through sexual transmission and blood transfusions are possible (23). In animals, in addition to respiratory and digestive routes, vertical transmission and sexual transmission can occur (16, 47). Arthropods, principally ticks, may be involved in Q fever transmission.

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, metritis and infertility (18). Nevertheless, serological responses in a given species or even the isolation of *C. burnetii* do not necessarily correlate with expression of the clinical disease (11, 18, 35). Indeed, the agent may persist in infected animals, be shed intermittently in milk, faeces, urine, and may be present in blood. The agent can be recovered in very high numbers in birth products (placenta, amniotic fluid and fetus), and non-pregnant animals are less of a risk. Domestic ruminants are considered the main reservoirs for *C. burnetii*, but cats, dogs, rabbits, birds, etc., have also been reported to be shedders involved in human contamination (14, 20, 21, 42). Thus, infected domestic and wild animals usually shed the agent with no outward signs of disease, and should be regarded as possible sources of infection for humans.

Abortions in ruminants have usually been investigated to determine if Q fever is present as it may affect the health of humans or other animals. Diagnosis of Q fever, and other abortive diseases, made on the basis of microscopy on clinical samples, coupled with positive serological results, is usually adequate for this purpose (18, 32, 34). The Q fever diagnostic is also required for epidemiological surveys of ‘at risk’ and suspected flocks...
in limited areas (following recent outbreaks in humans or animals), or for export purpose. However, identification of *C. burnetii* shedders and asymptomatic carriers is not currently practised (2).

Generally, when several animals are seropositive, an appropriate intervention is advised. The measures could be adapted according to the seroprevalence and epidemiological context. Proper pasteurisation of milk products must be ensured (44). The amount of agent can be reduced in the environment by regular cleaning and disinfection of animal facilities, with particular care of parturition areas, using 10% bleach. Pregnant animals must be kept in separate pens, and placentas and aborted fetuses must be removed quickly and disposed properly to avoid being ingested by dogs, cats or wildlife. Spreading manure from contaminated farms in suburban areas must be ensured (44). The amount of agent can be reduced in the environment by regular cleaning and disinfection of animal facilities, with particular care of parturition areas, using 10% bleach. Pregnant animals must be kept in separate pens, and placentas and aborted fetuses must be removed quickly and disposed properly to avoid being ingested by dogs, cats or wildlife. Spreading manure from contaminated farms in suburban areas must be ensured (44). The amount of agent can be reduced in the environment by regular cleaning and disinfection of animal facilities, with particular care of parturition areas, using 10% bleach. Pregnant animals must be kept in separate pens, and placentas and aborted fetuses must be removed quickly and disposed properly to avoid being ingested by dogs, cats or wildlife. Spreading manure from contaminated farms in suburban areas must be ensured (44). The amount of agent can be reduced in the environment by regular cleaning and disinfection of animal facilities, with particular care of parturition areas, using 10% bleach. Pregnant animals must be kept in separate pens, and placentas and aborted fetuses must be removed quickly and disposed properly to avoid being ingested by dogs, cats or wildlife.

Finally, it is important to remember that *C. burnetii* is extremely hazardous to humans, and laboratory infections are common. Because of its low infectious dose, resistance in the environment, and aerosol route of transmission, *C. burnetii* is considered a potential agent of bioterrorism (5). 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 Appendix I.1.6.1. of Chapter I.1.6. Human safety in the veterinary microbiology laboratory.

**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 diagnosis (6, 32, 34). Samples should be collected from aborted fetuses, placenta and vaginal discharges soon after abortion or parturition. Milk from the tank, individual milk or colostrum, and faeces samples can also be taken.

a) **Staining**

In a case of an abortion suspected of being caused by an infection, smears are prepared on microscope slides of placental cotyledon (33). Lung, liver and abomasal contents of the aborted fetus or vaginal discharge may be used in the same manner. These could be stained according to several rapid methods: Stamp, modified Ziehl–Neelsen, Gimenez, Giemsa, Macchiavello and modified Koster (8, 26, 32, 33, 35). For example, the Stamp staining method, which is very close to the modified Ziehl-Neelsen method, is performed with 2% basic fuchsin solution, followed by rapid decoloration 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). *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 due to their small size (0.3–1.5 µm long × 0.2–0.4 µm wide), 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 *Chlamydophila abortus* or *Brucella* spp. However, using the same staining procedure, *Chlamydomphila* have sharper outlines, are round, small and may resemble globules. *Brucella* 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, Chlamydomphila 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 (34). When biological staining is inconclusive, one of the other methods (below) may be used as a confirmatory test.

b) **Specific detection methods**

Detection of *C. burnetii* in samples can also be achieved by specific immunodetection (capture enzyme-linked immunosorbent assay [ELISA], immunohistochemistry), or DNA amplification (4, 6, 45). Immunohistochemistry may be used with paraffin-embedded tissues or on acetone-fixed smears (28). The method is an indirect immunofluorescence or immunoperoxidase assay using polyclonal *C. burnetii* antibodies, either a well characterised antiserum of human origin or a specific antiserum produced in laboratory animals (rabbit or guinea-pig). An anti-species (human, rabbit or guinea-pig) anti-IgG conjugate, labelled with fluorescein isothiocyanate (FITC) or peroxide, 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. Polymerase chain reaction (PCR) methods are currently under development, and PCR has been used successfully to detect *C. burnetii* DNA in cell cultures and...
biological samples. As the number of *C. burnetii* is likely to be lower in milk, colostrum, and faeces than in abortion material, PCR can be used for analysis of this large diversity of samples (2). This technique can be performed by suitably equipped laboratories using primers derived from the IS1111 gene coding for a transposase (accession number M80806), as the most popular employed (2). At least 19 copies of this gene are present in the genome (12). The other target genes to be used in the PCR for specific *C. burnetii* identification are: superoxide dismutase (sodB) gene (accession number M74242); comt encoding a 27 kDa outer membrane protein (accession number AB004712); and heat shock operon encoding two heat shock proteins (htpA and htpB) (accession number M20452). The recently developed real-time PCR provides an additional means of detection and quantification (43). There is an urgent need for the development of a molecular method for the assessment of bacterial viability, especially in milk samples. The development of a multiplex PCR constitutes another current challenge for screening all infectious abortive agents.

c) **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 (11). For example, a portion of placenta is homogenised in phosphate buffered saline (PBS) containing antibiotics (streptomycin 100–200 µg/ml and penicillin or gentamycin 50–100 µg/ml). After low-speed centrifugation, dilutions of the supernatant fluid are inoculated into 5-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 be used to confirm 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 strictly or facultatively intracellular bacteria, including *C. burnetii*. Such a method was described for *C. burnetii* in 1990 (6, 30). Suspensions of samples are inoculated into human embryonic lung (HEL) fibroblasts grown on a 1 cm² cover-slip within a shell vial. 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 HEL 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 anti-species conjugated to FITC. Cells of the remaining shell vial are harvested and transferred to 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 (41).

With heavily multi-contaminated samples, such as placentas, vaginal discharges, faeces, or milk, the inoculation of laboratory animals may be necessary. Mice and guinea-pigs are the most appropriate laboratory animals for this purpose (36). Following intraperitoneal inoculation with a dose of 0.5 ml per animal, body temperature and antibody status are monitored. This method should always 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* is done using immunoperoxidase staining of the collected spleens. Alternatively, PCR may be performed on spleens systematically collected 7–9 days post-inoculation (4).

Although characterisation of isolates seems necessary for understanding the varying epidemiology of Q fever in different geographical areas, no discriminatory typing methods are available currently. For this purpose, efforts should focus on genetic typing methods.

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 (CF) test (6). Three older serological tests are no longer used in routine diagnosis: the microagglutination technique, the capillary agglutination test

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¹ Sterilin, Bibby Sterilin Ltd, Stone, Staffordshire ST15 0SA, United Kingdom.
and the indirect haemolysis test. A high-density particle agglutination (HDPA) test has been evaluated (24). Serological assays are suitable for screening herds, but interpretation at the individual animal level can be difficult. Indeed, animals may remain seropositive for several years following an acute infection, some animals may shed C. burnetii and pose a risk for infection prior to the development of antibodies, and some infected animals seem not to seroconvert (1, 3, 4). Serological cut-off titres used to diagnose Q fever are given below; interpretation of the results requires at least ten animals (aborted or not). Both serological responses and bacterial evidence are necessary for establishing the presence of the infection.

a) Indirect immunofluorescence test

In human medicine, the IFA adapted as a microimmunofluorescence technique is the reference method for the serodiagnosis of Q fever (6, 46). The procedure can be adapted to perform an immunoperoxidase assay. Some commercial CF test antigens are suitable, but antigens prepared for the diagnosis in humans are preferred (6). This method of preparation has been demonstrated to yield antigens with the highest sensitivity for C. burnetii antibody detection. 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 (ATCC VR 615) 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 (46). In addition, antigen-spot slide wells may be purchased from a supplier providing the phase II form (OIE), 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 immunoglobulins.

• Antigen preparation

Antigen should only be prepared in facilities that meet the requirements for Containment Group 3 pathogens as outlined in Appendix I.1.6.1. of Chapter I.1.6. of this Terrestrial Manual.

Phase II C. burnetii Nine Mile (ATCC VR 615) are grown in confluent layers of Vero or L929 cells in 150 cm² culture flasks with minimal essential medium (MEM) supplemented with 2 mM L-glutamine and 4% fetal bovine serum. The infection is monitored by microscopic examination of Gimenez-stained cells scraped from the bottoms of the flasks. 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. Sodium azide is added as an antibacterial preservative at a final dilution of 0.1%. Antigen prepared in this manner is frozen at −20°C.

In order to obtain phase I antigen, mice are inoculated with C. burnetii Nine Mile 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 5–6 days of age, the microorganism is inoculated into the yolk sac of the embryonated eggs, which are harvested after death of the embryo at 12–15 days. 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 between 5 and 10 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 1.5% 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 the preparation is centrifuged for another 30 minutes. The resulting pellet is resuspended in 0.1% sodium azide and the suspension is used as antigen for the IFA.
added and centrifugation is repeated. The final suspension is diluted with PBS. Thiomersal is added as an antibacterial preservative at a final dilution of 1/10,000. 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 yolk passage (EP6).

Titration of antigen with at least three different known sera (with high, moderate and low titres, respectively) is sufficient to recover the appropriate dilution for further immunofluorescence tests.

- **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 ×40 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.

  The reaction is considered to be positive if there is obvious immunofluorescence at the 1/160 dilution and upwards. In human medicine, this method is used to determine antibodies against phases I and II in the IgG, IgM, and IgA fractions allowing acute and chronic Q fever to be differentiated. Rheumatoid factor absorbant is used for remove IgG before the determination of IgM and IgA. Screening of the sera is performed with phase II antigen, and positive sera are tested subsequently for the presence of the different classes of Ig directed against phases I and II antigens. However, neither phases I and II antibody responses nor Ig classes responses have been well studied in domestic animals.

  **b) Complement fixation test**

  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 CF test is specific but less sensitive than the ELISA or IFA (6, 25). Seroconversion is detected later by the CF test than by the IFA or ELISA, but CF antibodies can persist for long periods after illness, and the CF test gives excellent results for routine diagnosis at the flock level for abortive diseases (32, 34). The CF test is still largely used by many laboratories in many countries. This method often uses antigen in phase II prepared
from a mixture of two strains (Nine Mile and Henzerling)⁴. In France, this method has been standardised (AFNOR NFU47-006).

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.

*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. Results may vary from one antigen to another, and antigens prepared from various strains (ovine, bovine, human) or from indigenous strains should be preferred.

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 evolutive phase of the infection.

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⁴ Dade Behring, Marburg, Germany.
c) Enzyme-linked immunosorbent assay

This technique has a high sensitivity and a good specificity (6, 31, 48). It is easy to perform in laboratories that have the necessary equipment (a spectrophotometer) and reagents. The ELISA tends to replace the IFA and CF tests as the test of choice because it is convenient for large-scale screening and, particularly for veterinary diagnosis, as it is a reliable technique for demonstrating C. burnetii antibody in various animal species (13, 40). It requires a relatively pure antigen. Antibodies prepared for the CF test may be used for coating the plates. Ready-to-use kits are commercially available and can detect anti-phase II antibodies or both anti-phase I and II antibodies. ELISA kits able to distinguish anti-phase I and II antibodies are under development.

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 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 (OPD [orthophenylene diamine], ABTS [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)] for peroxidase); hydrogen peroxide.

Test procedure

1. 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.
2. 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.
3. Add the appropriate dilution of freshly prepared conjugate to the wells (0.1 ml per well).
4. Cover each plate and incubate as in step ii. Wash again three times.
5. Add 0.1 ml of freshly prepared chromogen substrate solution to each well (for example: OPD [0.1 mg/ml] in 0.1 M acetic acid, 0.2 M NaN₂HPO₄, pH 4.8, 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]).
6. Shake the plate; after incubation, stop the reaction by adding stopping solution to each well, e.g. 0.05 ml 3 M sulphuric acid for peroxidase or 10% sodium dodecyl sulphate for ABTS.
7. Read the absorbance of each well with the microplate reader at 405 nm (ABTS) or 492 nm (OPD). 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 (Abpos) and negative (Abneg) control sera, and for each serum, calculate the percentage:

\[
\frac{\text{Ab pos} - \text{Ab neg}}{\text{Ab pos} - \text{Ab neg}} \times 100
\]

Interpret the results as follows:
- Ab <30% negative serum
- Ab 30–40% doubtful serum
- Ab >40% positive serum

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5 CHEKIT-Q-Fever EIA kit, Intervet (Bommeli Diagnostics), Liebefeld-Bern, Switzerland.
6 Q fever IgG or IgM ELISA kits, PanBio Pty Ltd, Brisbane, Australia.
C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Vaccine

Vaccination is the most logical strategy for preventing Q fever in exposed subjects and livestock. A C. burnetii vaccine can only be prepared by trained staff only working in adequate conditions of protection (at a minimum in a biosafety level 3 laboratory). It is recommended to obtain the vaccine from manufacturers capable of completing and certifying tests for safety, inactivation and sterility.

Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 are intended to be general in nature and may be supplemented by national and regional requirements.

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 (19). Phase I vaccines are more 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, as the phase II vaccines are 100 times less effective against the colonisation of mouse spleen than phase I vaccines (7). An inactivated phase I vaccine is commercially available in Slovakia for vaccination of cattle. 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 during 10 years, together with improved veterinary control of domestic animal transport within the country (37).

This vaccine consists of highly purified antigen prepared from Nine Mile strain in the phase I (egg passage 2 to egg passage 6) and inactivated by formaldehyde. 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 (4). Ideally, vaccine efficacy must be demonstrated by tests on all the target species.

In the case of vaccination on already infected animals, there is a lack of information on possible adverse effects and on the shedding of the Q fever agent. Consequently, 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 (15). To date, no data are available for comparing the cost–benefit of this strategy with a nonselective strategy in the control of Q fever.

2. Diagnostic biologicals

See Section B.2.a. (Antigen preparation).

REFERENCES


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**CHAPTER 2.2.11.**

**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 infections with *L. tropica*, *L. major* and *L. braziliensis* have 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, in 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 now 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, no longer available commercially, 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 (38) and divided into three entities: visceral leishmaniosis (VL, kala azar), cutaneous leishmaniosis (CL, oriental sore, uta, pian bois, chiclero’s ulcer) and mucocutaneous leishmaniosis (MCL, espundia). In the New World¹, leishmanioses are caused by *L. braziliensis* complex (MCL and CL), *L. mexicana* complex (CL), *L. peruviana* (CL) and *L. chagasi* (VL and CL); in the Old World, leishmanioses are caused by *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 (20). The diseases are mainly zoonoses with a few exceptions. Canine leishmaniosis (CanL) is a chronic visceral-cutaneous disease caused by *L. infantum* (= *L. chagasi*), of which the dog acts as the source reservoir. In some instances, parasites belonging to *L. braziliensis* complex, *L. major* and *L. tropica* have been isolated from this host (27). The vectors of leishmanioses are phlebotomine sandflies belonging to the genera *Lutzomyia* (New World) and *Phlebotomus* (Old World).

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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.
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 (23). 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 invertebrate hosts 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 (34). **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 in strict sterile conditions; hence, it is not always 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 (7). 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 (Evan’s modified Tobie’s medium) should be used. For bulk cultivation of established isolates, suitable media are reported in Section B.1.a. (see ref. 7 for media composition). The organisms from patients with VL and MCL can be very difficult to cultivate. Sometimes, even when the initial isolation is successful, the parasites may die when subcultured. This seems especially common when the initial isolation has been 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 (27). 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 viscerotropic 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** is the most commonly used method (1, 13, 27, 34). This technique requires a large number of parasites (5 × 10^9–1 × 10^10). 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.

b) The monoclonal antibody (MAb) technique is applied to the analysis and classification of both New and Old World *Leishmania* species and subspecies (13, 27, 34). 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) Kinetoplast DNA restriction endonuclease analysis is based on the analysis, through gel electrophoresis, of fragments generated by endonucleases on the abundant mitochondrial DNA, which constitutes the kinetoplast (10, 13, 15, 27, 32). The electrophoretic pattern obtained, known as a restriction ‘fingerprint’, is a marker at the organelle genotype level that allows the identification and classification of *Leishmania* strains into schizodemes – populations having similar kinetoplast DNA sequences. This technique also requires a large number of parasites \((1 \times 10^{10})\).

d) DNA hybridisation probes are a promising 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 (13, 33). Only complementary DNA sequences will form double-stranded DNA, which can be detected by autoradiography if the probe is radiolabelled, 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.

e) Polymerase chain reaction (PCR)-based methods are now available for diagnosis and/or identification of *Leishmania* from human and canine samples. Essentially, techniques developed either to detect organisms from fresh or frozen 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 selected from the small-subunit rRNA gene as primers (19), kinetoplast DNA minicircles (16) or other highly repetitive genomic DNA sequences (25); (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 (88%), declining thereafter (50%) (26). In American CL and MCL, PCR appears to be consistently more sensitive than any previously recommended method of diagnosis (5).

2. Serological tests

Several serological tests are now used for detecting anti-leishmanial antibodies (2, 12, 13, 29). 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 (9).

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.3. 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 enzyme-linked immunosorbent assay (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**
  - 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).
  - 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 $4 \times 10^6$ 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, in CanL it ranges from 1/40 to 1/60. 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, which is called the Falcon assay screening test and enzyme-linked immunosorbent assay (FAST-ELISA) and 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 (2).

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%) (17).

The ELISA methods described above are all based on crude antigenic preparations. More recently, a recombinant antigen from a cloned protein of *L. chagasi*, 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 (3, 28). In HIV-positive patients, K39-ELISA showed higher sensitivity (82%) than the IFA test (54%) (14). 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 (4, 24). 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 (11, 12). 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) **Counter immuno-electrophoresis (CIEP)**

The general principle of this test is that the movement of most of the protein molecules is towards the anode, except for gamma-globulins, which move towards the cathode. This property is used in the counter immuno-electrophoresis (CIEP) test. The antibody moves towards the cathode under electroendosmotic flow and negatively charged antigen moves towards the anode and precipitates on contact with antiserum. The procedure is as follows: 3–5 ml of agarose is poured on to slides, and wells are punched when the agarose has set. Serum is placed in the anodal well and leishmanial antigen, prepared as for ELISA, in the cathodal well. The slide is placed in an electrophoresis tank filled with barbitone buffer, pH 8.2, and connected to the buffer chambers with filter-paper strips. A current of approximately 8 mAmp (milli-Ampere) is applied and after 2–3 hours the slide is examined for precipitation lines. The CIEP test is a reliable diagnostic test and in CanL has given better results than ELISA.

### 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 (18). 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. There is complete cross-reactivity among all strains of Leishmania, although heterologous antigens often give smaller reactions, but this may be caused by difficulty in standardisation. It 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 no longer 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 leishmaniae. This type of immunisation has been practised on a limited scale in hyperendemic areas of CL (caused by *L. major*) in Israel, Iran and the former Union of Soviet Socialist Republics (31). The immunising preparations consist of living, virulent and infective promastigotes. However, a killed *L. major* vaccine plus bacille Calmette-Guérin (BCG) against zoonotic and anthroponotic cutaneous leishmaniosis has recently undergone randomised, double-blind controlled trials in Iran (22, 30), but result have been disappointing.

*Leishmania major* causes cross-protection against *L. tropica*, but the reverse is not true. However, this species cannot be considered to be totally safe and may convert the immunised host into a reservoir that will be a potential source of infection as long as the lesion remains unhealed. Therefore, 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. It takes approximately 3 months before immunity is acquired. Standardisation and quality control of such vaccines, presently not available, are urgently needed. At present, a number of promising anti-leishmanial vaccines are under
development (8). First generation vaccines consist of killed Leishmania organisms mixed with a low concentration of BCG as adjuvant. Different preparations using either whole (autoclaved) or ultrasonic-disrupted promastigotes are currently undergoing phase I–II and phase III trials for immunisation against CL Leishmania agents in humans and against VL in humans and in dogs (6, 21). Therapeutic candidate subunit vaccines, consisting of several cloned antigens, one of which is a powerful adjuvant, have recently been used to treat drug-resistant mucosal L. braziliensis in humans in Brazil.

Second generation vaccines, which are currently at the predevelopment stage, consist of genetically reconstructed Leishmania parasites incapable of producing disease, recombinant molecules or their corresponding DNAs, or recombinant organisms carrying leishmanial genes and expressing parasite antigens.

2. Immunodiagnostic antigens

Neither the leishmanin used for skin tests nor the antigens employed in serodiagnosis in leishmaniosis are internationally standardised. 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

The commercial antigens for the IFA tests and ELISAs that 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 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 and the CIEP test a soluble form of the antigen is needed.

3. Seed management

a) Characteristics of the seed

Strains of 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 (27, 34, 38). 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 World Health Organization (WHO) Collaborating Centres in London, UK, Montpellier, France and Jerusalem, Israel. A list of Identification Centres has been published by WHO (38).

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) (34). 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 I.1.6. Human safety in the veterinary microbiology laboratory).

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 (36, 37). 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 (35). 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.

An international standard preparation has yet to be established. The Leishmaniosis Section of WHO’s Tropical Disease Research Programme is funding programmes aimed at reaching a satisfactory standardisation of leishmanin production.

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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SECTION 2.3.

BOVINE DISEASES IN LIST B

CHAPTER 2.3.1.

BOVINE BRUCELLOSIS

SUMMARY

Bovine brucellosis is usually caused by Brucella abortus, less frequently by B. melitensis, and rarely 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 recently developed 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, testes or epididymes. Species and biovars should be identified by phage lysis, and by cultural, biochemical and serological criteria. Molecular methods have recently been introduced as additional biotyping methods.

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 confirmed using an established confirmatory 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, and each batch must conform to minimum standards for viability, smoothness, residual virulence and ability to immunise mice against challenge with a virulent strain of B. abortus. Brucella abortus strain RB51 vaccine was produced from a laboratory-derived rough mutant of smooth B. abortus strain 2308. However, its efficiency in cattle and its innocuousness remain controversial. 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. Occasionally, B. suis may cause an infection in the mammary gland of cattle, but it has not been reported to cause abortion (16). The disease is usually asymptomatic in nonpregnant females. Following infection with B. abortus or B. melitensis, pregnant adult females develop a placentitis 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 cistic 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 also been reported in the one-humped camel (Camelus dromedarius) and in the two-humped camel (C. bactrianus), 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 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. 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. There is an occupational risk to veterinarians and farmers who handle infected animals and aborted fetuses or placentae. 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 safety precautions to be observed with Brucella-infected materials (for further details see refs 1, 28, 67, 68 and Chapter I.1.6. Human safety in the veterinary microbiology laboratory). 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 I.1.6., to minimise occupational exposure. At least containment level 3 is also recommended for handling large volumes of Brucella.

Genetic and immunological evidence indicates that all members of the Brucella genus are closely related and it has been proposed (but not yet accepted by the Taxonomy Subcommittee) that the genus contains a single species of which the classical species (abortus, melitensis, etc.) would be mere biovars (for a review see ref. 36). Nevertheless, there are real differences in host preference and epidemiology displayed by the major variants, as well as, molecular evidence of genomic variation. From a practical point of view, it is convenient to maintain the classification into the six classical nomenclature: Brucella abortus, B. melitensis, B. suis, B. neotomae, B. ovis and B. canis. The first three of these are subdivided into biovars based on cultural and serological properties (see Tables 1 and 2 at the end of this chapter). 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 the genus and it is proposed that they could be classified into two new species, B. cetaceae and B. pinnipediae (10, 18). 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).

B. DIAGNOSTIC TECHNIQUES

All abortions in cattle 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 and bacteriological methods is usually needed.
1. Identification of the agent (1, 28)

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 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 conjugation based technique could also be used (53). 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 currently under development can be used to demonstrate the agent in various biological samples (8).

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 nonsmooth 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, tryptase (or tryptone)—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 (1). 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 (17), which is prepared by the addition of six antibiotics to a basal medium. The following quantities are added to 1 litre of agar: polymixin 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, could have inhibitory effects on some *B. melitensis* strains (34). Therefore the sensitivity for *B. melitensis* isolation 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 collistin 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]) (34). 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. Enrichment can be carried out in liquid medium consisting of serum—dextrose broth, tryptase (or tryptone)—soy broth 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
Collection and culture of samples
directly into a sterile vessel. Care must be taken to avoid contact between the milk and the milker's hands. The milk is centrifuged at 2000 g for 15 minutes in sealed tubes (to avoid the risk of aerosol contamination of personnel), and the cream and deposit are spread on solid selective medium, either separately or mixed. If brucellae 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 in mice. This work must be carried out under appropriate biosecurity conditions as outlined in Chapter I.1.6. 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 nonsmooth 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 & Boneestell, or White & Wilson’s crystal violet method of staining colonies (1).

Identification of *Brucella* organisms can be carried out by a combination of the following tests: organism morphology and 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 A-, M- or R-specific antisera), 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 rough species 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 at the end of this chapter).

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 (25). These regulations are summarised in Chapter I.1.1. Sampling methods, and they must be followed. As *Brucella* cultures are infectious agents, they are designated UN2814 and a Declaration of Dangerous Goods must be completed. The requirements for submitting samples from suspected cases of brucellosis are similar and the IATA regulations should be reviewed before sending samples. Other international and national guidelines should also be followed (1, 28, 67, 68).

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 (see Appendix I.1.6.1. of Chapter I.1.6. International transfer and laboratory containment of animal pathogens).

d) Nucleic acid recognition methods

The recently developed PCR provides an additional means of detection and identification of *Brucella* sp. (8). 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 refs 8 and 36). Pulse-field gel electrophoresis has been developed that allows the differentiation of several *Brucella* species (26, 35). However none of these methods has been fully evaluated and standardised and none is widely available.

e) 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 (1).

*Brucella melitensis* strain Rev.1 has the normal properties of a biovar 1 strain of *B. melitensis*, but grows much more slowly on ordinary 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) (1, 11, 12).
Brucella abortus strain RB51 is identified by several characteristics; these are: rough morphology, growth in the presence of rifampicin (250 µg per ml of media), and inability to produce O-polysaccharide (OPS) (56). The inability to produce OPS can be demonstrated by reacting RB51 colonies with OPS-specific monoclonal antibodies (Mabs), in dot-blot assays or Western blots (53, 56). An indirect way of demonstrating lack of OPS is by injecting $4 \times 10^8$ viable RB51 organisms into BALB/c mice and testing for the induction of OPS-antibodies; the serology will be negative (56).

Vaccine strains S19, Rev.1 and RB51 may be identified using specific PCRs (8, 9, 55, 63).

2. Serological tests

No single serological test is appropriate in each and all epidemiological situations, all have limitations especially when it comes to screening individual animals (22, 42). 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. 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 (44, 69). 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 strategy.

In other species, for example, buffaloes (Bubalus bubalus), American and European bison (Bison bison, Bison bonasus), yak (Bos grunniens), elk/wapiti (Cervus elaphus), and camels (Camelus bactrianus and C. dromedarius), Brucella sp. infection follows a course similar to that in cattle. The same serological procedures may be used for these animals (40), but each test should be validated in the animal under study (19, 20).

- **Reference sera**

Primary bovine 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 (69):

- For RBT and CFT, the OIE International Standard Serum (OIEISS, previously the WHO Second International anti-Brucella abortus Serum) that contains 1000 IU and ICFTU (international complement fixation test units) is used.

- 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 (OIEELISANSS) standard.

- **Production of cells**

Brucella abortus strain 99 (Weybridge) (S99) (see footnote 1 for address) or 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 B. abortus strains is also used to test for B. melitensis or 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\(_2\)-independent strains of B. abortus.

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1. Obtainable from the OIE Reference Laboratory for Brucellosis at Veterinary Laboratories Agency (VLA) 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.
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.

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 (24), 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 must 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 g for 75 minutes.

a) Buffered *Brucella* antigen tests (prescribed tests for international trade)

- **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 (37).

- **Antigen production**
  Antigen for the RBT is prepared by depositing killed *B. abortus* S99 or S1119-3 cells by centrifugation at 23,000 g for 10 minutes at 4°C, and uniformly resuspending in phenol saline at the rate of 1 g 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 distilled water is added, and the mixture is stirred for 2 hours at room temperature. The mixture is filtered through 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 phenol saline, followed by 95 ml of lactic acid, and adjusted to 1056 ml with 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 never 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. 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 due to S19 vaccination or due to false-positive serological reactions (FPSR). Therefore positive reactions should be investigated using suitable confirmatory 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 a given time. 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 (2).

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*

Serum (80 µl) is mixed with a 30 µl volume of antigen on a glass plate marked in 4 × 4 cm squares. 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. The plate should be removed and rotated as above, and then returned for a second 4-minute incubation. At this point, the plate should be removed, rotated, and observed for agglutination (1). Any visible reaction is considered to be positive. Like the RBT, the test is very sensitive, especially for detection of vaccine-induced antibody, and positive samples should be retested using a confirmatory 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 a widely used and accepted 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 2.5% 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'H 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.

**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 (1, 24) 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 second 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.

ii) Volumes of 25 µl of antigen, diluted to working strength, and 25 µl of complement, diluted to the number of units required, are added to each well except in the first row.

iii) Control wells containing diluent only, serum + complement + diluent, antigen + complement + diluent, 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.
iv) 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.

v) 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.

vi) 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: $1000 \times \frac{1}{200} \times$ 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 OIEISS as described above and the results expressed in ICFTU/ml.

The CFT is 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 strategies. Females that have been vaccinated with *Brucella 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, antoglobulin-enzyme conjugates, and substrate/chromogens. 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 assay 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 (70). The threshold should be established in the test population using appropriate validation techniques (see Chapter I.1.3. Principles of validation of diagnostic assays for infectious diseases).

The I-ELISA is a highly sensitive test but it is sometimes not capable of differentiating between antibody resulting from S19 vaccination or other FPSR problems and that induced by pathogenic *Brucella* strains. The I-ELISA should therefore be considered more as a screening test than a confirmatory test in the testing of vaccinated cattle or herds affected by FPSR problems.

As far as the antigen is concerned, preparations rich in smooth lipopolysaccharide (sLPS) should be used. There are several protocols for preparing a suitable antigen.

Monoclonal or polyclonal antoglobulin-enzyme conjugates may be used depending on availability and performance requirements. An MAb specific for the heavy chain of bovine IgG1 may provide some improvement in specificity at the possible cost of some loss of sensitivity.

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.
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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 [0.2 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, 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 (4.6 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. abortus* S1119-3 or S99 is prepared 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 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 LPS 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)**

i) 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 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 a year. Frozen plates are thawed for 30–45 minutes at 37°C before use.

ii) 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 ethylene diamine tetra-acetic acid (EDTA) and ethylene glycol tetra-acetic acid (EGTA) (PBST/EDTA) are added to specified wells and incubated at ambient temperature for 30 minutes.

iii) 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.

iv) 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 IgG1 conjugated with HRPO and diluted in PBST 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 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.

vi) 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.²

Using this or another similar I-ELISA protocol calibrated against the OIE ELISA Standard Sera described above, the diagnostic sensitivity should be equal to or greater than the BBATs in the testing of infected cattle, and the diagnostic specificity should be equivalent to the CFT in the testing of unvaccinated cattle (22). It can be expected that the diagnostic specificity in the testing of S19 vaccinated cattle or in the case of FPSR will be significantly lower than for the CFT depending on where the I-ELISA positive/negative threshold is set.

**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 than the I-ELISA (33, 45, 58). This is accomplished by 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 (41, 66). 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. 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).

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 LPS 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 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 H₂O₂ and 4 mM ABTS) are added to each well, the plates are shaken for 10 minutes and 3 Obtainable from the OIE Reference Laboratory for Brucellosis at the Animal Diseases Research Institute, 3851 Fallowfield Road, Nepean, Ontario K2H 8P9, Canada.
d) Fluorescence polarisation assay (an alternative 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. At the time of publication, the FPA had been proposed as a prescribed test for international trade. Please consult the OIE Web site for the most recent version of this chapter.

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. Thus 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 B. abortus 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) using a fluorescence polarisation analyser (43).

The FPA can be performed in glass tubes or a 96-well plate format. The bovine serum is diluted 1/100 or, if EDTA-treated blood is used, 1/50 (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. 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 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 sulfoxide and a further incubation at 37°C for 1 hour. The conjugated OPS is applied to a 1 x 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.
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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).

- 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. 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% (19, 20, 43). However the specificity of FPA in FPSR conditions is currently unknown. The FPA should be standardised such that the OIE strong positive and weak positive sera consistently give positive results.

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 brucellin skin test has a very high specificity, such that serologically negative unvaccinated animals that are positive reactors to the brucellin test should be regarded as infected animals (15, 51). Also, results of this test may aid the interpretation of serological reactions thought to be FPSR due to infection with cross-reacting bacteria, especially in brucellosis-free areas (51, 54).

However, 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

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4 Brucellergène OCB®, Synbiotics Europe, 2 rue Alexander Fleming, 69007 Lyon, France.
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.

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 (21, 32, 46).

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 OIEISS contains 1000 IUs of agglutination. The antigen shall be prepared without reference to the cell concentration, but its sensitivity must be standardised in relation to the OIEISS in such a way that the antigen produces either 50% agglutination with a final serum dilution of 1/600 to 1/1000 or 75% agglutination 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 polyB tests

Native hapten and polyB tests are confirmatory tests that have been used successfully in an eradication programme in combination with the RBT as a screening test (3). The optimal sensitivity is obtained in a reverse radial immunodiffusion (RID) system in which the serum diffuses into a hypertonic gel containing the polysaccharide (14). However, the double gel diffusion procedure is also useful (30, 31). 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 (29). The conjunctival vaccination (both in young and adults) reduces the time to obtain a negative response in native hapten and polyB tests. A remarkable characteristic of the RID test is that a positive result correlates with *Brucella* shedding as shown in experimentally infected cattle (29) and in naturally infected cattle undergoing antibiotic treatment (27).

d) Milk tests

An efficient means of screening dairy herds is by testing milk from the bulk tank. 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 to that described for serum. The C-ELISA should not be used to test whole milk but may be used with whey samples.

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5 The detailed procedure could be obtained from the Departamento de Sanidad Animal, Servicio de Investigacion Agraria/DGA, Apartado 727, 50080 Zaragoza, Spain.
• **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. 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 OIE ISS so that a 1/500 dilution is positive and 1/1000 dilution is negative. The antigen must be stored at 4°C and not frozen.

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. 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). 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. 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. 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. The test is considered to be negative if the colour of the underlying milk exceeds that of the cream layer.

C. **REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS**

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.
Chapter 2.3.1. – Bovine brucellosis

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 (1)

*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, nontoxic 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.

2. Method of manufacture (1)

*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 I.1.5.

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 brucellin in Freund’s complete adjuvant from 1 to 6 months previously. The erythematous reactions are read and measured at 24 hours and the titre is calculated by comparison with a reference brucellin. 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 (1).

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.

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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, AFSSA, 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, AFSSA, BP67, 94703 Maisons-Alfort Cedex, France.
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 $5 \times 10^{10}$ viable organisms. A reduced dose of from $3 \times 10^8$ to $3 \times 10^9$ 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. Alternatively, it can be administered to cattle of any age as two doses of $5 - 10 \times 10^9$ 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.

*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. However its efficacy compared with S19 and its innocuousness remain controversial (38, 39, 57). 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 $1 - 3.4 \times 10^{10}$ 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 $1 \times 10^9$ viable strain RB51 organisms. 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.

It has been reported that full doses of RB51 when administered intravenously in cattle induce severe placentitis and placental infection in most vaccinated cattle (49), 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 placentitis lesions are produced in subcutaneously vaccinated cattle (50), but the vaccine strain can be shed by a significant proportion of vaccinated animals (61). However, this reduced dose does not protect against *B. abortus* when used as a calfhood vaccination (47), but does protect when used as an adult vaccine (48).

It should be emphasised that, as well as S19, RB51 could infect humans (65). The RB51 strain is highly resistant to rifampicin, one of the antibiotics of choice for treating human brucellosis. In addition, the diagnosis of the infection produced by RB51 requires special tests not available in most hospitals. The Centers for Disease Control, Department of Health and Human Services, Atlanta, Georgia, USA (CDC) established passive surveillance for accidental inoculation with the RB51 vaccine in the USA to determine if this vaccine is associated with human disease. This study included 26 participants that had been exposed to the vaccine during animal vaccination. Accidental human exposure resulted in both local and systemic adverse events; however, it remains undetermined if strain RB51 vaccine can cause systemic brucellosis in humans. The number of reported adverse event case-patients in this study (twenty-six) is small compared to the number of vaccination events (several million calves vaccinated), and estimated inadvertent RB51 inoculations predicted (8 per 11,000). The report indicated that appropriate antibiotic use should protect against infection, but it remains undetermined to what degree the organism versus other vaccine components contribute to the adverse events (60). This is in contrast to Strain 19 where development of undulant fever caused by accidental exposure is well documented to occur without preventive treatment.

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 (64). 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 only one report related to this issue that demonstrated that S19 is able to control *B. melitensis* at the field level (27). By contrast, no experiments have been conducted showing the efficacy of Rev.1 against *B. melitensis* infection in cattle. Moreover, the safety of this vaccine is practically unknown in cattle (62).
Until 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. 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 (1).

   *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 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 femoro-tibial joints (7, 13). The vaccine is safe for most animals if administered to calves between 3 and 8 months of age. It may also be used in adult animals at a reduced dose. It produces lasting immunity to moderate challenge with virulent *B. abortus* strains, but the precise duration of this is unknown. The length of protection against *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 concluded the value of *B. abortus* strain RB51 in protecting cattle from brucellosis. The organism is attenuated in calves and adults. As *B. abortus* strain RB51 contains minimally expressed sLPS and there is no serological conversion against sLPS in vaccinated animals. In addition, RB51 does not induce detectable antibodies, using current testing procedures, to the OPS antigen (59). It 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 in vivo or 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 $24 \times 10^8$ 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 \( B. \) 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 \( 50 \times 10^9 \) per standard 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 \( 1–3.4 \times 10^{10} \) 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 I.1.5.

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.

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 (6). Batches should also be checked for the number of viable organisms.

- **Identity**

The reconstituted S19 vaccine should not contain extraneous microorganisms. \textit{Brucella abortus} present in the vaccine is identified by suitable morphological, serological and biochemical tests and by culture: \textit{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 acriflavin agglutination test (1). 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) (6, 23)

  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 10^9 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 (10^8 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) (23). 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 ref. 4). 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 (52). Alternatively, the statistical calculations described in steps vi) to viii) can be avoided by an easy-to-use specific HTML-JAVA script program (Rev2) recently developed (52) and available free at: http://www.afssa.fr/interne/Rev2.html.

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 (5, 6)

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 $10^5$ 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 $10^6$ 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 $2 \times 10^5$ 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

There is no standardised potency test on *B. abortus* strain RB51 vaccine and such a test is not routinely carried out. A test in Balb/c female mice using $1 \times 10^4$ *B. abortus* strain 2308 organisms as the challenge strain has been proposed, but the usefulness of this test in predicting protection in cattle is questionable. In the USA plate counts of viable organisms have been approved and used.
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.

Vaccination of calves with B. abortus strain RB51 is believed to stimulate an immunity that will last for a period similar to the one induced with S19, although there are no specific studies to demonstrate this.

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 has its wboA gene disrupted by an IS711 element impeding synthesis of OPS. Unpublished data indicate that it also contains a second mutation affecting the export of OPS to the bacterial surface or the coupling of OPS to the core of the LPS, or both.

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. The 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; however, the CDC will provide treatment recommendations. If S19 contamination occurs, a combined treatment with doxicycline plus rifampicin could be recommended. In the case of contamination with RB51 (a rifampicin-resistant strain), the treatment with rifampicin should be avoided.

5. Tests of the final product

a) Safety

See Section C2.4.b.

b) Potency

For the lyophilised vaccine, potency must be determined on the final product. The procedure is as described in Section C2.4.c.

REFERENCES


brucellosis when aspecific serological reactions occur in the course of brucellosis testing. 


* * *

NB: There are OIE Reference Laboratories for Bovine brucellosis (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
Table 1. Differential characteristics of species of the genus Brucella

<table>
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<th>Serum requirement</th>
<th>Lysis by phages</th>
<th>Preferred host</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tb</td>
<td>Wb</td>
</tr>
<tr>
<td>B. abortus</td>
<td>S</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<tr>
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<td></td>
</tr>
<tr>
<td>B. suis</td>
<td>S</td>
<td>–</td>
<td>+</td>
<td>+&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>B. melitensis</td>
<td>S</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B. neotomae</td>
<td>S</td>
<td>–</td>
<td>–&lt;sup&gt;k&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>B. ovis</td>
<td>R</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B. canis</td>
<td>R</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

From refs 1, 28.

- Phages: Tbilisi (Tb), Weybridge (Wb), Izatnagar1(Iz1) and R/C
- Normally occurring phase: S: smooth, R: rough
- RTD: routine test dilution
- <sup>a</sup> Brucella abortus biovar 2 generally requires serum for growth on primary isolation
- <sup>b</sup> Some African isolates of B. abortus biovar 3 are negative
- <sup>c</sup> Intermediate rate, except strain 544 and some field strains that are negative
- <sup>d</sup> Some isolates of B. suis biovar 2 are not or partially lysed by phage Wb or Iz₁
- <sup>e</sup> Rapid rate
- <sup>f</sup> Some isolates are lysed by phage Wb
- <sup>g</sup> Slow rate, except some strains that are rapid
- <sup>h</sup> Minute plaques
- <sup>i</sup> Neotoma lepida

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### 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>
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</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>
</tr>
<tr>
<td></td>
<td>3</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></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. abortus</td>
<td>4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td></td>
<td>6</td>
<td>–</td>
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<td>+</td>
<td>+</td>
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<td>9</td>
<td>+ or –</td>
<td>+</td>
<td>+</td>
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<td>1</td>
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<td>–</td>
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<td>2</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>B. suis</td>
<td>3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>–</td>
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<td>+</td>
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<td></td>
<td>5</td>
<td>–</td>
<td>–</td>
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<td>+</td>
</tr>
<tr>
<td>B. neotomae</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<td>B. ovis</td>
<td>–</td>
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<td>–</td>
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</tr>
<tr>
<td>B. canis</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

From refs 1, 28.

- **a** Dye concentration in serum dextrose medium: 20 µg/ml
- **b** Usually positive on primary isolation
- **c** Some strains are inhibited by dyes
- **d** Some basic fuchsin resistant strains have been isolated
- **e** Negative for most strains
- **f** Growth at a concentration of 10 µg/ml thionin
CHAPTER 2.3.2.

BOVINE GENITAL CAMPYLOBACTERIOSIS

SUMMARY

Bovine genital campylobacteriosis is a venereal disease characterised by infertility, early embryonic mortality, and abortion. The causal agent of this sexually transmissible disease is Campylobacter fetus subsp. venerealis. The prepuce of healthy carrier bulls is the natural reservoir of this bacterium. Campylobacter fetus is divided into two subspecies: C. fetus subsp. venerealis and C. fetus subsp. fetus.

Bovine genital campylobacteriosis 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, but the presence of C. fetus subsp. venerealis in the semen of chronic carrier bulls creates the risk of spread of the disease through artificial insemination. Campylobacter fetus subsp. fetus can be recovered from the intestinal tract of cattle and other animal species. Its pathogenic role is minor compared with C. fetus subsp. venerealis, though C. fetus subsp. fetus is frequently isolated from aborted bovine fetuses showing that this subspecies has clinical relevance in cattle. Campylobacter fetus subsp. fetus has been shown to persist in virgin heifers, after experimental infection, for at least several weeks and up to 10 months or more.

Bovine genital campylobacteriosis can be diagnosed from samples taken from bulls, cows or aborted fetuses. Diagnosis is made by the demonstration of either the causal organism or of a specific immune response to it. In the case of bulls, samples of semen or of preputial smegma secretions can be collected. In cows, mucus samples are obtained by suction, vaginal lavage, or by use of tampons. Internal organs of aborted fetuses can be examined for the presence of the bacterium by culture, and wet preparations of the stomach contents can be examined for the organism by dark-field and phase-contrast microscopy.

Identification of the agent: The organism is a Gram-negative, spirally curved rod, approximately 1.5 µm long and 0.5 µm wide. It can be cultured when incubated microaerobically at 37°C for at least 3 days. 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.

Polymerase chain reaction based tests specific for C. fetus and for each of the two subspecies are described in the literature.

Serological tests: Agglutination tests on vaginal mucus provide a useful herd test, but are not useful for identifying individual infected animals. The animals to be tested should be selected carefully, as even in infected herds some animals may have escaped infection. After infection, there is a lag phase before antibodies develop. Also, agglutinins tend to disappear at the time of oestrus.

The enzyme-linked immunosorbent assay is a more sensitive test, but should also be used as a herd test rather than to test individuals.

Requirements for vaccines and diagnostic biologicals: A vaccine may be prepared from C. fetus subsp. venerealis 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

The genus *Campylobacter* (formerly included in the genus *Vibrio*) (38) comprises many species, among which *C. fetus*, *C. sputorum*, *C. jejuni* and *C. coli* can be isolated from cattle. There are two subspecies of *C. fetus*: *Campylobacter fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus*. It is *C. fetus* subsp. *venerealis* that causes bovine genital campylobacteriosis (14, 42). Glycine-tolerant variants of *C. fetus* subsp. *venerealis* have been described and are designated *C. fetus* subsp. *venerealis* biovar intermedius (34).

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 (39). On the basis of antigenic differences, several serotypes of *C. fetus* have been described (3, 32).

Studies of DNA homology have failed to reveal any major difference between the *venerealis* and *fetus* subspecies (18). However, on the basis of numerical analysis of pulsed-field gel electrophoresis (PFGE) patterns, separation of *C. fetus* into two subspecies clusters has been possible (28). Considerable agreement is seen between the PFGE clusters and identification based on phenotypic and polymerase chain reaction (PCR) methods. The recently developed amplified fragment length polymorphism analysis (AFLP) also discriminates between both subspecies (43), as does a recently described PCR assay (21). However, up to 10% of strains are not correctly subspeciated by this PCR assay as described in the original paper (21), and this observation was confirmed in a later study (43). Taxonomic differentiation of *C. fetus* into two subspecies is still justified because of the clinical and epidemiological differences between them. Epidemiologically, *C. fetus* subsp. *venerealis* is the most important of the two because of its genital tropism. *Campylobacter fetus* subsp. *fetus* can be recovered from the intestinal tract of cattle and other animals. Its pathogenic role is minor compared with *C. fetus* subsp. *venerealis*, though *C. fetus* subsp. *fetus* is frequently isolated from aborted bovine fetuses showing that this subspecies has clinical relevance in cattle. *Campylobacter fetus* subsp. *fetus* has been shown to persist in virgin heifers, after experimental infection, for at least several weeks and up to 10 months or more (25, 29, 35).

B. DIAGNOSTIC TECHNIQUES

1. Isolation and identification of the agent (the prescribed test for international trade)

Bovine genital campylobacteriosis is diagnosed bacteriologically by isolating *C. fetus* in culture, or by immunofluorescence. Bacteriological culture may be done directly from samples, or after transport and/or enrichment of samples. The use of a transport medium is essential if the samples are not processed in the laboratory within 6 hours 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 4–18°C), and protected from light.

a) Collection of samples

i) **In the male: preputial mucus or secretions, and semen**

In bulls, preputial mucus or smegma may be obtained by scraping (36), by suction (with Bartlett’s pipette) (2), or by preputial washing (10). Preputial smegma can also be collected from the artificial vagina after semen collection, by washing the artificial vagina with 20–30 ml of phosphate buffered saline (PBS).

For preputial washing, 20–30 ml of sterile PBS, pH 7.2, is introduced into the preputial sac. After vigorous massage for 15–20 seconds, the infused liquid is collected in a sterile flask, which is sealed immediately and then sent to the laboratory.

Semen is collected under conditions that are as aseptic as possible. The semen is transferred to a sterile tube, which is sealed immediately after filling. Samples of smegma, obtained by scraping or suction, and semen samples may be diluted with PBS, or sown directly on to culture medium or transport and enrichment medium. Dishes containing transport medium are sealed, and then sent to the laboratory in an insulated container (preferably at 4–18°C), protected from light (13).

ii) **In the female: vaginal mucus, cervicovaginal mucus**

Samples may be obtained by swabbing, suction (Bartlett’s pipette), or by washing the vaginal cavity. The use of a sterile speculum, preferably of a disposable type, is vital for obtaining samples of good quality (37).

After cleaning the vulval region, the vaginal cavity is washed by infusing 20–30 ml of sterile PBS into the cavity through a syringe attached to a sterile catheter. The fluid is sucked out and reinfused four to five times before being collected in a sterile flask, which is then sealed immediately and sent to the laboratory. Washing fluid in the vaginal cavity may also be collected by a tampon of sterile gauze held
inside the vagina for 5–10 minutes after PBS infusion. Samples of vaginal mucus obtained by suction may be diluted with PBS, or sown directly on to culture medium or transport and enrichment medium.

### iii) Aborted fetuses, placentas

When abortion occurs, the placenta, and the stomach contents, lungs and liver of the fetus provide the best samples. They are removed under conditions that are as aseptic as possible, and sent to the laboratory in a cooled insulated container (at 4–8°C).

#### b) Transport of samples

i) **Transport and enrichment media**

Various transport and enrichment media are available, such as Clark’s (Australia), Lander’s (United Kingdom [UK]), SBL medium (France), and Foley’s and Clark’s media (used in the United States of America) (16, 20, 24). A comparison of other culture and transport media has recently been published (26).

Some of the transport and enrichment media mentioned above contain cycloheximide. Because of its potential toxicity, this antifungal agent will soon no longer be available. Isolation of *C. fetus* subsp. *fetus* has been shown to be possible on media containing amphotericin B (1).

- **Clark’s medium**

Clark’s medium is an excellent medium, but its preparation is long and difficult, and this hampers its routine use when many samples have to be processed. It contains sterile bovine serum plus 5-fluorouracil (300 µg/ml), polymyxin B sulphate (100 IU/ml), brilliant green (50 µg/ml), nalidixic acid (3 µg/ml), and cycloheximide (100 µg/ml). This mixture is distributed into 10 ml bottles provided with a rubber stopper and a screwed metal lid that has a hole in the top through which a needle can be inserted into the stopper. The filled bottles are placed in a water bath at 100°C. When the medium has solidified, the rubber stopper is pierced with a hypodermic needle, and the air inside is replaced by a mixture of 5% oxygen, 5% carbon dioxide and 90% nitrogen. This step is performed inside an anaerobic jar. The prepared medium must be stored in a refrigerator for 1 week before use. Its storage life is 3 months at 4°C.

Approximately 1 ml of the sample under test is injected into the medium using a syringe, with the needle passing through the rubber stopper. The bottle is stored at approximately 18°C and sent to the laboratory. The time taken for transport to the laboratory should not exceed 48 hours.

When the bottle is received at the laboratory, it is incubated at 37°C for 4 days, and then 3 ml of normal saline is introduced. After the medium has been shaken vigorously, 1 ml of fluid is withdrawn for further testing for *Campylobacter* by isolation in culture or by immunofluorescence.

- **Lander’s medium**

This is a Mueller–Hinton broth with 5 g of bacteriological charcoal/litre, added before sterilisation. When required for use, the following are added to each litre, under sterile conditions: two bottles of ‘*Campylobacter* growth supplement’ (Oxoid), haemolysed horse blood (70 ml), vancomycin (40 mg), polymyxin B sulphate (10,000 IU), cycloheximide (100 mg), trimethoprim (20 mg) and 5-fluorouracil (500 mg). The medium is distributed into 26 ml universal containers in 10 ml volumes. It can be stored for at least 2 weeks at 4°C.

This medium is inoculated with samples under test, volumes of which may have been passed through a 0.65 µm pore size filter. After incubation at 37°C for 3 days, the medium is distributed into culture and isolation media.

- **Modified SBL medium**

SBL medium, as modified by Clark (9), contains in each litre: agar (8 g), sodium thioglycollate (0.5 g), sodium glycerophosphate (10 g), 1% aqueous calcium chloride (10 ml), cysteine hydrochlorhydrate (250 mg), and 0.1% aqueous methylene blue solution (2 ml). After sterilisation by autoclaving, the medium is rendered selective by adding, to each 1 ml, polymyxin (1 IU), novobiocin (5 µg), bacitracin (15 units), and cycloheximide (20 µg). The medium is then distributed under sterile conditions into completely filled anaerobic tubes.

Swabs soaked in various samples are placed in this transport medium and dispatched in an insulated container (at 18–30°C), to reach the laboratory within 24–48 hours.

c) **Treatment of samples**

On arrival at the laboratory, samples should be inoculated directly on to culture medium, or processed further if required (i.e. membrane filtration to reduce contamination).
Chapter 2.3.2. – Bovine genital campylobacteriosis

i) **Genital tract samples**

As vaginal mucus can be 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 on to isolation medium. If the mucus is not very viscous, it can be inoculated directly or diluted with an equal volume of PBS, pH 7.2.

Preputial washings may be centrifuged (3500 g) to concentrate the sample. The final sample (reduced to 250 µl) may be inoculated on to the culture medium (directly or using the filter method).

ii) **Aborted fetuses, placentas**

Fetal stomach contents are inoculated directly on to suitable culture medium. Internal organs or pieces of organs are flame sterilised to sterilise the surface, and then homogenised. The homogenate is inoculated on to culture medium.

After washing placental membranes with sterile normal saline or PBS, to eliminate most of the surface contamination, the chorionic villi are scraped and the scrapings are transferred to culture media.

d) **Isolation of Campylobacter fetus**

i) **Culture media for isolation**

Many media are in current use for the bacteriological diagnosis of bovine genital campylobacteriosis (7). Most culture media contain cycloheximide. Because of its potential toxicity, this antifungal agent will soon no longer be available. Isolation of *C. fetus* is possible on media containing amphotericin B (1).

Examples of media are the following:

- **Skirrow medium with cycloheximide**

  This medium contains as selective agents: polymyxin B sulphate (2.5 IU/ml), trimethoprim (5 µg/ml), vancomycin (10 µg/ml), and cycloheximide (50 µg/ml). It contains 5–7% lysed defibrinated horse blood. However, addition of 5–7% defibrinated sheep blood gives good results.

- **Clark’s selective medium**

  Peptone (10 g), sodium chloride (5 g), beef extract (5 g), agar (15 g), and distilled water (1000 ml). Dissolve the ingredients, except the agar, in water by shaking if necessary. Adjust the pH to 7.4–7.6, then add the agar and bring to the boil. Sterilise by autoclaving (121°C for 15 minutes). Cool to 55°C and add 10% sterile, defibrinated ovine or bovine blood, then bacitracin (15 IU/ml), polymixin B sulphate (1 IU/ml), novobiocin (sodium salt) (5 µg/ml) and cycloheximide (10 µg/ml).

- **Nonselective media**

  As nonselective medium, any blood-based medium can be used (Columbia agar, blood agar base No. 2) supplemented with either sheep blood or horse blood.

ii) **Inoculation of the media and incubation**

Each sample is inoculated directly on to a selective medium or, after passage through a 0.65 µm pore size filter, on to a selective and/or a basic medium. The material is spread out with a loop to facilitate isolation of single colonies.

The plates are incubated at 37±1°C. Microaerobic atmospheres of 5–10% oxygen, 5–10% carbon dioxide (and preferably 5–9% hydrogen) are required for optimal growth (40). Appropriate microaerobic atmospheric conditions may be produced by a variety of methods. In some laboratories, (repeated) gas jar evacuations followed by atmosphere replacement with bottled gasses are used. Gas generator kits are available from commercial sources. Variable atmosphere incubators are more suitable if large numbers of cultures are undertaken.

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. However, it is unnecessary to set up more than one control per day, unless different batches of medium are used, in which case each batch has to be tested.

iii) **Reading the results**

Colonies of *C. fetus* usually appear in the recommended media after 2–5 days. Growth may be slow, particularly in the presence of contaminating bacteria in the samples. To prevent overgrowth of specific colonies by contaminants, it is recommended to check the media daily and to subculture colonies suspected of being *C. fetus*. 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. Cultures should be incubated for a minimum of 6 days.
e) **Confirmation of the organism**

i) *Microscopic morphology:* *Campylobacter fetus* is motile, a property that may disappear during subculturing. *Campylobacter fetus* 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. The bacteria are invariably separated from each other. Old cultures may contain coccoid bacteria.

ii) *Biochemical tests:* *Campylobacter fetus* is oxidase and katalase positive.

iii) *Campylobacter fetus* does not grow under aerobic conditions.

f) **Identification of Campylobacter to the species level**

These tests (see Table 1) must be done on pure cultures. Ideally, they should be performed using a standardised suspension with turbidity no greater than a McFarland No. 1, or equivalent.

<table>
<thead>
<tr>
<th></th>
<th>Oxidase</th>
<th>Catalase</th>
<th>H₂S (a)</th>
<th>25°C</th>
<th>42°C</th>
<th>Glycine 1%</th>
<th>NaCl 3.5%</th>
<th>Cephalothin</th>
<th>Nalidixic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. fetus subsp. venerealis</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>V</td>
<td>–</td>
<td>S</td>
<td>V</td>
</tr>
<tr>
<td>C. fetus subsp. fetus</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>V</td>
<td>–</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>C. jejuni</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>C. sputorum biovar sputorum</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>S</td>
<td>V</td>
</tr>
<tr>
<td>C. sputorum biovar faecalis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>S</td>
<td>V</td>
</tr>
<tr>
<td>C. sputorum biovar paraureolyticus</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>S</td>
<td>V</td>
</tr>
</tbody>
</table>

(a) = On triple sugar iron agar medium; (b) = Although *C. fetus* does not belong to the thermophilic *Campylobacter* spp., growth of this species at 42°C has been reported (12, 41); (+) = positive reaction or growth and (–) = negative reaction or absence of growth of the strain on an appropriate medium under specified conditions; V = variable results; S = sensitive; R = resistant.

i) **Test for growth in the presence of glycine**

The test is done on each of the strains that appear to be *C. fetus* (suspected strains) by preparing a suspension in buffer solution at pH 7.2. This is inoculated on to a glycine medium (15 ml of blood medium + precisely 1.65 ml of 10% aqueous solution of glycine), and on to basic blood medium. Incubation is performed under the specified atmospheric conditions. 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 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 (34).

ii) **Test for growth in the presence of sodium chloride**

A suspension of the suspected strain in buffer solution, pH 7.2, is inoculated on to 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. Two control strains are tested in parallel.

iii) **Test for hydrogen sulphide production**

The hydrogen sulphide (H₂S) test is done on triple sugar iron agar (TSI) medium containing 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 1000 ml). This medium is sterilised after distribution into tubes by autoclaving at 115°C for 15 minutes. It can be prepared as required, or can be made and kept under the usual storage conditions for approximately 3 weeks.

Just before use, melt the medium in a bath of boiling water and incline the tubes in order to obtain a slope with a depth of 3 cm. This ready-to-use medium can be stored under the usual conditions for 8–10 days before use.
iv) **Test for sensitivity to cephalothin and nalidixic acid**

Sensitivity to cephalothin (CN) and nalidixic acid (NA) is tested by the disk technique (31). Disks, each containing CN (30 µg) or NA (30 µg) are prepared in the laboratory by soaking filter paper disks in solutions of CN (3 mg/ml) and NA (3 mg/ml). The disks are then dried and stored until required.

For the test, 72-hour cultures of the strains under test are suspended in PBS, pH 7.2, at a concentration of 10^9 bacteria/ml. Using this suspension, 100 µl portions are placed as an even layer on to basic blood medium. The culture dishes are dried before the culture is deposited on the surface. The sensitivity disks are then placed on top. These cultures are incubated at 37°C in an appropriate atmosphere, and examined after 48 and 96 hours. A zone of inhibition of at least 3 mm around a disk indicates that the strain under test is sensitive to that antibiotic. All *C. fetus* subsp. *fetus* strains and most of the *C. fetus* subsp. *venerealis* strains are resistant to NA (27). All *C. fetus* are sensitive to CN (27).

g) **Immunofluorescence**

This test can be applied to identify the organism by the direct technique or to confirm the identification of a strain after isolation. It will not differentiate between different subspecies (30).

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 separately on blood agar medium microaerobically at 37°C for 3 days. The organisms are harvested into PBS, pH 7.2, 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. The inoculum is 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 (8). Monoclonal antibodies that can be used for immunodiagnostic detection of *C. fetus* have been described (6).

ii) **Preparation of conjugates**

The IgG fraction is separated by precipitation with sodium sulphate. The serum is adjusted to pH 8.0 with 0.1 M PBS, and 18 g of anhydrous sodium sulphate is added to each 100 ml. The precipitate is collected by centrifugation and redissolved in distilled water to its original volume. It is reprecipitated by the addition of 12 g of sodium sulphate/100 ml. The precipitate is collected, dissolved in distilled water to half its volume, and dialysed against PBS, pH 7.2, at 4°C until free of sulphate. The protein content is determined and the concentration is adjusted to 1.0–1.5 g protein (e.g. bovine serum albumin) per 100 ml with PBS. This solution is adjusted to pH 9.0 with 1 M sodium carbonate. Fluorescein isothiocyanate isomer 1 (FITC) in a minimum volume of 0.1 M sodium carbonate is added to a final concentration of 15 mg FITC/1.0 g protein. This is stirred for 18 hours at 4°C. The mixture is adjusted to pH 7.0 with 0.1 M hydrochloric acid, and dialysed against frequent changes of PBS at 4°C. Traces of free FITC are removed by gel filtration on Sephadex G25, and the final product is stored at -20°C or lower in small aliquots.

The working dilution of the conjugate is determined by testing various dilutions in PBS against smears of a *C. fetus* culture, and selecting twice the lowest concentration that produces brilliant fluorescence with these test organisms. Under high-power microscopy, the fluorescent organisms have a typical morphology, and are most often concentrated at the edges of the smear.

iii) **Immunofluorescence test**

The samples are fixed on to glass slides, stained with the FITC-conjugated antiserum, and examined for the fluorescing organisms. Staining is carried out in a humid chamber at 37°C for 30 minutes. The slides are then rinsed free of conjugate, washed in two changes of PBS (10 minutes each wash) and mounted in buffered glycerol (80% [v/v] glycerol: 20% 0.1 M phosphate buffer, pH 8.0).

The cover-slips are sealed to prevent drying, and the slides are examined under ultraviolet light microscopy.

b) **Molecular identification of *Campylobacter fetus***

PCR-based methods for the specific identification of *C. fetus* (21) and for each of the subspecies are described in the literature (21, 44).

2. **Serological tests/antibody detection**

Tests for antibodies include the vaginal mucus agglutination test and the enzyme-linked immunosorbent assay (ELISA).
a) **Vaginal mucus agglutination test**

This test is useful in herds, but not for identifying individual animals with *C. fetus* infection. Only around 50% of infected animals are identified. The test is best done on vaginal mucus taken 37-70 days after infection, but the presence of antibodies may be delayed for up to 3-4 months. Some cows may remain positive for several years, while others become negative within 2 months. Approximately 50% of positive cows will become negative in 6 months (15).

Samples taken by vaginal lavage are assumed to have been diluted 1/5 with the saline wash. When samples are obtained by the use of tampons, the vaginal mucus is extracted with 7 ml of physiological saline, and left overnight at 4°C. The fluid is pressed from the tampon and used as the test fluid for the vaginal mucus agglutination test.

The antigen for the vaginal mucus agglutination test is a 48-hour growth of *C. fetus* subsp. *venerealis* on fresh (less than 2 weeks old) 7% sheep blood agar, preferably containing 0.1% cycloheximide. This is either subcultured on to 20–30 blood agar plates, or a suspension of the bacteria in sterile PBS is pipetted into Roux flasks of blood agar and spread over the surface of the medium by gentle rocking. The cultures are incubated microaerobically for 2–3 days at 37°C in 85% nitrogen, 10% carbon dioxide and 5% oxygen. The growth is harvested and suspended in 0.5% formol saline. If Roux flasks are used, 10 ml of formol saline and a few glass beads are used to remove the growth. The suspension is filtered through muslin to remove coarse debris, washed three times by centrifugation at 6000 g for 20 minutes, and the final wash is resuspended in 0.25% formol saline and stored for 1 week. To titrate the antigen, a dilution series is prepared in formol saline. Each tube should contain 0.5 ml of serum and 0.5 ml of antigen. The tube contents are mixed well before incubation at 37°C for 18 hours. The antigen titre is determined by the highest dilution that gives at least 50% agglutination with the positive serum sample.

Vaginal mucus samples are homogenised with four volumes of PBS (or 5% phenol saline) using ground glass beads. If a vaginal lavage has been carried out, the sample is already suitable. For the homogenate, 2 ml is transferred to a test tube placed in a water bath at 57°C. Approximately 10 ml of molten Oxoid agar No. 1 (20 g/litre) is also placed in the same water bath. When the contents of both tubes have equilibrated to 57°C, 2 ml of the agar is pipetted into the homogenate. The mixture is shaken vigorously, and quickly poured into a 45 mm wide-mouth screw-cap container. When the mixture has solidified, 2 ml of 5% phenol saline is layered over the top of the agar before the lid is secured. The container is then incubated for 18 hours at 37°C to extract the antibody.

The clear fluid is aspirated off the agar surface and used as the test sample in a three-tube agglutination test. The first tube is left empty, while 0.5 ml of 0.5% phenol saline is pipetted into the others. Then, 0.5 ml of the test sample is put into both the first and second tubes. The contents of the second tube are mixed, and 0.5 ml is transferred into the third tube. The contents of the third tube are mixed and 0.5 ml is discarded. Next, 0.5 ml of a standardised dilution of the antigen is added to each tube and mixed well. After incubation at 37°C for 18 hours, the tests are read by oblique light against a dark background. Each dilution is scored as follows:

- Water clear = ++++
- 75% clearing = +++
- 50% clearing = ++
- 25% clearing = +
- No clearing = –

A positive control titration should be carried out at the same time, using the same method as that for determining the titre of the antigen against a known positive antiserum. The antigen is used at the same standardised dilution against dilutions of positive serum. The results can be validated if the expected titre is obtained using the positive control samples.

A positive result is one where 50% agglutination has occurred in the third tube (dilution 1/80), or beyond if more than three tubes are used. The reaction is inconclusive if there is less evidence of agglutination. It is generally agreed that the mucus test is valuable for the diagnosis of genital campylobacteriosis, but it must be emphasised that interpretation of the results should be made on a herd basis. To use the test to its best advantage, it is necessary to select the animals with care, as even in herds where widespread infection exists, it is probable that a variable proportion of the animals may have escaped infection. There is a variable lag period between infection and the development of a positive agglutination test, and at the time of oestrus, agglutinins tend to disappear, either partially or entirely, from the mucus, although they may be present in high concentrations at other stages of the cycle. In any case, agglutination titres tend to decline with time.
b) Enzyme-linked immunosorbent assay

An ELISA is available to detect antigen-specific secretory IgA antibodies in the vaginal mucus following abortion due to *C. fetus* subsp. *venerealis* (17, 19). These antibodies are long lasting, and their concentration remains constant in the vaginal mucus for several months (22).

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 was recently described (33).

- **Antigen preparation and coating**

  *Campylobacter fetus* subsp. *venerealis* is grown on 7–10% blood agar in microaerobic conditions at 37°C for 3 days. The plates are checked for purity, and colonies are transferred to 0.5% formol saline for 1 hour, centrifuged at 17,000 g, washed twice with PBS, pH 7.5, and then resuspended in 0.05 M carbonate buffer, pH 9.6. The final absorbance is adjusted to 0.21 at 610 nm. 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.

- **Test procedure**

  i) Diluted vaginal mucus (100 µl) is added to each well, and the plate is incubated at 37°C for 2 hours.

  ii) The plates are then washed as before, and 100 µl of rabbit anti-bovine IgA is added.

  iii) After 2 hours’ incubation at 37°C, the plates are washed and 100 µl of goat anti-rabbit IgG conjugated to horseradish peroxidase is added to each well.

  iv) After a further 2 hours’ incubation at 37°C, the plates are washed, and 100 µl of substrate is added (5 amino-salicylic acid [0.8 mg/µl], pH 6.0, immediately activated by the addition of 2% 1 M hydrogen peroxide [H₂O₂]).

  v) The plates are left at room temperature for 30 minutes and the reaction is then stopped by the addition of 50 µl of 3 M sodium hydroxide (NaOH). The absorbance is measured on an ELISA reader at 450 nm.

  vi) **Interpretation of the results:** 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

*Campylobacter fetus* subsp. *fetus* vaccine confers immunity against *C. fetus* subsp. *venerealis* because both strains share common antigens (5). Two groupings of antigens of *C. fetus* are recognised: the thermolabile ‘H’ flagellar antigens and the thermostable ‘O’ somatic antigens. In addition, a capsular or ‘K’ antigen is present (23). The vaccine must incorporate these antigens. It is an oil-emulsion vaccine of one or more strains that have been inactivated by formaldehyde. Other vaccine preparations have also been described (11).

The addition of a second strain of *C. fetus* subsp. *venerealis* to the biological product is widely practised. 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 (4).

In infected herds, all breeding animals, including bulls, cows and heifers, are vaccinated following the diagnosis of bovine genital campylobacteriosis. Additional antibiotic treatment of an infected bull at the time of the second vaccination is recommended, because 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 noninfected herds, only the bulls are vaccinated, and this is done once a year.

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% Bacto 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 in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂. The growth is transferred to additional tubes with semisolid medium and incubated for 48 hours. The resulting growth is used for vaccine production.

   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.

      The vaccine should be stored at 4°C.

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%.

   The vaccine is mixed with an oil-emulsion adjuvant to extend the period of immunity.

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 I.1.5.

   b) **Safety**

      The inactivation process must be complete. This 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 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 subcutaneously with half the dose used in cattle, on two occasions 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.

- **Acknowledgement**

Parts of this chapter were taken from or based on the chapter on bovine genital campylobacteriosis in previous editions of the *Terrestrial Manual*.

**REFERENCES**


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CHAPTER 2.3.3.

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 among 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 of this disease and might include weakness, anorexia, emaciation, dyspnoea, enlargement of lymph nodes, and cough, particularly with advanced tuberculosis. After death, it is diagnosed by post-mortem examination and histopathological and bacteriological techniques. DNA probe and polymerase chain reaction (PCR) techniques may also be used. These are demanding techniques and only validated procedures should be used. Traditional bacterial culture remains the routine method for confirmation of infection.

Identification of the agent: Bacteriological examinations may comprise the demonstration of acid-fast bacilli by microscopic examination (provides presumptive confirmation), and the isolation of mycobacteria on selective culture media and their subsequent identification by cultural and biochemical tests or DNA probe and PCR techniques. Animal inoculation is slightly more sensitive than culture, but should only be used when histopathology lesions are compatible with mycobacteriosis infection and isolation in culture is negative.

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 3 days 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 choice of which of the two tests to use 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 appropriate schemes.

Blood-based laboratory tests: New diagnostic blood tests are now available, e.g. the lymphocyte proliferation assay, the gamma-interferon assay, and the enzyme-linked immunosorbent assay. The logistics and laboratory execution of these tests may be a limiting factor. Further comparative studies of these new tests and skin testing under different field conditions are required. Data on some situations are becoming available. 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.

**Requirements for vaccines and diagnostic biologicals:** Vaccines are being developed and tested, but at this time are not routinely administered. 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, during diagnostic examination, 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*, and is usually characterised by formation of nodular granulomas known as tubercles. Although commonly defined as a chronic debilitating disease, bovine tuberculosis can occasionally assume an acute, rapidly 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.

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, the signs associated with tuberculosis were commonly observed (6).

These signs vary with the distribution of tubercles in the body but, with few exceptions, the course of the disease is chronic. In many instances, characteristic signs are lacking, even in advanced stages of the disease when many organs may be involved. 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.

Tubercles of cattle are most frequently seen at necropsy in bronchial, mediastinal, cranial and portal lymph nodes, which may be the only tissue affected. In addition, the lung, liver, spleen and the surfaces of body cavities are commonly affected. Other anatomical sites must be considered as having the potential to become infected.

At necropsy, a tuberculous granuloma usually has a yellowish appearance and is caseous, caseo-calcareous, or calcified in consistency. Occasionally, its appearance may be purulent. Some nontuberculous granulomas occur in which purulent content with a greenish lustre is replaced by granulation tissue, which may have a resemblance to tuberculous granulomas. The caseous centre is usually dry, firm, and covered with a fibrous connective capsule of varying thickness. Fixed tissues in a tubercle are not easily removed intact, as is the case with some nontuberculous granulomas. 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 is vital to detect lesions contained within the tissue.

*Mycobacterium bovis* has been identified in humans in most countries where isolates of mycobacteria from human patients have been fully typed. 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, llamas, kudus, elands, tapirs, elks, elephants, sitatungas, oryxes, addaxes, rhinoceroses, possums, ground squirrels, otters, seals, hares, moles, raccoons, coyotes and several predatory felines including lions, tigers, leopards and lynx (7, 21).
Bovine tuberculosis in wildlife was first reported in 1929 in greater kudu (Tragelaphus strepsiceros) and common duiker (Sylvicapra grimmii) in South Africa. During the 1940s greater kudu in the same region were found to be endemically infected. In 1982 in Uganda, a prevalence of 10% in African buffalo and 9% in warthog (Phacochoerus aethiopicus) was found. In Zambia, M. bovis infection has been reported in Kafue lechwe (Kobus leche kafuensis) and in a single eland (Traurotragus 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 (3), and more recently spill over to other species such as chacha 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. 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 (31) and the Republic of Ireland (27), the brush-tail possum (Trichosurus vulpecula) in New Zealand (2), and several wild living species in Africa have been shown to be capable of harbouring M. bovis infection. Control of transmission from the wildlife population to farmed species is complex and, to date has relied on the 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 (proliferative granuloma, caseation, granulation and calcification with ageing). The lesions may take the form of thin-walled abscesses with little calcification and containing purulent material. In cervids, tuberculosis should be considered when abscess-like lesions of no known aetiology are observed. The lymph nodes affected are usually those of the head and chest. 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 very carefully, with hair clipping at the site of testing, accurate intradermal injection, and careful pre- and post-inoculation skin thickness measurement using callipers to obtain results that are valid (5).

Mycobacterium bovis can cause severe economic losses due to its effects on domesticated livestock and zoonotic infections. In addition, the presence of infection in wildlife populations poses a threat to the survival of endangered wildlife species.

**B. DIAGNOSTIC TECHNIQUES**

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 are being studied in an attempt to develop improved or alternative diagnostic methods, as skin testing sometimes has practical drawbacks. However, there is no universally accepted diagnostic blood test for tuberculosis in cattle or other animals.

The presence of M. bovis in clinical and post-mortem specimens may be demonstrated by examination of stained smears 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, one-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 I.1.1. Sampling methods. Prompt delivery of specimens to the laboratory greatly enhances the chances of cultural recovery of M. bovis, but 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 I.1.6 Human safety in the veterinary microbiology laboratory). 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). The presence of acid-fast organisms in histological sections may not be detected although *M. bovis* can be isolated in culture.

b) Culture of *Mycobacterium bovis*

In order to process specimens for culture, the tissue is first homogenised using a pestle and mortar, stomacher or blender followed by decontamination with either an acid or an alkali, such as 5% oxalic acid or 2–4% sodium hydroxide. The mixture is shaken for 10 minutes at room temperature and then neutralised. The suspension is centrifuged, the supernatant is discarded, and the sediment is used for culture and microscopic 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 glycerol or both. An agar-based medium such as Middlebrook 7H10 or 7H11 should also be used.

Cultures are incubated for 8 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 after 3–6 weeks' incubation. *Mycobacterium bovis* will grow on Lowenstein–Jensen medium without pyruvate, but will grow less well when glycerol is added. Characteristic growth patterns and colonial morphology can provide a presumptive diagnosis of *M. bovis*, which can be confirmed by polymerase chain reaction (PCR) and molecular typing techniques such as spoligotyping.

Characteristic growth patterns and colonial morphology can provide a presumptive diagnosis of *M. bovis*; however every isolate needs to be confirmed by biochemical evaluation (niacin and nitrate) or Gen Probe TB complex DNA probe to confirm the isolate is in the tuberculosis complex.

If gross contamination of culture media occurs, or a specimen shows a negative culture result and a positive macroscopic and histopathology result, the culture process should be repeated using the retained inocula with further decontamination in the case of gross contamination occurring. 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.

Growth considered to be mycobacterial is subcultured to egg-based and agar-based media or into Tween albumin broth, and incubated until visible growth appears. In some laboratories, sterile ox bile is used before inoculation to facilitate the dispersion of the bacterial mass into small viable units.

Identification of isolates is usually carried out by determining 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 thiopehen-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. Additional tests may be used for identification. A number of DNA analysis techniques offer a rapid method of determining the identity of isolates and may also provide molecular-typing information on the isolate that is of epidemiological value.

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*), and *M. microti* (the ‘vole bacillus’, a rarely encountered organism). The above-mentioned tests will serve to separate *M. bovis* from the other species.
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. *Mycobacterium tuberculosis* may sensitise cattle to bovine tuberculin without causing distinct tuberculous lesions.

Liquid culture systems such as Bactec are used routinely in some hospital and veterinary laboratories. Growth is assessed by radiometric or fluorometric means. The manufacturers (BD-Diagnostic systems) no longer support the radiometric system.

c) **Nucleic acid recognition methods**

The 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, including primers that have amplified sequences from 16S–23S rRNA, the insertion sequences IS6110 and IS1081, and genes coding for *M. tuberculosis*-complex-specific proteins, such as MPB70 and the 38 kDa antigen b. 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 (25). 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. PCR is not only used for direct detection in material, or strain characterisation or differentiation within the TB complex, but it is also widely used as a method of initial identification (selection of organism being done on the basis of colony morphology and AP staining). Commercial kits that are quite ‘robust’ are available, for example the Gen probe accuprobe. Although the number of species that can be identified by these kits is limited, their primers for the *M. tuberculosis* complex are widely used in both the human and veterinary field. Laboratories have also developed their own ‘in-house’ methods. 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. This type of application has been overlooked. Usually some biochemical tests are done to confirm the finding. However, PCR is now being used on a routine basis to detect the *M. tuberculosis* group and distinguish it from *M. avium* in formalin-fixed, paraffin-embedded tissues (23, 24). Optimal results are obtained when both PCR and isolation methods are used.

DNA analysis techniques may prove to be faster and more reliable than biochemical methods for the differentiation of *M. bovis* from other members of the *M. tuberculosis* complex. A mutation at nucleotide position 285 in the oxyR gene has been found to be specific for *M. bovis* in all *M. tuberculosis* complex isolates examined to date (10). It is of practical significance that the specific gene probe for hybridisation, needed for visualisation of the amplified segment, can be labelled with biotin or digoxigenin instead of isotopes.

Genetic fingerprinting allows laboratories to distinguish between different strains of *M. bovis* and will enable patterns of origin, transmission and spread of *M. bovis* to be described. 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* (19). Other new techniques are currently under development in order to differentiate more accurately the strains that have the same spoligotype. These include restriction fragment length polymorphism (RFLP) using IS6110, the direct repeat (DR) region and the PGRS probe (poly G repeat sequence) (30) RFLP using a combination of the DR and puce probes (26) and characterisation of the VNTR profile (variable number tandem repeat) (8, 9, 13, 20). The genome of *M. bovis* is currently being sequenced and this information should lead to improved methods of genetic fingerprinting.

2. **Delayed hypersensitivity test**

- **The tuberculin test (the prescribed test for international trade)**

In the past, heat-concentrated synthetic medium (HCSM) tuberculin was used, but, in most countries, HCSM tuberculin has been replaced by purified protein derivative (PPD) tuberculin. The HCSM tuberculins can have a good potency if correctly standardised for biological activity, but their specificity is inferior to PPD tuberculins. Moreover, it has been shown that bovine PPDs prepared with the *M. bovis* production strain AN5 are more specific for detecting bovine tuberculosis than human PPDs prepared with *M. tuberculosis*. 

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The standard method for detection of bovine tuberculosis is the tuberculin test, which involves the intradermal injection of bovine tuberculin PPD and the subsequent detection of swelling (delayed hypersensitivity) at the site of injection 3 days 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.

It is not recommended to use this test when the epidemiology suggests that the herd/animal is suspected to have been in contact with infected animals as it may result in false-negative responses and a decrease in the sensitivity of the test. Complete eradication is difficult if only a single tuberculin test is used, as false negative responses may occur in the early stage of the disease and in severely infected animals.

The comparative intradermal tuberculin test is used to differentiate between animals infected with *M. bovis* and those sensitised to bovine tuberculin as a result of exposure to other mycobacteria. This sensitisation can be attributed to the large 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**
  
  1. **i)** A correct injection technique is important. The injection sites must be clipped and cleansed. 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, is inserted obliquely into the deeper layers of the skin. The dose of tuberculin is then injected. 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. 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 remeasured 72 hours after injection. The same person should measure the skin before the injection and when the test is read.
  
  2. **ii)** 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 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. 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 an intradermal comparative test. Retesting can be performed in accordance with the national or local control programmes.
  
  3. **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 from 1 to 4 mm greater than the avian reaction. 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 (11). Sometimes a more stringent interpretation is used.
  
  4. **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
3. Blood-based laboratory tests

Besides the classical intradermal tuberculin test, a number of new diagnostic blood tests have become available (16). Due to the cost and the more complex nature of laboratory-based assays, they are usually used as ancillary tests to confirm or negate the results of an intra-dermal skin test. The lymphocyte proliferation assay and the gamma-interferon assay correspond to cellular immunity, while the enzyme-linked immunosorbent assay (ELISA) corresponds to humoral immunity.

a) 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 (4) or purified lymphocytes from peripheral blood samples (14). These tests endeavour to increase the specificity of the assay by removing the response of lymphocytes to ‘non-specific’ 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). However, 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 (14). The test is relatively expensive and has not yet been subject to inter-laboratory comparisons.

b) Gamma-interferon assay

In this test, the release of a lymphokine (interferon gamma) in a whole-blood culture system is measured. The assay is based on the release of gamma-interferon from sensitised lymphocytes during a 16–24-hour incubation period with specific antigen (PPD-tuberculin). The test makes use of the comparison of gamma-interferon production following stimulation with avian and bovine PPD. The quantitative detection of bovine gamma-interferon is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine gamma-interferon. The blood sample must be transported to the laboratory and the assay set up within 24–30 hours of collection. The test is considered to have a high sensitivity compared with the skin test, but it has proven to be less specific in a number of trials. However, the use of defined mycobacterial antigens promises to improve specificity (4). In animals that are difficult or dangerous to handle, such as excitable cattle or other bovidae, the advantage over the skin test is that the animals need only be captured once.

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 best choice 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 both specificity and sensitivity are limited in cattle, mostly due to the late and irregular development of the humoral immune response in cattle during the course of the disease. 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% (15). Improvement may be possible by using different antigens, including proteins (e.g. MPB 70, which is very specific but lacks sensitivity). Moreover, in M.-bovis-infected animals, an amnestic rise has been described, resulting in better ELISA results 2–8 weeks after a routine tuberculin skin test. A comparison of antibody levels to PPD-B and PPD-A has also been shown to be useful in increasing specificity in the ELISA (15). 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.

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 (29). 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 DNA of the tuberculosis organism is now being studied in detail and the entire genome sequence has recently been published. This may be particularly useful in identifying genes associated with virulence and in advancing towards a DNA 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. 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 (33). It is important to recognise that use of vaccine will compromise tuberculin skin tests or other immunological tests. Cattle vaccination should not therefore be used in countries where control or trade measures based on such testing are in operation. BCG vaccines may also be used to reduce spread of \(M. bovis\) in wildlife reservoirs of infection. It is essential before doing so to validate the delivery system for the particular wildlife species.

Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 are intended to be general in nature and may be supplemented by national and regional requirements.

Tuberculins were preparations made from the heat-treated products of growth and lysis of \(M. tuberculosis\) or \(M. bovis\) (known as human and bovine tuberculins, respectively). At the beginning the culture medium used for their production was 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. Currently, both the old and HSCM tuberculins have been replaced, almost world-wide, with the purified protein derivatives or PPD.

- **Production of tuberculin**
  
1. **Seed management**
   
   a) **Characteristics of the seed**
   
   Strains of \(M. bovis\) used to prepare seed cultures must be identified as to species 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 \(M. bovis\) AN5 or Vallee are the most commonly used.

   b) **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 (1, 17).

   The production culture substrate must be shown to be capable of producing a product that conforms to recognised international standards (World Health Organization [WHO], European Pharmacopoeia or other recognised control authorities). It must be free from ingredients known to cause toxic or allergic reactions.

   c) **Validation**
   
   The strains of \(M. bovis\) 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. **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.

   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 (1, 17).

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 I.1.5.).

b) Safety

Two guinea-pigs, each weighing not less than 250 g, 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.

In the 1970s, countries in the then European Economic Community (EEC, now the EU) recognised a standard for bovine HCSM tuberculins. This EEC standard for bovine HCSM has a potency of 65,000 provisional Community tuberculin units per ml.

As early as the 1960s, the EEC 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 tuberculin 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 (22, 28, 32).

- **Standardisation in guinea-pigs**

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 (12).

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* (e.g. 1 mg wet weight) 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, but less reliable, 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. 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.

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 42 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 (12). 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 not 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 (32). 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 (18).

The results are statistically evaluated using the same standard methods for parallel-line assays as employed in the potency tests in guinea-pigs.
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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. Freeze-dried preparations may be stored at higher temperatures (but not exceeding 25°C) 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


* * *

NB: There are OIE Reference Laboratories for Bovine tuberculosis (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.3.4.

ENZOOTIC BOVINE LEUKOSIS

SUMMARY

Enzootic bovine leukosis (EBL) is a disease of adult cattle caused by the retrovirus, bovine leukaemia virus (BLV). 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 buffaloes, sheep 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 isolated by cultivation of peripheral lymphocytes, followed by demonstration of the virus by electron microscopy or by BLV antigen detection tests. Proviral DNA can be detected in peripheral blood or tumours by the polymerase chain reaction.

Serological tests: The methods most widely used are agar gel immunodiffusion (AGID) on serum and the enzyme-linked immunosorbent assay (ELISA) on 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 available commercially.

Requirements for vaccines and diagnostic biologicals: 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 leukemia (SBL) is usually reserved for calf, cutaneous and thymic types of lymphoma, which are defined by the age of the animal affected and the distribution of the tumours. The cause or causes of SBL are 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.

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% 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 as a cause of immunological deficiency or increased cull rate. In one study it was demonstrated that BLV-infected herds have lower milk production (2.5–3% on a herd level), an increased cull rate, and are more susceptible to other diseases with infectious aetiology, e.g. mastitis, diarrhoea and pneumonia, but the effect on fertility is only minor (9).

B. DIAGNOSTIC TECHNIQUES

Virus can be detected by in-vitro cultivation of peripheral blood lymphocytes. The virus is present in blood lymphocytes and in tumour cells as provirus integrated into the DNA of the cell. 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. In regions where blood-sucking insects occur in large numbers, especially tabanids, these may transmit the virus mechanically.

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. Persistent antibody can also be detected after experimental infection in deer, rabbits, rats, guinea-pigs, cats, dogs, sheep, rhesus monkeys, chimpanzees, antelope, 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 on into other countries for the first time, by the importation of cattle from North America (11). 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. There is, however, no conclusive evidence of transmission, and it is now generally thought that BLV is not a hazard to humans.

1. **Identification of the agent**

BLV is an exogenous retrovirus related structurally and functionally to human T-lymphotropic viruses 1 and 2 (HTLV-1 and HTLV-2). The major target cells of BLV are B lymphocytes. The virus particle consists principally of single-stranded RNA, nucleoprotein p12, capsid (core) protein p24, transmembrane glycoprotein gp30, envelope glycoprotein gp51, and several enzymes, including reverse transcriptase. Proviral DNA, which is generated by reverse transcription of most of the virus genome, integrates at random into the nuclear DNA of the host cell where it remains, but does not give rise to free virus in vivo. When infected cells are cultured in vitro, usually by co-cultivation of lymphocytes and an indicator cell line, infectious virus is produced, most readily through stimulation with mitogens (16).

**a) Virus isolation**

Mononuclear cells from 1.5 ml of blood in ethylene diamine tetra-acetic acid (EDTA) are separated out on a ficoll/sodium metrizoate density gradient, then cultured with 2 \( \times \) 10^6 fetal bovine lung (FBL) cells, and 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 sheet. Short-term cultures can be prepared by culturing mononuclear cells in the absence of FBL cells in 24-well plastic trays for 3 days (13). The p24 and gp51 antigens can 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 and of the BLV-provirus can be demonstrated by electron microscopy and by PCR, respectively.

**b) Polymerase chain reaction**

The use of the polymerase chain reaction (PCR) to detect BLV provirus has been described by various workers (3–5, 19). Primers constructed to match the *gag*, *pol* and *env* regions of the genome have all been used with variable success. Double (nested) PCR followed by gel electrophoresis and staining is the most rapid and sensitive method (4, 12). The method described is 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 Chapters I.1.4. and I.1.8.). Several other PCR protocols for the detection of BLV provirus sequences have been published (3, 4, 19).

The nested PCR is applicable to the detection of BLV infection in individual animals 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.

PCR is not suitable for use as a herd test, but may be used as an adjunct to serology for confirmatory testing.

**Sensitivity and reliability of the method**

i) *Analytical sensitivity*

Although the nested PCR assay has a theoretical sensitivity of one target molecule, in practice the analytical sensitivity is somewhat lower, around five to ten target molecules of proviral DNA per sample.

ii) *False-positive samples*

The high sensitivity of the nested PCR method may cause problems of false-positive samples due to contamination between samples. To minimise this, several special procedures are adopted during the analysis, such as the use of laminar air-flow hoods, separate rooms for different steps of the analysis, new gloves or the use of special tube openers for each individual assay, negative controls (water blanks), etc. These precautions against contamination have been described (5).

iii) *False-negative samples*

It should be noted that only a small proportion of the peripheral lymphocytes 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, internal controls (mimics) 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 longer PCR product, which can be visualised by agarose gel electrophoresis. The mimic is added at a low concentration, and this, together with its larger PCR-product size, favour an amplification of the real target (2). 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**

Peripheral blood lymphocytes (PBL) are separated from EDTA blood samples by using the Ficoll-Paque separation method (Pharmacia & Upjohn, Uppsala, Sweden). 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, e.g. NucleoSpin (Macherey-Nagel) or Chelating resin treatment (BioRad).

The following method is based on studies by Singer-Sam *et al.* (17) and Walsh *et al.* (20). Special precautions should be taken during all steps to minimise the risk of contamination (5).

i) Approximately 100 µl chelating resin (Sigma C-7901 or Chelex from Bio-Rad) is added for each sample in a 1.5 ml eppendorf tube.

ii) 100 µl of the samples and 10 µl of the mimic are added to the tubes with chelating resin. The samples are vortexed.

iii) The eppendorf tubes are closed and incubated at 56–60°C for 20 minutes.

iv) The tubes are vortexed for 10 seconds.

v) The tubes are incubated at 98°C for 8–10 minutes.

vi) The tubes are vortexed for 10 seconds and immediately put on ice.

vii) *Optional: all samples are equilibrated to a standard amount of DNA (500 ng/reaction) applying, for example, the Beta Globin-method (19).*

viii) The tubes are centrifuged at 15,000 g for 2 minutes.

ix) 5 µl is used in the PCR assay.
• Nested PCR procedure

i) Primer design and sequences

Several PCR protocols for the detection of BLV provirus sequences have been published (3, 4, 19). As an example, a PCR assay based on the one developed by Ballagi-Pordany et al. (3) is 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. The sequences of the primers are:

<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^I^-product size: 440 bp; PCR^II^-product size: 341 bp; Mimic-product size: 761 bp.

ii) Reaction mixtures

Reaction mixtures are blended (except 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} (2) should be added in separate rooms in the laboratory: laboratory room 1 for DNA preparations and mimics, and laboratory room 2 for PCR^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^I^-reaction</th>
<th>PCR^II^-reaction</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):**

<table>
<thead>
<tr>
<th>Oligo</th>
<th>PCR^I^-reaction</th>
<th>PCR^II^-reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>OBLV1A</td>
<td>1.5 µl</td>
<td>–</td>
</tr>
<tr>
<td>OBLV6A</td>
<td>1.5 µl</td>
<td>–</td>
</tr>
<tr>
<td>OBLV3</td>
<td>–</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>OBLV5</td>
<td>–</td>
<td>1.5 µl</td>
</tr>
</tbody>
</table>

In total: 38 µl

The following should be added just before starting the PCR

<table>
<thead>
<tr>
<th>Reagents per reaction (conc.)</th>
<th>PCR^I^-reaction</th>
<th>PCR^II^-reaction</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

\textsuperscript{1} Available from Dr S. Belák, Department of Virology, National Veterinary Institute, Box 585, Biomedical Centre, S-751 23, Uppsala, Sweden.
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b) **Reagents added in laboratory room 1 (DNA) or 2 (PCR²)**

<table>
<thead>
<tr>
<th>Reagents per reaction (conc.)</th>
<th>PCR¹ reaction</th>
<th>PCR² reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA sample* (or water*)</td>
<td>5 µl</td>
<td>–</td>
</tr>
<tr>
<td>PCR¹ 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**

**PCR¹-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

**PCR²-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

iv) **Laboratory procedure**

Mix PCR¹-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. Put samples in the thermoblock and start the PCR¹-programme (step iii).

Mix PCR²-reagents as described in step ii. Use separate gloves or tube openers for each individual tube when adding the PCR¹-product. Put the samples on ice. Heat the thermoblock to 80°C. Put samples in the thermoblock and start the PCR²-programme (step iii).

- **Agarose gel electrophoresis**

  Take the PCR²-products to the electrophoresis laboratory. Load approximately 10–15 µl of the samples and 23 µl loading buffer on a 2% agarose gel containing ethidiumbromide at 0.01%. 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**

  Positive samples should have PCR products of the expected size (341 bp), similar to the positive control.

ii) **Negative samples**

  Negative samples should have no PCR products of the expected size (341 bp), 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 (10).
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 (6, 8).

Weak positive and negative OIE Standard Sera for use in ELISA are available in freeze-dried, irradiated form from the OIE Reference Laboratory in the UK (see Table given in Part 3 of this Terrestrial Manual). These sera may be used to establish ELISA sensitivity. It should be noted that, although the weak positive serum has been calibrated to be equivalent to E4 diluted 1/10; it is not suitable for use in the AGID test. Because supplies are limited, it is suggested that ELISA working standards, calibrated against the Standard Sera, be derived locally.

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 in a similar manner to the antigen used in the AGID test and 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.

• 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 positive Standard Serum (E4 1/10) 1/25 in negative milk. A weak positive control should be prepared by diluting, in negative milk, the OIE positive Standard Serum (E4 1/10) 25 times the number of individual milk samples in the pool under test. The milk used for diluting the Standard 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 \times 0.750 = 0.30
   the inconclusive range in this example would be 0.30–0.39
   and samples of \geq 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).

• **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 orthophenylamine 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 negative Standard Sera. Assays should give a positive result on E4 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, E4 should be diluted 1/250 × 60 = 1/15000. Using the weak positive OIE Standard Serum currently available (E4 1/10) in the above example, the dilution used should therefore be 1/25 × 60 = 1/1500. For individual milk samples the weak positive OIE Standard Serum diluted 1/25 (E4 1/250) in negative milk must be positive.

Where pooled serum samples are tested, the weak positive OIE Standard Serum must test positive at a dilution equal to the number of individual animals in the pool. For example, for a pool of 50 individual samples, the weak positive OIE Standard Serum diluted 1/50 in negative serum should give a positive result. In assays where serum samples are tested individually, undiluted weak positive OIE Standard Serum must be positive.

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, but there are no OIE Standard Sera currently available for this test. Further advice may be obtained from the OIE Reference Laboratories listed in Part 3 of this *Terrestrial Manual*.

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 fetal lamb kidney (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 is 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 E4 as follows: a twofold dilution of the antigen preparation is made. The highest dilution that, when tested against undiluted standard serum E4, 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 AND DIAGNOSTIC BIOLOGICALS

In young calves born to a BLV-infected mother, maternal antibodies to BLV gp51 are important in protection against BLV infection (12). However, experiments using inactivated BLV, fixed infected FLK cells, and purified gp51 have indicated that these give only short-term protection. It was also found that vaccination of cattle with live cells from a cell line BL3, established from an animal with sporadic bovine leukosis, resulted in short-term protection. The nature of the antigen conferring protection was possibly a tumour-associated transplantation antigen (18). Ovine cells synthesising only the env gene products gp51 and gp30 and the main structural protein p24, induced a serological response in cattle (1); cattle were protected after repeated vaccination with these cells.

Vaccination in sheep with a recombinant vaccinia virus expressing BLV gp51 induced protection (14). However, protection was achieved without production of detectable levels of neutralising antibodies. Also, Portetelle et al. found that vaccinia virus recombinants protected sheep even though anti-gp51 antibody waned (15). It is therefore thought that a cell-mediated immune response may play a role in protective immunity against BLV infection. Expression of gp51 has been obtained by recombinant vaccinia virus and yeast containing the coding sequence of BLV env gene. These have resulted in protection of sheep (7). Despite these advances in knowledge, there is as yet no vaccine available commercially for the control of EBL.

### REFERENCES


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NB: There are OIE Reference Laboratories for Enzootic bovine leukosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.3.5.

INFECTIONOUS BOVINE RHINOTRACHEITIS/INFECTIOUS PUSTULAR VULVOVAGINITIS

SUMMARY

Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis, caused by bovine herpesvirus 1 (BHV1), is a disease of domestic and wild cattle. The virus is distributed worldwide, but has been eradicated from Austria, Denmark, Finland, Sweden and Switzerland and control programmes have started in some other countries.

The disease is characterised by clinical signs of the upper respiratory tract, such as a (muco)purulent nasal discharge, and by conjunctivitis. Signs of general illness are fever, depression, inappetance, 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. Many infections run a subclinical course. Secondary bacterial infections can lead to more severe respiratory disease.

Identification of the agent: The virus can be isolated from nasal swabs taken during the acute phase of the infection, and from various organs collected at post-mortem. Post-mortem examinations reveal a rhinitis, laryngitis, and tracheitis.

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. 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. The BHV1 isolates can be further subtyped by DNA restriction enzyme analysis.

Viral DNA detection methods have been developed, and the polymerase chain reaction technique may prove to be particularly useful for testing semen samples.

Serological tests: The virus neutralisation test and various enzyme-linked immunosorbent assays (ELISA) are most widely used for antibody detection. Antibodies can be detected in milk with an ELISA.

Requirements for vaccines and diagnostic biologicals: Attenuated and killed vaccines are available. The vaccines must protect cattle clinically in case of infection and markedly reduce the subsequent shedding of field virus. The vaccines must not induce disease, abortion, or any local or systemic reaction, and must be genetically stable. In 1995, deletion mutant marker vaccines became available. The use of a gE ELISA makes it possible to distinguish infected cattle from cattle vaccinated with such a marker vaccine.

A. INTRODUCTION

Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV), caused by bovine herpesvirus 1 (BHV1), is a disease of domestic and wild cattle. BHV1 is a member of the genus Varicellovirus in the subfamily alphaherpesvirinae, which belongs to the Herpesviridae family. The viral genome consists of double-stranded DNA that codes for about 70 proteins, of which 33 structural and up to 15 nonstructural proteins have been demonstrated. The viral glycoproteins, which are located in the envelope on the surface of the virion, play an important role in pathogenesis and immunity. Based on differences in DNA restriction enzyme analysis, BHV1 can be differentiated into subtypes 1.1 (respiratory infections), 1.2 (respiratory and genital infections) and 1.3 (neurological infections; BHV5), 2a and 2b (17). Subtype 2 virus may be less virulent than subtype 1 virus (7). There is nevertheless only one antigenic type of BHV1.
The name of the disease indicates the most prominent clinical signs. After an incubation period of 2–4 days, a serous nasal discharge, salivation, fever, inappetence, and depression become evident. Within a few days the nasal and ocular discharges change to mucopurulent. The necrotic lesions in the nose may progress to pustules and ulcers covered by a pseudomembrane that obstructs the upper airways and leads to mouth breathing. The infection may also induce abortion and a reduction in milk yield (10). Where natural mating is practised, genital infection can lead to pustular vulvovaginitis or balanoposthitis. These are characterised by mild to severe necrotic lesions in vaginal or preputial mucosae. After artificial insemination with infected semen, endometritis can arise (12). In calves infected with BHV1, a systemic disease may develop, with focal necrotic lesions in visceras and possibly a prominent gastroenteritis. Many infections run a subclinical course (32). Meningoencephalitis usually appears to be the result of an infection with a related, but distinct, herpesvirus, recently proposed as BHV5 (27), although BHV1 infection may also sporadically cause meningoencephalitis. BHV1 can affect all ages, but disease is more common in animals over 6 months of age.

Uncomplicated cases of respiratory or genital disease caused by BHV1 last 5–10 days. Secondary bacterial infections with, for example, Pasteurella spp., can give rise to more severe clinical signs due to the deeper airways being affected.

The virus enters the animal via the nose and replicates to high titres in mucous membranes of the upper respiratory tract and in the tonsils. It subsequently disseminates to conjunctivae and by neuronal axonal transport reaches the trigeminal ganglion. A low level viraemia can occasionally occur. After genital infection, BHV1 replicates in mucous membranes of the vagina or prepuce, and becomes latent in sacral ganglia. The viral DNA remains in the neurons of the ganglia, probably for the entire life of the host. Stress, such as transport and parturition, can induce reactivation of the latent infection. Consequently, the virus may be shed intermittently into the environment.

The primary lesion is a focal necrosis of nasal, laryngeal, tracheal or genital mucous membranes. This lesion is probably the direct sequel of virus replication and its subsequent cytopathic effect (CPE). The animal reacts with an intense inflammatory response. The lesions may coalesce to form large pustules that consist of massive infiltrates of leukocytes. Peripheral blood leukocytes can harbour BHV1 (9, 19). An acute BHV1 infection induces apoptosis in lymphoid cells (35). When secondary bacterial infections are involved, a pneumonia may develop. In aborted fetuses, tiny necrotic foci are present in various tissues, particularly in the liver.

An infection normally elicits an antibody response and a cell-mediated immune response within 7–10 days. The immune response is presumed to persist for life, although it may fall below the detection limit of some tests. However, the protective immunity after infection is not lifelong: cattle can be reinfected. Maternal antibodies are transferred via colostrum to the young calf, which is consequently protected against BHV1-induced disease (16). 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, paralleling the distribution of domestic cattle. Other ruminants may be infected with BHV1, but this probably has no influence on the spread of BHV1 among domestic cattle. Apart from ruminants, no other reservoir of BHV1 exists. The minimal infective dose of BHV1 is not known. After infection, nasal viral shedding is detected for 10–14 days, with peak titres of $10^8$–$10^{10}$ TCID50 (50% tissue culture infective doses) per ml of nasal secretion. Airborne transmission of BHV1 is likely only over short distances (15). The semen of an infected bull may contain BHV1 and the virus can thus be transmitted by natural mating and artificial insemination (21).

The control of BHV1 is based on the normal hygienic measures taken on a farm. Ideally, a 2–3-week quarantine period is imposed for newly introduced cattle. Only cattle that are BHV1-seronegative are then admitted to the herd. Vaccines usually prevent the development of severe clinical signs and reduce the shedding of virus after infection, but do not prevent infection. Only vaccines with demonstrated efficacy and safety should be used (see Section C).

BHV1 infection may be suspected as the cause of disease on the basis of clinical, pathological and epidemiological signs. To make a definite diagnosis, however, laboratory examinations 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.

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% 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 portions of the tonsil, lung, and bronchial lymph nodes, are collected for virus detection. In cases of abortion, the fetal liver, lung, spleen, kidney and a placental cotyledon are examined. Samples should be sent to the laboratory as quickly as possible, on ice.

After arrival at the laboratory, swabs are agitated in the transport medium to elute virus and left at room temperature for 30 minutes. 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, various cell cultures 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, are all suitable. 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 BHV1. 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.

To identify the virus that produces the CPE as BHV1, the supernatant of the culture should be neutralised with a monospecific BHV1 antiserum or neutralising monoclonal antibody (MAb). For this purpose, serial tenfold dilutions of the test supernatant are made, and to each dilution monospecific BHV1 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 BHV1. 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 BHV1.

An alternative method of virus identification is by direct demonstration of BHV1 antigen in cells around the CPE by an immunofluorescence or immunoperoxidase test (11) with conjugated monospecific antiserum or MAb.

• Virus isolation from semen (a prescribed test for international trade)

At least 0.05 ml of raw semen should be tested, with two passages in cell culture. For extended semen, an approximation should be made to ensure that the equivalent of 0.05 ml raw semen is examined. Raw semen is generally cytotoxic and should be prediluted before being added to cell cultures. A similar problem may sometimes arise with extended semen. A suitable test procedure is given below.

• Test procedure

i) Dilute 200 µl fresh semen in 2 ml fetal bovine serum (free from antibody against BHV1) with added antibiotics.

ii) Mix vigorously and leave for 30 minutes at room temperature.

iii) 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.

iv) Incubate the plates for 1 hour at 37°C.
v) Remove the mixture, wash the monolayer twice with 5 ml maintenance medium, and add 5 ml maintenance medium to each well.

vi) Include BHV1 negative and positive controls in the test. Extreme 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.

vii) Observe plates under a microscope daily for the appearance of a CPE. If a CPE appears, confirmatory tests for BHV1 are made by specific neutralisation or immunolabelling methods (see above).

viii) 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.

ix) The sample is considered to be negative if there is no evidence of a CPE after 7 days’ incubation of the passaged cultures.

c) Viral antigen detection

Nasal, ocular or genital swabs can be directly smeared on to glass cover-slips, or, following centrifugation, the cell deposit (see Section B.1.a.) may be spotted on to 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 fluorescein isothiocyanate, whereas in the indirect procedure it is the anti-bovine immunoglobulin second antibody that is conjugated to fluorescein isothiocyanate. To obtain the best 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 within 24 hours. Smears from nasal swabs from cattle with a purulent or haemorrhagic nasal discharge are often negative (28). 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 (5). Positive and negative controls must be included in each test.

Tissues collected at post-mortem can be examined for the presence of BHV1 antigen by the immunofluorescence test on frozen sections. Immunohistochemistry may also be used. The advantage is that the location of the antigen can be determined. MAbs are increasingly being used for detecting BHV1 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 BHV1.

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 the well of a microplate. Amounts of antigen equivalent to $10^4$–$10^5$ TCID$_{50}$ of BHV1 are required in order to have a high rate of positive results (4). 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 BHV1. Sensitivity can be increased by amplification systems (see ref. 6 for an example).

The advantages of antigen-detection methods versus virus isolation are that no cell culture facilities are required and a laboratory diagnosis can be made in 1 day. The disadvantages are the lower sensitivity of direct antigen detection and the extra requirement to perform virus isolation, if the isolate is required for further study.

d) Nucleic acid detection

During the past decade, various methods of demonstrating BHV1 DNA in clinical samples have been described, including DNA–DNA hybridisation and the polymerase chain reaction (PCR). The PCR is also increasingly used in routine diagnostic submissions (18). 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 can also detect DNA in latently infected sensory ganglia (29). The disadvantage is that it is prone to contamination and therefore precautions have to be taken to prevent false-positive results.

So far the PCR has been used mainly to detect BHV1 DNA in artificially (37) or naturally (29, 30) infected semen samples. These workers found that it was important to thoroughly optimise the PCR conditions, including the preparation of the samples, the concentration of Mg$^{2+}$, primers and Taq polymerase, and the cycle programmes. The target region for amplification must be present in all BHV1 strains, and its nucleotide sequence must be conserved. The TK, gB, gC, gD and gE genes have been used as targets for PCR amplification. PCRs based on detection of gE sequences can be used to differentiate between wild-type virus and gE-deleted vaccine strains (9, 26). Discrimination between infection with virulent IBR strains and infection with other live attenuated strains is not possible with the PCR technique. PCRs have been developed that discriminate between BHV1 and BHV5 (1, 25).

Experimentally, the PCR was found to be more sensitive than virus isolation: it detected five times as many positive samples as did virus isolation. In addition, it had a detection limit of only three molecules. 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 (25, 29, 30).

Before the PCR can be internationally recognised as a suitable diagnostic tool for animal trade, it would have to be validated by an inter-laboratory comparative test.

e) Differentiation of bovine herpesvirus 1 subtypes

By using MAbs and immunofluorescence, radioimmunoprecipitation, immunoperoxidase or immunoblot assays, BHV1 subtype 1 and subtype 2b can be differentiated (24, 36). Restriction endonuclease analysis makes it possible to differentiate among all the recognised BHV1 subtypes (17). The DNA is first extracted from virions or infected cells, digested by restriction endonucleases, and the resulting fragments are separated by agarose gel electrophoresis. The number and size of the fragments indicates the subtype of the virus. Such techniques are of limited diagnostic value, but may be useful in epidemiological studies.

f) Interpretation of results

The isolation of BHV1 from an animal does not unequivocally mean that this virus is the cause of the disease outbreak. 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 titre rise in antibodies to BHV1. Cattle from which the nasal swabs are to be collected must be bled twice, 2–3 weeks apart. These paired serum samples are examined together in a serological test for the presence of specific antibody (see Section B.2).

2. Serological tests

Serological tests can be used for several purposes:

i) To diagnose an acute infection: serum samples from the acute and convalescent stages of infection in the same animals are examined in one test. A seroconversion from negative to positive or a four-fold or higher increase in antibody titre is considered to indicate infection.

ii) To demonstrate the 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 and various ELISAs (13) are usually used for detecting antibodies against BHV1 in serum. An alternative serological test is the indirect fluorescent antibody test (33). Because virus latency is a normal sequel to BHV1 infection, the identification of serologically positive animals provides a useful and reliable indicator of infection status. Any animal with antibody to the virus is considered to be a carrier and potential intermittent excretor of the virus. The only exceptions to this are young calves that have acquired passive colostral antibody from their dam, and noninfected cattle vaccinated with inactivated vaccines.

ELISAs, including the gE-ELISA, are increasingly used for the detection of antibodies in (bulk) milk samples, but have some limitations. A negative bulk milk test indicates that not more than 20% of the adult milking herd has antibodies to BHV1. Many individual seropositive cows have a milk antibody titre less than 1/5. This titre may vary somewhat, depending on the detection level of the ELISA used. Consequently, it is not possible to declare a herd to be free from BHV1 infection on the basis of bulk or pooled milk tests, and a negative bulk milk test should be followed up with individual serum samples from all cattle in the herd. For general surveillance purposes, bulk milk tank tests can give an estimate of BHV1 prevalence in a herd, an area or country (20). These should be supplemented by serum testing (individual or pooled) from nonmilking herds.

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%) (22). Of these variables, the virus/serum incubation period has the most profound effect on the antibody titre. A 24-hour incubation period may score up to 16-fold higher antibody titres than a 1-hour incubation period (2), 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 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 two 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 BHV1 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 18–24 hours at 37°C.

vi) Add 100 µl per well of the cell suspension at $3 \times 10^4$ 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 $\pm 1$ twofold dilution ($\pm 0.3 \log_{10}$ units) from its target value. The weak positive serum should be positive. The negative serum should give no neutralisation when tested undiluted (equivalent to a final dilution of 1/2 at the neutralisation stage). In the cell control wells, the monolayer 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 serum toxicity 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 there is cytotoxicity with a serum from which it is critical to obtain a result, changing the medium in the wells of the lowest two or three dilutions 16–24 hours after the addition of cells will remove the cytotoxic effect with many problem sera.

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

ELISAs for the detection of antibody against BHV1 appear to be gradually replacing VN tests. A standard procedure for ELISA has not been established. Several types of ELISA are available, including indirect and blocking ELISAs. Indirect ELISA procedures are more commonly used. Various ELISA kits are available commercially, most of which are also suitable for detecting antibodies in milk. For reasons of standardisation in a country or state, it may be desirable to compare the quality of the kits and to test each batch by previously defined criteria in one national reference laboratory, before it is used in 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. For this purpose, a panel of well defined (e.g. by VN test) strong positive, weak positive and negative sera should be tested.

- **Indirect enzyme-linked immunosorbent assay**

An example of an indirect ELISA procedure is given below:

i) Prepare the antigen by growing BHV1 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 the supernatant is used at an appropriate dilution to coat the plates. Many alternative methods of antigen production may be found in the published literature.
ii) Coat the microtitre plate with antigen by adding 100 µl of diluted antigen (in 0.05 M carbonate buffer, pH 9.6) to each well of a microplate. Seal the plates with tape, incubate at 4°C overnight or 37°C for 1–2 hours, and store at −20°C.

iii) Before the test is performed, wash the plates. To prevent nonspecific binding in the test, resulting in high background levels, a second coating with a nonrelevent protein (e.g. albumin) may be necessary. Add buffer, the serum sample and positive, weak positive and negative control sera. Usually, serum samples are diluted 1/10 to 1/100 in PBS with 0.05% Tween 20. Shake and seal the plates and incubate for 1 hour at 37°C.

iv) Wash the plates thoroughly, add anti-bovine immunoglobulin/horseradish peroxidase conjugate at a predetermined dilution, and incubate again for 1 hour at 37°C.

v) Add freshly prepared substrate/chromogen solution (e.g. 0.1 M citrate phosphate buffer, pH 4, containing 2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]).2NH₄[ABTS; 0.5 mg/ml] and a 3% solution of freshly added H₂O₂ [2 µl/ml], and incubate for the appropriate time.

vi) Measure the absorbance of the plates on a microplate photometer at the appropriate wavelength.

vii) A test sample is considered to be positive if it has an absorbance value of 0.2 higher than the negative control serum. The test is valid if the positive and weak positive sera are positive and if the negative serum has an absorbance value less than 0.2. Invalid tests should be repeated. The acceptable limits for control and cut-off values must be determined for the individual assay.

- **Blocking enzyme-linked immunosorbent assay**

  The principle of a blocking or competitive ELISA is based on blocking the binding of antigen to an enzyme-labelled BHV1 antiserum or anti-BHV1 MAb by antibodies in the test sample. The presence of antibodies in the test sample will block binding, resulting in reduced colour development after addition of the substrate/chromogen solution. A comparison of indirect ELISAs and blocking ELISAs showed the latter to be generally more sensitive (22). Recently, gE-ELISAs have been described that can be used in conjunction with marker vaccines to detect infected cattle in vaccinated populations (31, 34). In one gE-ELISA, milk can replace serum (34).

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 recently agreed on the use of a strong positive, a weak positive and negative serum for standardisation of BHV1 tests in laboratories that routinely examine samples from artificial insemination centres (23). These sera have been adopted as OIE international standards for BHV1 tests and are available at the OIE Reference Laboratories for IBR/IPV1. 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.

### C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Several attenuated and inactivated BHV1 vaccines are available currently. The vaccines contain virus strains that 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.

Marker vaccines are now available in various countries. These attenuated or inactivated marker vaccines are based on deletion mutants or on a subunit of the virion, for instance glycoprotein D. The use of such marker vaccines in conjunction with companion diagnostic tests makes possible the distinction between infected and vaccinated cattle, and may thus provide the basis for novel eradication programmes of BHV1. Intensive vaccination programmes can reduce the prevalence of infected animals (3, 14), which could be monitored by using a companion diagnostic test. In situations where it is economically justifiable, the residual infected animals could then be culled, if necessary, resulting in a region free from BHV1. Some countries recently implemented such an eradication programme and vaccination is no longer allowed.

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1 Obtainable from the Central Institute for Animal Disease Control, Infectious Diseases Section, P.O. Box 2004, 8203 AA Lelystad, The Netherlands, and AFSSA Lyon, Laboratoire de pathologie bovine, 31 avenue Tony Garnier, BP 7033, 69342 Lyon Cedex 07, France.
Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 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 vaccine is prepared using a seed-lot system. The 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 BHV1 strains that have been 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 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.

b) Method of culture

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 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.

c) Validation as a vaccine

Irrespective of the method of preparation of the seed-lot vaccine virus, the seed-lot virus destined for incorporation in a live vaccine must be shown to be efficacious, safe and pure.

i) Efficacy

This must be shown in a vaccination challenge experiment under laboratory conditions. Example guidelines are given in a monograph of the European Pharmacopoeia (8). Briefly, the vaccine is administered to five 2–3-month-old BHV1 seronegative calves. Two calves are kept as controls. All the calves are challenged intranasally 3 weeks later with a virulent strain of BHV1 that gives rise to typical signs of a BHV1 infection. The vaccinated calves should show no or only mild signs. The maximum virus titre found in the nasal mucus of vaccinated calves should be at least 100 times lower than that found in control calves. The virus excretion period should be at least 3 days fewer in vaccinated than in control calves.

ii) 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.

iii) Purity

The seed lot is tested for absence of extraneous viruses and absence from contamination with bacteria, fungi or mycoplasmas. The following extraneous viruses should be specifically excluded in BHV1 vaccines: adenovirus, Akabane virus, bovine coronavirus, bovine herpesviruses 1, 2, 4 and 5, bovine parvovirus, bovine respiratory syncytial virus, bovine rotavirus, vaccinia virus, and the viruses of Aujeszky's disease, bluetongue, bovine ephemeral fever, bovine leukaemia, bovine papilloma, bovine parvovirus, bovine viral diarrhoea, cowpox, foot and mouth disease, lumpy skin disease, malignant catarrhal fever, parainfluenza 3, rabies, rinderpest, and vesicular stomatitis. As bovine viral diarrhoea virus has regularly been found to be a contaminant of vaccines, special attention should be paid to ensure that it is absent.

2. Method of manufacture

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 detecting complete inactivation of the virus is carried out. The test should consist of 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).

3. In-process control

Working cell seed and working virus seed must have been shown to be free from contaminants. The cells must have their normal morphology before being inoculated with virus. They are checked for CPE 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 should be tested for freedom from contaminants.

4. Batch control

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.

a) Sterility

Bacteria, fungi, mycoplasmas and extraneous viruses must not be present. Tests for sterility and freedom from contamination of biological material may be found in Chapter I.1.5.

b) 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 BHV1 seronegative calves.

c) Potency

It is sufficient to test one representative batch for efficacy, as described in Section C.1.c.i. In the case of live vaccine, 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.

d) Duration of immunity

It is sufficient to test this on the seed lot of vaccine virus. An efficacious BHV1 vaccine should induce protective immunity for at least 1 year, although many existing vaccines have not been tested to this standard.

e) 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.

f) Preservatives

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.

g) Precautions (hazards)

No special precautions need to be taken. BHV1 is not pathogenic for humans.

5. Tests on the final product

a) Safety

Each product must be shown to be safe in at least two susceptible calves that receive a twofold (inactivated vaccine) or a tenfold (live vaccine) dose of vaccine.

b) Potency

See Section C.4.c.
REFERENCES


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**NB:** There are OIE Reference Laboratories for Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
Bovine venereal trichomonosis is caused by Tritrichomonas foetus, a flagellate protozoan. 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. In areas of the world where there is widespread use of artificial insemination, the prevalence is much reduced, though it is still of importance in beef herds or in other circumstances 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.

Identification of the agent: Tritrichomonas foetus is a flagellate, pyriform eukaryotic protozoan, approximately 8–18 µm long and 4–9 µm wide, with three anterior and one posterior flagellae and an undulating membrane. 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. Organisms can be cultured in vitro, and may be viewed in a wet mount or stained slide. The standard diagnostic method for bulls involves the 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 more recently a commercially available field culture test has been introduced that allows for trichomonad growth and direct microscopic examination.

Alternative tests: The infection may also be detected by polymerase chain reaction (PCR) 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 and diagnostic biologicals: 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 trichomonosis is caused by the flagellate protozoan, Tritrichomonas foetus. The natural hosts of T. foetus are cattle (Bos taurus, B. indicus). Nonpathogenic species of trichomonads occur in the intestine of cattle; T. suis of pigs is indistinguishable morphologically, serologically and genetically from T. foetus.

Tritrichomonas foetus is pyriform, 8–18 µm long and 4–9 µm wide, with three anterior and one posterior flagellae, and an undulating membrane. Living 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 by Warton & Honigberg (47). It is important to differentiate T. foetus from other contaminant flagellated...
protozoa that may be present in the bovine reproductive tract (3, 6, 33, 45). Under phase illumination, the number of flagella 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 (25).

The organism multiplies by longitudinal binary fission; there is no sexual reproduction and environmentally resistant stages of the parasite have not been observed.

In a few early studies, three serotypes were recognised based on agglutination (42): the ‘belfast’ strain, reportedly predominated in Europe, Africa and the USA (23); the ‘brisbane’ strain in Australia (12); and the ‘manley’ strain, which has been reported in only a few outbreaks (42). Little further work has been done in this area. More 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 made with surety.

Organisms may be cultured in vitro, preferably in Diamond’s medium (10), Clausen’s medium (28) or *Trichomonas* medium, which is available commercially (40). A field culture test that allows for growth of the trichomonads and direct microscopic examination without aspiration of the inoculum has been developed in the USA (41, 46) (InPouch™ TF, see footnote 2).

Transmission of infection under natural conditions is by coitus, by artificial insemination, or by gynaecological examination of cows. The site of infection in bulls is primarily the preputial cavity (1, 35), and little or no clinical manifestation occurs. For bulls older than 3-4 years, spontaneous recovery rarely occurs, and the bull becomes a permanent source of infection. In bulls under 3–4 years old, infection may be transient.

Organisms are present in small numbers in the preputial cavity of bulls, with some concentration in the fornix and around the glans penis (20). The chronically infected bull shows no gross lesions. In the 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 placentalitis 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 exhibit irregular oestrous cycles, uterine discharge, pyometra, and early abortion (1, 15, 42). The cow usually recovers and generally becomes immune, at least for that breeding season, after infection or abortion (1, 15, 44).

**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 trichomonositis is based on the clinical history, signs of early abortion, repeated returns to service, or irregular oestrous cycles. Confirmation depends on the demonstration of organisms in placental fluid, stomach contents of the aborted fetus, uterine washings, pyometra discharge, or vaginal mucus. In infected herds, the most reliable material for diagnosis is either preputial or vaginal washings or scrapings (24, 29, 31, 41). In Western Europe, European Union (EU) Directives require collection of preputial washings, or in the case of female animals, a vaginal mucus agglutination test (13).

   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. Thereafter the number of organisms varies according to the phase of the oestrous cycle, being highest 3–7 days after ovulation. In the infected bull *T. foetus* organisms are present in highest numbers on the mucosa of the prepuce and penis, apparently not invading the submucosal tissues. It is generally recommended to allow 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* (45). 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 (31, 41) or metal brush (30, 31), by preputial lavage (41) or by washing the artificial vagina after semen
collection (19). The latter technique is not recommended as its sensitivity may be lower (19). Samples from cows are collected by washing the vagina, or by scraping the cervix with an artificial insemination pipette or metal brush (24, 27).

Where samples must be submitted to a laboratory and cannot be delivered within 24 hours, a transport medium should be used (e.g. Winters’ medium, buffered saline solution with 5% fetal bovine serum, or skim milk, with or without antibiotics [37] or in thioglycollate [5]), or the field culture plastic pouch (5, 46). 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 (5).

**Culture**

Where organisms are too few to allow for direct detection and accurate identification, cultures should be prepared. Culture of the organisms 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. The CPLM (cysteine/peptone/liver-infusion maltose) medium, BGPS (beef-extract/glucose/peptone serum) medium, Clausen’s medium (Neopeptone-Lemco-liver extract glucose), Diamond’s trichomonad medium, Oxoid’s *Trichomonas* medium and the commercial culture kit are the media of choice (11, 28, 32, 40). 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 centrifuging. The sediment is then examined and inoculated into culture media. 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™ (InPouch™ TF system, see footnote 2) using the specially provided plastic clip. The organisms may be seen under a standard light microscope using a magnification of 100 or more. An inverted microscope may be useful for examining tubes containing (Diamond’s) culture medium. Culture media should be examined microscopically at intervals from day 1 to day 7 after inoculation (26). 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 recently developed rapid-staining procedure may also be used (25). 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 trypsinase 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 K$_2$HPO$_4$ and KH$_2$PO$_4$, 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 (1, 26). The incorporation of agar into the medium confines contaminating organisms largely to the upper portion of the test tube, while helping to maintain microaerophillic conditions at the bottom of the tube where the trichomonads occur in largest numbers.

- **Field culture test (InPouch™ TF)**

  Where a combination of convenience and sensitivity is required, a field culture test (InPouch™ TF system) may be used (1, 4, 32, 41, 46). This 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 (1, 41). 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 (4). Diagnostic results with samples from bulls using either Diamond’s medium or the InPouch™ TF system have shown that the two methods give comparable results or that there are some advantages (in convenience and in test results) with the InPouch™ system (4, 5, 24, 32, 41).
• 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, 84–96%) (31). Estimates for Diamond’s and related media have been variable, possibly due to variation in composition and preparation, but range from 78% to 99%. 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 (31). With samples from females, there are few estimates of the diagnostic sensitivity of the standard sample and culture method.

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 88% was achieved through a 10-week period after infection (24).

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 can be used to demonstrate tissue-invasive T. foetus organisms in aborted fetuses.

b) Polymerase chain reaction

Molecular-based techniques that use polymerase chain reaction (PCR) technology have been exploited for the identification of T. foetus (14, 21). Development of 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. Initial research (14, 21) demonstrated that DNA primers are capable of detecting very low numbers of parasites from laboratory cultures of the organism with no preputial material present. However, in preputial samples, 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 (14, 21, 34) and it is likely that the sensitivity of the diagnostic test will be influenced by the efficiency of the extraction method and the procedures to overcome contaminating inhibitors. The diagnostic sensitivity of PCR tests has been estimated to be similar to that of the InPouch™ TF culture kit (14, 21). However, too few samples from only a small population of infected bulls have been analysed to determine a sound estimate of the sensitivity.

Diagnostic specificity of the PCR test depends on the specificity of the primers. One set of primers (21) yielded similar sized nonspecific products in approximately one-third of negative control samples. A set of primers based on the 5.8s rRNA sequence demonstrated good diagnostic specificity in samples from negative animals (14), but further work with these primers using different populations of animals is needed. These primers 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 (14, 18). It is possible that some of these species are synonymous with T. foetus. Recent work has demonstrated that these primers can be used to differentiate between T. foetus and a non-T. foetus trichomonad sometimes found in preputial samples (3, 6, 33). DNA-based techniques have potential as an ancillary or primary test (3, 6, 14, 29, 34). Although this technology shows promise in research, further work evaluating and validating its application in the clinical diagnosis of T. foetus infections needs to be done before it replaces the culture method as the routine diagnostic test.

2. Alternative tests

In the 1940s, mucus agglutination tests and intradermal diagnostic tests were developed and are still in limited use, but problems with sensitivity and specificity restrict their usefulness. Other immunological tests based on the antigen-trapping enzyme-linked immunosorbent assay (ELISA) are now being developed (1, 17). Immunohistochemical techniques using monoclonal antibodies have been shown to reveal T. foetus organisms in formalin-fixed tissues (38).

a) Mucus agglutination test

A mucus agglutination test was developed in the 1940s (23, 36) that detects about 60% of naturally infected cows, antibody levels varying according to stage of oestrus. Mucus samples are collected from the cervical
region of the vagina, preferably a few days after oestrus. Antibodies appear in cervical mucus about 6 weeks after infection, and persist for several months. Antibodies may also be found in preputial secretions (39, 43). The mucus agglutination test is most useful as a herd test, being capable of detecting latent or recently cleared infections. It is specific and does not cross-react with *Campylobacter foetus* or *Brucella abortus*, but lacks sensitivity.

A sterile glass tube, 30 cm in length, 9 mm in diameter, and bent at an angle of 15° approximately 9 cm from one end, or an artificial insemination pipette, should be used for taking the cervical sample. Any mucus containing blood should not be used and the animal should be re-sampled. Serum contains nonspecific antibodies and will cause agglutination to occur. The mucus is diluted 1/5 with physiological saline and emulsified in a Griffith's tube. Duplicate samples, diluted to 1/10 and 1/20, are prepared by pipetting 2 ml of mucus and 2 ml of melted agar (56°C in a water bath) into tubes for the 1/10 dilution, and 1 ml mucus, 1 ml saline and 2 ml melted agar for the 1/20 dilution. Duplicate controls containing 2 ml saline and 2 ml melted agar are also prepared. All tubes are kept in a water bath at 56°C during mixing and then poured individually into 5 cm Petri dishes and allowed to cool. The test antigen is made up by slowly adding a trichomonad culture to a 2/1 mixture of saline and 1% glucose broth to achieve a concentration of approximately 100,000 organisms/ml (approximately six trichomonad/microscope field at ×400). Next, 1.5 ml of antigen is added to each Petri dish, the dishes are incubated for 1.5 hours at 37°C and then left at room temperature for a further 1.5 hours. Agglutination at a dilution of 1/10 is considered to be positive.

b) **Intradermal ‘Tricin’ test**

An intradermal test for diagnosis of bovine trichomonosis has been reported (22). The injection site is in the skin of the neck, similar to the site used for the tuberculin test. A dose of 0.1 ml of the ‘Tricin’ antigen is injected intradermally and the reaction is measured 30–60 minutes later. The reaction consists of a shallow plaque observed visually and showing an increase of >2 mm in skin thickness.

c) **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 (38). 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 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-amino-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 AND DIAGNOSTIC BIOLOGICALS**

Whole cell vaccines for cows have been shown to offer protection and are available commercially (9) as either a monovalent ‘bacterin’ or part of a polyvalent vaccine also containing *Campylobacter* and *Leptospira* spp. (CL-vaccine) (1) (see footnote 3). These products show efficacy in the female but not in the bull (2). This is in contrast to earlier studies in Australia in which protection or even clearance was afforded to bulls receiving membrane or glycoprotein fractions of *T. foetus* (7, 8). Specific antibodies have been demonstrated in serum and vaginal mucus of young cows inoculated with a vaccine containing *T. foetus* (17). In this study a partially effective killed whole-cell vaccine did not prevent infection, but appeared to allow clearance of the infection from vaccinated females before the time in gestation when the fetus is generally most at risk from abortion. More effective vaccines that make use of membrane surface antigens from *T. foetus* are being sought and offer the potential of a recombinant vaccine (9, 16).

Whole cell vaccine is produced by growing *T. foetus* (culture VMC-84) in modified Diamond’s medium (9) 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**


4 DAKO Corporation, Carpinteria, California, USA.


Chapter 2.3.6. — Trichomonosis


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CHAPTER 2.3.7.

BOVINE ANAPLASMOSIS

SUMMARY

Bovine anaplasmosis results from infection with Anaplasma marginale. A second species, A. centrale, has long been recognised. Whether it truly represents a separate species is unclear. Anaplasma marginale is responsible for almost all outbreaks of clinical disease. The organism is classified in the genus Anaplasma belonging to the family Anaplasmataceae of the order Rickettsiales. Genetically, A. marginale clusters within genogroup II of the Ehrlichiae.

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. 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: The most widely used serological tests have been the complement fixation (CF) and the card agglutination tests. However, recent data confirm that the CF test has an unacceptably low sensitivity (20%) in identification of persistently infected cattle. Therefore the CF test is considered an unreliable test for disease certification of individual animals. A competitive enzyme-linked immunosorbent assay (C-ELISA) that is commercially available has been demonstrated to have much improved sensitivity (96%) in detecting carrier animals. Indirect ELISA, dot ELISA and indirect fluorescent antibody tests also can be used.

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. Caution is warranted with polymerase chain reaction-based assays when used diagnostically, as a nested reaction is necessary to identify low-level carriers and nonspecific amplification can occur.

Requirements for vaccines and diagnostic biologicals: Live vaccines are used in several countries to protect cattle against A. marginale infection. 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 usually 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. Appendages associated with the Anaplasma body have been observed in certain isolates of A. marginale (17); although this parasite has been termed A. caudatum, it is not considered to be a separate species.

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 some 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 1957 they have been classified in the family Anaplasmataceae of the order Rickettsiales. Reorganisation of the family, which in the past has included the genera Anaplasmataceae, Aegyptianella, Haemobartonella and Eperythrozoon (31), has been proposed recently based on a combination of 16S ribosomal RNA, groesl, and surface protein gene sequence analysis (6, 37). In the reorganised family, Anaplasmataceae would now include all alpha subdivision Proteobacteria presently in the genera Ehrlichia, Anaplasma, Cowdria, Wolbachia, and Neorickettsia, with provisional retention of Aegyptianella. Moreover, the genus Anaplasma would be expanded to include Ehrlichia phagocytophila, Ehrlichia bovis, and Ehrlichia platys, with the newly named Anaplasma phagocytophila to include both Ehrlichia equi and Ehrlichia ‘HGE agent’. Haemobartonella and Eperythrozoon are now considered most closely related to the mycoplasmas.

Anaplasma species are transmitted either mechanically or biologically by arthropod vectors. A review based on a careful study of reported transmission experiments lists 14 different ticks as capable of transmitting A. marginale experimentally (18). These are: Argas persicus, Omithodorus lahorensis, Boophilus annulatus, B. decoloratus, B. microplus, Dermacentor albipictus, D. andersoni, D. occidentalis, D. variabilis, Hyalomma excavatum, Ixodes ricinus, Rhipicephalus bursa, R. sanguineus and R. simus. The authors concluded that some of these reports, including those of R. bursa, H. excavatum and O. lahorensis, were not entirely convincing, and that the ticks identified as A. persicus were probably either A. sanchezi or A. radiatus. In addition, Rhipicephalus e. evertsi and Hyalomma m. rufipes have been listed as experimental vectors in South Africa (28). Intrastadial or transstadial transmission is the usual mode, even in the one-host Boophilus species. Male ticks may be particularly important as vectors. 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 (30). The importance of biting insects in the natural transmission of anaplasmosis has not been well documented, and 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 multihost 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.

B. DIAGNOSTIC TECHNIQUES

The most marked clinical signs of anaplasmosis are anaemia and jaundice, the latter occurring late in the disease. Haemoglobinemia 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.

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 Anaplasma 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*, *Anaplasma* do 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 (14), 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 (14).

Both blood and organ smears are fixed in absolute methanol for 1 minute and stained in 10% Giemsa stain for 30 minutes. After staining, the smears are rinsed three or four times with tap water to remove adhering stain, and are then air-dried. Smears are examined under oil immersion at a magnification of ×700–1000.

In Giemsa-stained smears, *A. 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. Commercial stains that give very rapid staining of *Anaplasma* are available in some countries.

The percentage of infected erythrocytes varies with the stage and severity of the disease. Maximum parasitaemias in excess of 50% may occur with *A. marginale*. Multiple infections of individual erythrocytes are common during periods of high parasitaemias.

The infection becomes visible microscopically 2–6 weeks following transmission. During the course of clinical disease, the parasitaemia 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.

Fluorescent antibody staining may be used as an alternative staining technique for detecting *Anaplasma* in smears taken at post-mortem. A direct fluorescent antibody procedure for the post-mortem diagnosis of anaplasmosis has been described in detail by Johnston *et al.* (14). While it has been shown to be more sensitive than Giemsa staining for this purpose, nonspecific fluorescence is a significant problem.

An expensive procedure, but one that may occasionally be justified to confirm infection, particularly in latently infected 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 recently (8, 9, 11, 12, 33). A radioactive RNA probe, which detected parasitaemias as low as 0.000025%, has been described (8). Infected ticks have also been identified using a cloned DNA probe (13). 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 (9, 11). A sensitive and potentially specific nested PCR has recently been used to identify *A. marginale* carrier cattle (33). This technique is capable of identifying as few as 30 infected erythrocytes per ml of blood, equivalent to a parasitaemia of approximately 0.000001%, well below the lowest levels in carriers (33). However, nested PCR poses significant quality control problems for routine use (33). Laboratories running this assay should recognise problems in specificity due to nonspecific amplification.

An additional step such as restriction enzyme analysis, Southern hybridisation, or sequencing can confirm the specificity of the amplicon.

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1. Commercial stains include Camco-Quik and Diff-Quik, Baxter Scientific Products, McGaw Park, Illinois, USA, and Hema-Quik, Curtin-Matheson, Houston, Texas, USA.
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) is the preferred method of identifying infected animals.

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 an acute parasitaemic episode. Thus, a number of serological tests have been developed with the aim of detecting latently 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 rarely been adequately addressed. An exception is the recently described C-ELISA (see below), which has been validated using true positive and negative animals defined by nested PCR (33). 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.

It should be noted that there is a high degree of cross-reactivity between *A. marginale* and *A. centrale* in serological tests. 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 (16, 23, 33, 36). All *A. marginale* strains tested, *A. ovis* and *A. centrale*, express the MSP5 antigen and induce antibodies against the immunodominant epitope recognised by the MSP5-specific MAb. The test 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 (16). 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% (33). An independent study using an indirect ELISA (I-ELISA) validates the use of rMSP5 as a diagnostic antigen (29). However, initial studies suggest that in its current format the indirect rMSP5 ELISA is less sensitive than the C-ELISA (29).

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 × MAb/peroxidase conjugate,
  - 10 × 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.

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2 VMRD, Inc., 4641 Pullman–Albion Rd., P.O. Box 502, Pullman, Washington 99163, USA. Tel.: (1.509) 334.58.15; Fax: (1.509) 332.53.56; http://www.vmrd.com
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) 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:

\[
\frac{100 - \text{Sample OD} \times 100}{\text{Mean negative control OD}} = \text{Per cent inhibition}
\]

Samples with <30% inhibition are negative. Samples with ≥30% inhibition are positive.

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. The CAT antigen is a suspension of A. marginale particles. Splenectomised calves are infected by intravenous inoculation with blood containing Anaplasma-infected erythrocytes. When the parasitaemia 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 (1, 2) 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 antigen3. 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.

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

Detailed manuals for the conduct of both the standard (34) and the microtitre techniques (35) for the CF test for anaplasmosis have been produced. While the CF test using both techniques has been used extensively for many years, recent data confirm that it lacks sensitivity and fails to detect a significant proportion of carrier cattle (3). Therefore, the CF test can no longer be recommended as a reliable assay to detect infected animals.

3 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).
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**d) Indirect enzyme-linked immunosorbent assay**

An I-ELISA based on the use of a normal red blood cell antigen (negative antigen) and an *A.-marginale*-infected red blood cell antigen (positive antigen) has been found to be reliable for the detection of *A.-marginale*-positive sera (7). Although more cumbersome than tests using only one antigen, this test eliminates those sera that have high levels of nonspecific activity due to iso-antibodies to normal red blood cell components. The test correctly identified all 100 known positive sera taken from cattle up to 3 years after infection, while 3% of negative sera, 2% of *Babesia bovis* and 4% of *B. bigemina* sera gave false-positive results.

The two antigen test is conducted as follows.

- **Negative antigen**

Blood is collected into Na₂-ethylene diamine tetra-acetic acid (EDTA) from a splenectomised calf. The cells are washed three times with phosphate buffered saline (PBS), and centrifuged at 5000 g for 10 minutes at 5°C. After the final centrifugation, white blood cells are removed by diluting the cells tenfold in PBS and passing them through a Whatman CFI 1 cellulose column. Following further centrifugation, one volume of cells is diluted in two volumes of PBS and stored as 2.5 ml aliquots in the vapour phase of liquid nitrogen. An aliquot is thawed at room temperature, as required, and sonicated for 30 seconds at 100 W with a small probe at 0°C. The whole sonicate is then diluted as required for use in the ELISA.

- **Positive antigen**

A calf previously used to produce negative antigen is infected with *A. marginale* by blood inoculation. When the parasitaemia reaches 80–85%, 500–1000 ml of blood is collected, processed and stored as described above. When required, aliquots are sonicated, as before, and diluted appropriately.

- **Test procedure**

Flat-bottomed 96-well micro-ELISA plates are used. Standard antigen checkerboard titrations are performed with each new batch of antigen, both positive and negative, to determine the working range, which is usually 1/400 (150–200 µg protein/ml). Diluted antigen (200 µl) is added to each well on the plate, one-half of the plate for positive antigen, the other half for negative antigen. The plates are sealed with plate sealers and incubated at 4°C overnight. After incubation, the antigen solution is removed and the plates are washed three times in PBS containing 0.1% Tween 20 (PBST), and then blocked with a 1% solution of normal horse serum in PBS for 60 minutes at 37°C.

After blocking, the plates are washed five times in PBST prior to the addition of 200 µl of test sera. (Test sera are diluted 1/400 to 1/800 in PBST containing 1% horse serum.) The plates are sealed with plate sealers and incubated for 2 hours at 37°C. The wells are then washed five times with PBST, and 200 µl of conjugated second antibody (goat anti-bovine IgG conjugated with horseradish peroxidase), at a dilution of 1/400 in PBS containing 1% horse serum, is added. The plates are then sealed and incubated for 2 hours at 37°C. After incubation, the conjugate is removed and the wells are washed five times in PBS prior to the addition of 200 µl of freshly prepared substrate.

Substrate is prepared as follows; recrystallised 5-amino salicylic acid is dissolved at 1 mg/ml in phosphate buffer, pH 6.8, at 37°C; 2 µl of 30% hydrogen peroxide is then added to each 1 ml of solution. After the addition of the substrate, the plates are sealed and gently agitated for 30 minutes at 22°C. Plates are then read immediately at 492 nm with a plate reader. Column one of each plate is always used as a blank.

Net readings are calculated for each sample by subtracting the negative antigen result from the positive antigen result. A group of 20 negative sera and a standard positive *A. marginale* serum are routinely tested with each batch of assays. A ‘threshold’ is calculated as the mean net absorbance reading (plus two standard deviations) of the 20 standard negative sera. Ratios of all other net absorbances are determined against the ‘threshold’ as unity. The positive serum is used to determine that each assay batch is performing normally.

**e) Dot enzyme-linked immunosorbent assay**

Compared with the I-ELISA, the dot ELISA has the potential advantages of being rapid, inexpensive and simple to perform. The dot ELISA described below has been reported to have a sensitivity of 93% and a specificity of 96% (21).

- **Antigen preparation**
  
i) Collect high parasitaemic blood in EDTA.
  
ii) Remove the plasma and wash the erythrocytes in 0.1 M glycine buffer, pH 3.0.
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iii) Wash the erythrocytes in PBS, pH 7.4, in the presence of protease inhibitors (3.5 mM sodium tetrathionate) and 0.01% thimerosal.

iv) Remove theuffy coat after each wash.

v) Resuspend the erythrocytes in PBS at a 1/5 dilution, sonicate, subject to three freeze–thaw cycles, and resonicate the lysate.

vi) Mix the material with an equal volume of 1% Nonidet P-40 in PBS, and incubate for 3 minutes at 25°C.

vii) Recover the Anaplasma bodies by differential centrifugation.

eviii) Wash the pellet three times in cold buffer (155 mM choline chloride, 5 mM HEPES [N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid], pH 7.4).

ix) Resuspend the pellet in PBS, and measure the protein concentration.

ix) Dot the antigen (1.0 µl/disc; 25 µg protein/ml) on to discs (6 mm in diameter) cut from nitrocellulose using a paper punch. Air dry the antigen-spotted discs. Antigen on these discs remains stable for over 2 years when held at –20°C or at 4°C, and for at least 3 months when held at 25°C.

- Test procedure
  i) Place the antigen-spotted discs in wells in flat-bottomed microtitration plates.
  ii) All procedures are undertaken at 25°C and using 200 µl reaction volumes.
  iii) Include known positive and negative sera and antigen controls.
  iv) For blocking, add PBS containing 0.5% Tween 20 to each of the wells, which are then incubated for 15 minutes.
  v) Test the sera at an initial concentration of 1/200 in PBS containing 0.05% Tween 20, and incubate for 1 hour.
  vi) Wash three times for 10 minutes each time with PBS containing 0.1% Tween 20.
  vii) Add alkaline-phosphatase/protein-A conjugate, diluted 1/500 in PBS containing 0.5% Tween 20, and incubate the wells for 30 minutes, followed by washing as before, except that the last wash is in PBS alone.
  viii) Add nitroblue tetrazolium/5-bromo-4-chloro-indoxyl phosphate in 0.1 M Tris buffer, pH 9.5, and allow the colour to develop for 10–20 minutes.
  ix) A purple-coloured dot of variable intensity indicates a positive reaction; no colour is seen on discs with negative reactions.

f) 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 2.3.8., except that A. marginale-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 parasitaemia (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 (22). 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.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Several immunisation methods have been used to protect cattle against anaplasmosis in countries where the disease is endemic, but none is ideal (19). 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. Another method involves the use of a strain of A. marginale attenuated by passage in nonbovine hosts, such as deer or sheep (32).

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 (4, 10, 25).
Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 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.

### 1. Seed management

#### a) Characteristics of the seed

*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) (10). In the humid tropics where *A. marginale* appears to be a very virulent parasite, 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.

Rapid passage of *A. centrale* in splenectomised calves appears to reduce its virulence (10).

#### b) Preparation and storage of stabilate

Infective material is readily stored as frozen stabilates of infected blood in liquid nitrogen or dry ice. Dimethyl sulphoxide (DMSO) is the recommended cryopreservative, as it allows for intravenous administration after thawing of the stabilate. A detailed account of the freezing technique is reported elsewhere (10), 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.

#### c) Validation as a vaccine

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 parasitaemias in stained blood films and the depression of packed cell volumes of inoculated cattle during the vaccination and challenge reaction periods. Evidence of purity of the 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 (26).

### 2. Method of manufacture

#### a) Production of frozen vaccine

Quantities of the frozen stabilate (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 parasitaemia of the donor calf is monitored daily by examining stained films of jugular blood, and the blood is collected for vaccine production when suitable parasitaemias are reached. A parasitaemia of $1 \times 10^6$/ml (approximately 2% parasitaemia in jugular blood) is the minimum required for production of vaccine. If a suitable parasitaemia 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 parasitised 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 (5).
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 stablate (20, 24).

If glycerolised vaccine is to be diluted, the diluent should consist of PBS with 1.5 M glycerol and 5 mM glucose (15). Vaccine cryopreserved with DMSO should be diluted with diluent containing the same concentration of DMSO as in the original cryopreserved blood (27).

**b) Production of chilled vaccine**

Infective 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 salt solution containing the following quantities per litre: NaCl (7.00 g), MgCl$_2$$\cdot$6H$_2$O (0.34 g), glucose (1.00 g), Na$_2$HPO$_4$ (2.52 g), KH$_2$PO$_4$ (0.90 g), and NaHCO$_3$ (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.

**c) Use of vaccine**

In the case of frozen vaccine, vials should be thawed by immersion in water, preheated 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 (5). If DMSO is used as a cryoprotectant, the prepared vaccine should be kept on ice and used within 15–30 minutes (24). The vaccine is most commonly administered subcutaneously.

Chilled vaccine should be kept refrigerated and used within 6 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 between weeks 4 and 6 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.

### 3. In-process control

**a) 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 (10).

**b) Surgery**

Donor calves should be splenectomised to allow maximum yield of organisms for production of vaccine. This is best carried out under general anesthesia in young calves. Details of the technique, including pre- and post-operative procedures, are reported elsewhere (10).

**c) Screening of vaccine donors before inoculation**

Donor calves 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 (5, 24, 26).

d) Monitoring of parasitaemias following inoculation

It is necessary to determine the concentration of parasites in blood being collected for vaccine. There are accurate techniques for determining the parasite count (10) but, in the absence of these, the parasite concentration can be estimated from the erythrocyte count and the parasitaemia (percentage of infected erythrocytes).

e) Collection of blood for vaccine

All equipment should be sterilised before use (e.g. by autoclaving). Once the required parasitaemia 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.

f) 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.

4. Batch control

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.

a) Sterility and freedom from contaminants

Standard tests for sterility are employed for each batch of vaccine and diluent (see Chapter I.1.5.).

The absence of contaminants is confirmed 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 (5).

b) Safety

Vaccine reactions of the cattle inoculated in the test for potency (see Section C.4.c.) are monitored by measuring parasitaemia and depression of packed cell volume. Only batches with pathogenicity levels equal to or lower than a predetermined standard are released for use.

c) Potency

Vaccine is thawed and diluted 1/50 with a suitable diluent (15). The diluted vaccine is then incubated for 8 hours at 30°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 at 1/50 is recommended for use at a dilution of 1/5 with isotonic diluent.

d) Duration of immunity

Partial but long-lasting immunity results from one inoculation. There is no evidence that repeated vaccination will have a boosting effect.
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e) 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.

f) Preservatives
No preservatives are added. Penicillin (500,000 IU/litre) and streptomycin (370,000 µg/litre) are added to the vaccine at the time of dispensing.

g) Precautions (hazards)
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.

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 2.3.8.

BOVINE BABESIOSIS

SUMMARY

Babesiosis is a tick-borne disease of cattle caused by the protozoan parasites Babesia bovis, B. bigemina, B. divergens and others. 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 Rhipicephalus spp.

Identification of the agent: Demonstration of parasites in dead animals is possible by microscopic examination of smears of blood, brain, kidney, liver and spleen, provided decomposition is not advanced. The smears are fixed with methanol, stained with 10% Giemsa for 20–30 minutes, and examined at ×800–1000 magnification under oil immersion. In the case of live animals, thick and thin films of capillary blood should be taken from, for example, the tip of the tail. Sensitive polymerase chain reaction assays are available that can detect and differentiate Babesia species in cattle.

Serological tests: The indirect fluorescent antibody (IFA) test is the most widely used test for the detection of antibodies to B. bovis and B. divergens, but enzyme-linked immunosorbent assays are gaining popularity. The IFA test has been used for detection of antibodies to B. bigemina, but serological cross-reactions make species diagnosis difficult.

Requirements for vaccines and diagnostic biologicals: 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. The vaccines are provided in frozen or chilled forms. Production of frozen vaccine is usually recommended as it allows thorough post-production control of each batch. The risk of contamination of this blood-derived vaccine makes thorough quality control essential, but may be prohibitively expensive.

Live Babesia vaccines are not entirely safe. A practical recommendation is to limit their use to calves, 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 reactions occur.

Protective immunity develops in 3–4 weeks and lasts for several years after a single vaccination.

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 B. 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.

The vector of Babesia is the tick (18). 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, Rhipicephalus and other Boophilus spp. Babesia bigemina has the widest distribution.

Generally, B. bovis is more pathogenic than B. bigemina and B. divergens. 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. 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
Infections (7, 35). PCR assays to differentiate isolates of cases for regulatory testing. Assays generally do not lend themselves well to large-scale testing and are unlikely to supplant serological tests. Number of PCR techniques have been described that can detect and differentiate species of Babesia. Detection levels as low as three parasitised erythrocytes in 20 µl of packed cells have been claimed (36). A quantitative Buffy Coat method using acridine orange to stain parasites in capillary tubes was developed to demonstrate Plasmodium in human blood and could potentially also detect low Babesia parasitaemias, but differentiation is likely to be poor (6).

2.4.3.2. B. bigemina infections. The parasitaemia and clinical appearance of B. divergens infections are somewhat similar to B. bigemina infections (20).

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 (8, 10, 11).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The traditional method of identifying the agent in infected animals is by microscopic examination of thick and thin blood films stained with, for example, Giemsa. The sensitivity of this technique is such that it can detect parasitaemias as low as 1 parasite in 10^7 red blood cells (RBCs) (6). 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 (19). A Quantitative Buffy Coat method using acridine orange to stain parasites in capillary tubes was developed to demonstrate Plasmodium in human blood and could potentially also detect low Babesia parasitaemias, but differentiation is likely to be poor (6).

Samples from live animals should preferably be 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 smears from capillary blood, sterile jugular blood should be collected into an anticoagulant such as ethylene diamine tetra-acetic acid (EDTA) (e.g. 1 mg/ml). Heparin may affect the colour characteristics of the staining and is not recommended. The sample should be kept cool, preferably at 5°C, until delivery to the laboratory, again preferably within hours of collection. Thin blood films are air-dried, fixed in absolute methanol for 1 minute, and stained in 10% Giemsa stain for 20–30 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 drop (approximately 50 µl) of blood on to a clean glass slide. This droplet is then air-dried, heat-fixed at 80°C for 5 minutes, and stained in 10% Giemsa for 15–20 minutes. Unstained blood smears should not be stored with formalin solutions as it may affect staining quality.

Samples from dead animals should consist of thin blood films, as well as smears from (in order of preference), cerebral cortex, kidney, liver, spleen and bone marrow. 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 between two clean microscope slides drawn lengthwise to leave a film of tissue on each slide. The smear is then air-dried (assisted by gentle warming in humid climates), fixed for 5 minutes in absolute methanol, and stained for 20–30 minutes in 10% Giemsa. This method is especially suitable for the diagnosis of B. bovis infections, but is unreliable if samples are taken 24 hours or longer after death has occurred. However, parasites can often be detected in blood taken from veins in the lower limb region one or more days after death.

All stained smears are examined under oil immersion using (as a minimum) a ×8 eyepiece and a ×60 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 a somewhat larger parasite and is very similar morphologically to B. bovis. However, obtuse-angled pairs are often located at the rim of the erythrocyte. Babesia bigemina is a much longer parasite, and is often found as pairs at an acute angle to each other. Babesia bigemina is typically pear-shaped, but many diverse single forms are found. It is 3–3.5 µm long and 1–1.5 µm wide, and paired forms often have two discrete red-staining dots in each parasite (B. bovis and B. divergens always have only one). In acute cases, the parasitaemia of B. bovis seldom reaches 1%, but with B. bigemina and B. divergens much higher parasitaemias are the norm. Thick blood films are especially useful for the diagnosis of low level B. bovis infections, as are organ smears (1).

Probes have been used to detect DNA of some Babesia spp., but generally are not more sensitive than direct microscopy and application in routine diagnostics is limited (19). Polymerase chain reaction (PCR) assays have proven to be very sensitive particularly in detecting B. bovis and B. bigemina in carrier cattle (7, 11, 17, 35). Detection levels as low as three parasitised erythrocytes in 20 µl of packed cells have been claimed (36). A number of PCR techniques have been described that can detect and differentiate species of Babesia in carrier infections (7, 35). PCR assays to differentiate isolates of B. bovis have also been described (5). However, PCR assays generally do not lend themselves well to large-scale testing and 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.
In-vitro culture methods have been used to demonstrate the presence of carrier infections of Babesia spp. (22), 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 (6), but could be as low $10^{-10}$ (19), making it a very sensitive method for the demonstration of infection. An added benefit is that it is 100% specific.

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) can, however, be used to demonstrate the presence of B. divergens.

2. Serological tests

The indirect fluorescent antibody (IFA) test is widely used 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 are a particular problem in areas where the two parasites coexist. The IFA test has the disadvantages of low sample throughput and subjectivity. An internationally validated enzyme-linked immunosorbent assay (ELISA) for the diagnosis of B. bovis infection has been developed (13, 29, 38) but, despite the efforts of several investigators in different laboratories, there is still no similarly validated ELISA for B. bigemina. ELISAs for antibodies to B. bigemina typically have poor specificity. In one study (16), B. bigemina antiserum appeared to react nonspecifically with fibrinogen. However, an ELISA recently developed and validated in Australia (30) shows considerable promise. In the absence of any other workable test for B. bigemina, the procedure for that assay has been included here. ELISAs have also been developed for B. divergens (9) using antigen derived from culture, Meriones or cattle, but there does not appear to be one that has been validated internationally.

a) Babesia bovis enzyme-linked immunosorbent assay

Antigen preparation is based on a technique described by Waltisbuhl et al. (38). Infected blood (usually 5–10% parasitaemia) is collected from a splenectomised calf into EDTA. The blood is washed three times in five volumes of phosphate buffered saline (PBS), and then 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. A series of hypotonic saline solutions are prepared, ranging from 0.35% to 0.50% NaCl, in 0.025% increments. To find the best concentration, five volumes of each saline solution is then added to one volume of packed RBC, which are gently mixed and allowed to stand for 5 minutes.

The mixtures are then centrifuged and the supernatants are aspirated. An equal volume of plasma (retained from the original blood) is added to each tube containing packed RBC, 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 RBC but leaves infected RBC intact. It should be possible to achieve >95% infection in the remaining intact RBC. The bulk of the packed RBC is then differentially lysed with the optimal saline solution and centrifuged. The sediment (>95% infected RBC) is lysed in distilled water at 4°C, and parasites are pelleted at 12,000 g for 30 minutes. The pellet is washed three times in PBS by resuspension and centrifugation at 4°C. 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 ultracentrifuged, (105,000 g for 60 minutes at 4°C) and the supernatant 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
  i) 100 µl of this antigen, diluted 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) Antigen is removed and the wells are then blocked for 2 hours at room temperature by the addition of 200 µl of a 2% solution of sodium caseinate in carbonate buffer.
  iii) After blocking, the wells are rinsed briefly with PBS containing 0.1% Tween 20 (PBST) and 100 µl of bovine serum diluted 1/100 in PBST containing either 5% normal horse serum or 5% skim milk powder is added, and the plates are incubated for 2 hours at room temperature.
  iv) The washing step consists of a brief rinse with PBST, followed by three 5-minute washes with the same buffer (during which the plate is shaken vigorously), and finally the plates are given a further brief rinse.
  v) Next, 100 µl of peroxidase-labelled anti-bovine IgG diluted appropriately in PBST containing horse serum or skim milk 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),

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. The substrate reaction is allowed to continue until the absorbance of a strong positive control serum included on each plate approaches 1. At this point the absorbance at 414 nm is read on a microtitre plate reader.

To control for inter-plate variation, known positive and negative sera are included in each plate (38). Test sera are then ranked relative to the positive control. ELISA results are expressed as a percentage of this positive control (percentage 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 tetramethyl benzidine (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 bigemina enzyme-linked immunosorbent assay

This ELISA is based on an immunodominant 58 kDa antigen identified by a number of groups in B. bigemina isolates from Australia, Central America and Texas, United States of America, Egypt and Kenya (30). A monoclonal antibody (MAb) (D6) (Tick Fever Research Centre, Qld, Australia) directed against this antigen has been used to develop a competitive inhibition ELISA (30). The antigen used in the ELISA is a 26 kDa peptide (Tick Fever Research Centre, Qld, Australia), encoded by a 360 bp fragment of the p58 gene, expressed in Escherichia coli and affinity purified. This antigen can also be used in an indirect ELISA format, but some cross-reactivity of antibodies to B. bovis should be expected.

- Test procedure
  i) The recombinant 26 kDa antigen is diluted in 0.1 M carbonate buffer, pH 9.6, to a concentration of approximately 2 µg/ml and 100 µl is added to each well of a 96-well microtitre plate. The plates are incubated overnight at 4°C.
  ii) Excess antigen is removed and the wells are then blocked for 1 hour at room temperature by addition of 200 µl per well of a 2% solution of sodium caseinate in carbonate buffer.
  iii) Following a brief rinse (3 ‰ 200 µl) with PBS containing 0.1% Tween 20 (PBST), 100 µl of undiluted serum is added and the plates are incubated for 30 minutes at room temperature with gentle shaking.
  iv) The plates are then washed with PBST (5 ‰ 200 µl rinse, 5-minute soak with shaking), and 100 µl of peroxidase-labelled MAb D6 diluted to a concentration of 0.03 µg/ml in PBST containing 2% skim milk powder is added to each well. The plates are then incubated at room temperature for 30 minutes with gentle shaking.
  v) Plates are washed again, 100 µl TMB peroxidase substrate is added to each well, and the plates are incubated in the dark until the absorbance of the conjugate control wells (no serum) approaches 1. At this point the reaction is stopped by the addition of 50 µl of 1 M sulphuric acid and the absorbance is read at 450 nm. Positive and negative control sera should be included on each test plate.

The per cent inhibition (PI) for test sera is calculated relative to the conjugate control (PI = 100 – [100 × test absorbance/conjugate control absorbance]). Positive and negative threshold values should be determined in each laboratory by testing as many known positive and negative sera as possible.

The specificity of the ELISA has been estimated at 97.0% and the sensitivity for detection of antibodies in experimentally infected cattle is 95.7% (30).

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 from 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 adhere RBCs 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 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 and control 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.
To each test square 5–10 µl of each serum dilution is added using a fine pipette. The preparations are then
incubated at 37°C for 30 minutes, in a humid chamber. For controls, dilutions of weak positive and negative
sera are used on each test slide.

After incubation, the slides are gently rinsed once with PBS, and given two 10-minute washes with PBS
followed by water. An appropriate dilution of anti-bovine IgG antibody labelled with fluorescein
isothiocyanate (which is available commercially) is then added to each test square. Every new batch of
conjugate must be titrated, 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 1:1 glycerol and PBS, and examined by standard fluorescence microscopy.
A competent operator can examine approximately 150 samples per day.

d) **Other tests**
  Other serological tests have been described in recent years, and include a dot ELISA (31), a slide ELISA
(25), and latex and card agglutination tests (3, 26). 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 AND DIAGNOSTIC BIOLOGICALS

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 (8, 14, 27, 34). 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 services to the livestock industries, in particular in
Australia, Argentina, South Africa, Israel and Uruguay. An experimental *B. divergens* vaccine prepared from the
blood of infected *Meriones* has also been used successfully in Ireland (21).

A killed *B. divergens* vaccine is prepared in Austria from the blood of infected calves (15), but little information is
available on the level and duration of the conferred immunity. Experimental vaccines containing antigens
produced *in vitro* have also been developed (2, 32), but the level and duration of protection against heterologous
challenge are unclear. Parasite proteins have been characterised and there has been some progress towards
the development of subunit vaccines (11, 33). No effective subunit vaccine is available commercially.

Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine
production. The guidelines given here and in Chapter I.1.7 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 blood
as vaccine (8, 12, 14). Calves used for infection with these strains 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 unguculatus*)
can be used instead of bovine blood. *In-vitro* culture methods have also been used to produce parasites for vaccine (24, 27).
However, the relatively high cost of production from culture and evidence of possible antigenic drift during long-term maintenance in culture, make mass culture of *Babesia* impractical in most laboratories at present.

* 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 is
preferred (12, 14, 27, 34), as it allows for thorough post-production control of each batch. However, it is more
costly to produce and more difficult to transport than chilled vaccine. The potential risk of contamination of this
blood-derived vaccine makes post-production control essential, but may put production beyond the financial
means of some countries in endemic regions (14). A production facility supplying an annual market of fewer than
50,000 doses is unlikely to operate without financial support.

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1. Seed management

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 (12, 14). Tick-transmissible and nontransmissible strains are available. A strain of *B. divergens* with reduced virulence for *Meriones* has also been developed (39).

- 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 (18).

*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* (21). Maintenance of isolated strains in vitro (23) can be used to eliminate most contaminants, but not to separate *Babesia* spp. Selective chemotherapy can be used to obtain pure *B. bovis* from a mixed *Babesia* infection, while rapid passaging in susceptible calves will allow isolation of *B. bigemina* (1).

- 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 (8).

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 after 3 months and then using the ensuing relapse parasites to repeat the procedure (8).

Attenuation of *B. divergens* for *Meriones* followed long-term maintenance in vitro (39).

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 stabilitate for safety testing and for future use as master seed in the production of vaccine.

b) Preparation and storage of master seed

Avirulent strains are readily stored as frozen infected blood in liquid nitrogen or dry ice. Dimethyl sulphoxide (DMSO) (28) and polyvinylpyrrolidine MW 40,000 (37) 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 (28). Briefly, it involves the following:

Infected blood is collected and chilled to 4°C. Cold cryoprotectant (4 M DMSO in PBS) is then added, while stirring slowly, to a final blood:protectant ratio of 1:1 with the final concentration of DMSO being 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* have been known to remain viable for 20 years.

c) Preparation and storage of working seed

Working seed is prepared in the same way as master seed (Section C.1.b.) using master seed as starting material.

d) Validation of safety and efficacy of working seed

The suitability of a working seed is determined by inoculating suitable numbers of susceptible cattle with vaccine prepared from it and then challenging them and susceptible controls with a virulent, heterologous strain. Both safety and efficacy can be judged by monitoring fever, parasitaemias in stained blood films, and
depression of packed cell volumes. The purity of the working seed is tested by monitoring the cattle used in the safety test for evidence of possible contaminants as mentioned in Section C.4.b.

2. Method of manufacture

a) Production of frozen vaccine concentrate

First, 5–10 ml quantities of working seed are rapidly 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 if DMSO is used) to infect a susceptible, splenectomised calf (free of potential vaccine contaminants) by intravenous inoculation.

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 $1 \times 10^8$/ml (approximately 2% parasitaemia in jugular blood) is usually adequate for production of vaccine. If a suitable *B. bovis* parasitaemia is not obtained, passage of the strain by subinoculation of 100–500 ml of blood into a second splenectomised calf may be necessary. Passage of *B. bigemina* is not recommended.

Blood from the infected donor calf is collected by jugular cannulation using preservative-free heparin as anticoagulant (5 International Units [IU] heparin/ml blood).

In the laboratory, the parasitised blood is mixed in equal volumes with 3 M glycerol in PBS supplemented with 5 mM glucose (final concentration of glycerol 1.5 M) 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 (12, 14).

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 (34).

If glycerolised frozen vaccine is to be diluted, 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.

Frozen vaccine containing both *B. bovis* and *B. bigemina* can be prepared (27) by mixing equal numbers of the parasites obtained from different donors.

The recommended dose of vaccine after reconstitution and dilution ranges from 1 to 2 ml depending on local practices and requirements.

b) 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 $10 \times 10^7$). A suitable diluent is 10% sterile bovine serum in a balanced salt solution containing the following ingredients per litre: NaCl (7.00 g), MgCl$_2$·6H$_2$O (0.34 g), glucose (1.00 g), Na$_2$HPO$_4$ (2.52 g), KH$_2$PO$_4$ (0.90 g), and NaHCO$_3$ (0.52 g).

Blood containing *B. divergens* may be diluted in Hanks’ solution. If diluent is not required, sterile acid citrate dextrose or citrate phosphate dextrose should be used as the anticoagulant, at a rate of one part to four parts blood, to provide the glucose necessary for parasite survival.

3. In-process control

a) 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 (8).

b) Surgery
Donor calves should be splenectomised to allow maximum yield of parasites for production of vaccine. This is best done in young calves and under general anaesthesia.

c) 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, 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. The absence of other infective agents endemic in the country should also be confirmed; these may include the agents of enzootic bovine leukosis, bovine immunodeficiency virus, bovine pestivirus, infectious bovine rhinotracheitis, Akabane disease, ephemeral fever, bluetongue, foot and mouth disease, 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, antigen or DNA detection (12, 34).

d) Monitoring of parasitaemias following inoculation
It is necessary to determine the concentration of parasites in blood collected for vaccine. There are accurate techniques for determining the parasite count (1), but the parasite concentration can be estimated from the RBC count and the parasitaemia (% infected RBCs).

e) 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 with, for example, 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 is indicated. Alternatively, the calf should be killed immediately after collection of the blood.

f) 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.

4. Batch control
The potency, safety and sterility of vaccine batches cannot be determined in the case of chilled vaccine, and 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.

a) 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 testing of donor cattle and by inoculating donor lymphocytes into sheep and then monitoring them for evidence of viral infection. Potential contaminants include the agents of enzootic bovine leukosis, infectious bovine rhinotracheitis, bovine pestivirus, ephemeral fever, Akabane disease, Aino virus, bluetongue, Brucella abortus and Leptospira, foot and mouth disease, lumpy skin disease, rabies, Rift Valley fever, rinderpest, contagious bovine pleuropneumonia, heartwater, Jembrana disease, and pathogenic Theileria and Trypanosoma spp. (12, 14).

b) Safety
Vaccine reactions of the cattle inoculated in the test for potency (see Section C.4.c.) are monitored by measuring parasitaemia, fever and depression of packed cell volume. Only batches with pathogenicity levels equal to or lower than a predetermined standard are released for use.
Chapter 2.3.8. – Bovine babesiosis

c) **Potency**

Frozen, glycerolised vaccine concentrate is thawed and diluted 1/5 with isotonic diluent (12, 14). The prepared vaccine is then incubated for 8 hours at 30°C, and five cattle are inoculated subcutaneously with 2 ml doses each. The inoculated cattle are then monitored for the presence of infections by examination of stained blood smears. Only fully infective batches are released for use at a working dilution of 1/5.

d) **Duration of immunity**

Long-lasting immunity usually results from one inoculation. Evidence of *B. bovis* vaccine failures have been reported (4) and are related to the choice of vaccine strains, the presence of heterologous field strains, and host factors. There is little evidence of time-related waning of immunity (8).

e) **Stability**

When stored in liquid nitrogen, the 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.

f) **Preservatives**

Penicillin (500,000 IU/litre) and streptomycin (370,000 µg/litre) are added to the vaccine at the time of dispensing.


g) **Use of vaccine**

In the case of frozen vaccine, vials should be thawed by immersion in water preheated to 40°C. Glycerolised vaccine should be kept cool and used within 8 hours (9, 10), while vaccine with DMSO as cryoprotectant should be kept on ice and used within 15–30 minutes of thawing (34).

Chilled vaccine should be kept refrigerated and used within 4–7 days of preparation, depending on the viability of the parasites.

The strains of *B. bovis*, *B. divergens* and *B. bigemina* used in the vaccine may be of reduced virulence, but will not be entirely safe. A practical recommendation is, therefore, to limit the use of vaccine to calves, when nonspecific immunity will minimise the risk of vaccine reactions. If older animals are to be vaccinated, there is a risk of severe 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 10–16 (8).

Protective immunity develops in 3–4 weeks, and lasts at least 4 years in most cases.

Babesiosis and anaplasmosis vaccines are often used concurrently, but it is not advisable to use any other vaccines at the same time (8).

h) **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 deep-frozen material applies.

5. **Tests on the final product**

a) **Safety**

See Section C.4.b.

b) **Potency**

See Section C.4.c.
REFERENCES


Chapter 2.3.8. — Bovine babesiosis


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CHAPTER 2.3.9.

BOVINE CYSTICERCOSIS

See Chapter 2.10.1. Cysticercosis

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CHAPTER 2.3.10.
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. *Dermatophilus cheloniae* may be found in crocodiles.

**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.

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.

**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:** 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. It is the commonest skin disease of crocodiles in Australia and has an impact on farming of this species (2). Dermatophilosis is caused by the bacterium *Dermatophilus congolensis*, the type species of the genus *Dermatophilus*, which is a member of the Actinomycetes (*Dermatophilus cheloniae* has been isolated from crocodiles). 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.
The severe disease in ruminants is promoted by immunomodulatory effects induced by infestation with adults of the tick species *Amblyomma variegatum* (1).

It must be remembered that both scabs and cultures can cause human infections.

### B. DIAGNOSTIC TECHNIQUES

#### 1. Identification of the agent

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 on to 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.

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 (3) 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.

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 (4) is preferable. Thin, heat-fixed smears of scab emulsions, or impression smears, are stained. Known positive and negative control specimens should always be included.
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 (5). At present, the ELISA remains only suitable as a research and investigation method; it is not used for routine diagnosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Vaccines are under development, but so far no commercial vaccines are available.

REFERENCES


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CHAPTER 2.3.11.
THEILERIOSIS

SUMMARY

Tick-transmitted Theileria parasites of cattle are important constraints to the improvement of the livestock industry in large parts of the Old World. Theileria annulata and T. parva, the most economically important species, are responsible for mortality and losses in production that can only be guessed at, but these species are consistently recorded as the cause of major disease entities. Bovine theileriosis is generally controlled by the use of acaricides to kill ticks, but this method is not sustainable because acaricides are expensive, resistance has developed to many of them, regulations regarding cattle movement and quarantine are not strictly enforced, and management and maintenance of dips and spray races are often poor. More reliable systems are desirable, and vaccination should provide better control of the disease entities caused by these pathogens.

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 schizont is a characteristic diagnostic feature of infections with T. parva and T. annulata in lymph node biopsy smears. 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, splenic enlargement, and froth in the trachea, lymph node enlargement, haemorrhages in internal organs, abomasal erosions and 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 \(-20^\circ\text{C}\), 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 these species overlap. 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 are a recent development. 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 contains schizont-infected cells and 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

1 In this chapter, the term ‘New World’ refers to the Americas and the term ‘Old World’ refers to Europe, Africa and Asia.
subcutaneous dose of tick-derived sporozoites and a simultaneous treatment with a long-acting tetracycline formulation. This treatment results in a mild or unapparent East Coast fever reaction followed by recovery. Recovered animals have a robust immunity to homologous challenge, which usually lasts for the lifetime of an animal. More than one stock of the parasite may need to be used in the live vaccination method in order to induce a broad protective immunity. 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 involved and to avoid contamination of the stabilitates. 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 occurs in 13 countries in sub-Saharan Africa causing East Coast fever, 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. velfera 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 and T. buffeli/T. orientalis – the latter may be referred to as T. buffeli (14).

Most T. parva stocks produce a carrier state in recovered cattle, and recent 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 East Coast fever 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 have become less reliable because 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.

Chemotherapy using parvaquone, buparvaquone and halofuginone is used to treat T. parva and T. annulata, although treatments with these agents do not sterilise theilerial infections.

**B. DIAGNOSTIC TECHNIQUES**

Diagnosis 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 piroplasmic 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)

The schizont is a characteristic diagnostic feature of acute infections with T. parva and T. annulata in Giemsa-stained biopsy or tissue impression smears of lymph nodes, liver and spleen. Schizonts are transitory in T. mutans and the T. sergenti/T. buffeli/T. orientalis group, in which the piroplasmic stage may be pathogenic. Theileria taurotragi schizonts are not readily detected in Giemsa-stained blood smears. A veil to the side of the schizont may distinguish T. velfera. 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, and, in longer standing cases, lymphocytic infiltrations of the kidneys that resemble infarcts. In animals that recover, occasional relapses occur. A nervous syndrome called ‘turning sickness’ is sometimes seen observed in *T. parva*-endemic areas, and is considered to be associated with the presence of intravascular and extravascular aggregations of schizont-infected lymphocytes, thrombosis and ischaemic necrosis.

In *T. annulata*, both the schizont and piroplasm stages may be pathogenic. Schizonts are scarce in the peripheral blood of acutely sick animals. The gross pathology caused by schizonts of *T. annulata* resembles that of *T. parva*, while anaemia and jaundice are features of the 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.

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.

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**

       i) **Schizont antigen slides**

       The antigens used for the schizont IFA tests are obtained from schizont-infected cell lines prepared as described below:

       Cultures of from 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 smears of the cell suspension are made on Teflon-coated multispot 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 multichannel or a 100-µl pipette. By dispensing and immediately sucking up the schizont suspension, a monolayer of schizonts remains on 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 smears 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

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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.
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 infected animals. Experimental infections are induced by infecting cattle subcutaneously with sporozoites, or using ticks infected with *T. parva*, *T. annulata* or *T. taurotragi*. Peak parasitaemias are of short duration and if animals survive the disease the percentage of infected red blood cells (RBC) decreases considerably in a few days. Infections of the parasite group referred to as *T. sergentii/T. buffelli/T. orientalis, T. mutans or T. velifera* 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 10% 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 taken to be the dilution 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 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. (15), for use in tests with antigens of T. parva in suspension. Briefly, a 3-month-old calf is splenectomised and maintained under tick- and tsetse fly-free conditions. To exclude the possibility of latent infection, blood smears from the animal are taken daily for a period of 4 weeks, stained with Giemsa's stain, and examined for parasites. The 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 x 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**

  Schizont and piroplasm antigen slides are removed from -20°C storage and allowed to thaw for 30 minutes at 4°C and for 30 minutes at room temperature, before being unpacked. The frozen schizont antigen, fixed in suspension and stored at -20°C, is thawed at room temperature, while the piroplasm antigen, kept at 4°C, is resuspended by agitation, passed through a 25-gauge needle to break the clumps (not necessary for schizont antigen), and diluted to previously standardised dilutions. The antigens are distributed on to the slides using multichannel or a 100 µl pipette. By dispensing and immediately sucking up the schizont or piroplasm suspension, a monolayer of schizonts or piroplasms remains on each well. The slides are allowed to dry either at 37°C or at room temperature.

  For initial screening using T. parva antigen in suspension, 1/40 dilutions of test and control sera are made in lymphocyte lysate (195 µl lymphocyte lysate + 5 µl serum), and incubated for 30 minutes at room temperature. For antigen slides, 1/40 and 1/60 dilutions are used. Further doubling or five-fold dilutions in PBS can be made if end-point antibody titre is desired. Of the diluted sera, 25 µl are then transferred to the antigen slides. For each slide, the positive and negative control sera, diluted 1/40, are included. The slides are incubated at room temperature in a humid chamber for 30 minutes, and then washed twice in PBS for 15 minutes. To each well, 20 µl of anti-bovine immunoglobulin fluorescein isothiocyanate (FITC) conjugate at recommended optimal dilution, is added. Evans blue is incorporated into the conjugate at a final dilution of 1/10,000 as a counterstain. The slides are incubated for 30 minutes at room temperature in a humid chamber, washed, as before, mounted with a cover-slip in 50% glycerol in PBS, pH 8.0, and examined for fluorescence, for example, using 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.

- **Characteristics of the indirect fluorescent test**

  Using the test as described, the fluorescence is generally specific for the particular *Theileria* species (see below). The incorporation of Evans blue provides a good contrast, making reading easy. 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.

  Following infection with sporozoites, antibodies to *T. parva* and *T. annulata* are first detected between days 10 and 14 using the schizont antigen, while using the piroplasm 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 rechallenge. Following recovery from either East Coast fever or tropical theileriosis, some animals may have low antibody titres in the IFA test after 4–6 months, but the antibodies may persist for more than 1 year following a single challenge.

  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 IFA antigens have failed to detect antibody in some animals carrying patent infection with piroplasms (10).

  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.

  If the IFA test is used routinely for the detection of antibodies to one of the bovine *Theileria* spp., it is sensitive and requires little standardisation. 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. This is not important in the field, as the diseases do not overlap. Such cross-reactivity does not seem to occur between **T. parva** and **T. mutans** or between **T. annulata** and **T. mutans**. There is low titre cross-reactivity between **T. parva** and **T. taurtragi**, which is a problem as the two infections overlap throughout much of the **T. parva** distribution. The IFA test has been modified so that a panel of monoclonal antibodies (MAbs) can be used to detect theilerial antigens prepared as schizont-infected lymphocytes fixed on Teflon-coated multispot slides (see footnote 2). This MAb panel can detect differences between certain stocks of **T. parva** and between **T. parva** and other theilerial species. It is a useful test and forms part of the characterisation process for **T. parva**, especially for field isolates and for laboratory quality control during stabilate preparation (7).

**Future tests for Theileria diagnosis**

The IFA test is easy to perform and reasonably sensitive and specific. However, because of the problems of cross-reactivity among different **Theileria** spp. described above, the test has limitations for large-scale serological surveys, particularly in areas where several species overlap. There is a need for tests that are more specific, are 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 has been successfully adapted for the detection of antibodies to **T. annulata** (16), and has been shown to detect antibodies for a longer period of time than the IFA (20, 21). An ELISA for **T. mutans** has also been described (22). 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. The expressed products are fusion proteins with glutathione S transferase, and are directly coated on to ELISA plates. These ELISAs provide higher sensitivity and specificity than the IFA tests (24, 27) 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 (2, 6). DNA probes specific for **T. parva** (1, 9, 24) and **T. mutans** (25), 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 (3). A specific PCR was developed to test whole blood samples from **T. annulata**-carrier cattle (12). 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 recently introduced (17). 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** (23).

### 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 much 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 (13, 18, 30), and the vaccine is widely used in Israel, Iran, Turkey, India, northern Africa, central Asia and the People’s Republic of China.

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 theuffy coat of heparinised peripheral blood separated on a density gradient (Ficoll Hypaque), or using lymphocytes harvested from lymph node biopsy material using a simple plastic syringe method (8, 13).
Seed cultures are prepared from cryopreserved cell lines that have been isolated from cattle and attenuated as described below. Cultivation for vaccine production is recommended from the seed culture following a maximum of 20–30 passages, 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 10^8 cells are introduced into a 25 cm^2 flask, and the same seeding rate in 100–200 ml is used in larger flasks. The general culture technique is as described by Brown (8).

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 (30). The mechanism of attenuation is unknown, but the number of cell divisions is likely to be more important than time in culture or number of passages. Field isolates have required passage for periods of from 4 to 30 months for complete attenuation. 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 (31). 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. To prepare master seed, schizont-infected cells that have proved to be safe for cattle are propagated to obtain in a single culture passage approximately 5 x 10^8 cells. The cells are cryopreserved in about 100 cryotubes each containing 5 x 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 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 supplementary subcultivations, enough cells are available to start the production run.

*Production seed:* Schizont-infected cells from a specific passage level, which is used without further propagation, for 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 from 150 cm^2 to 175 cm^2 flasks that usually provide an average of from 7 x 10^7 to 8 x 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 x 10^9 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 seeded with 10^8 cells and roller bottles with up to 4x10^9 cells/vessel in L-15 medium with 20% serum followed by 7 days cultivation without change of medium showed the best large-scale cell production (34).

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
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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 dispensed in 1.8-ml aliquots into 2-ml plastic vials, each vial containing ten doses of concentrated vaccine. The proper number of schizont-infected cells per dose is a subject of controversy. A recommended practical approach is to prepare doses of $10^6$–$10^7$ infected cells in order to counteract variable environmental conditions in the field.

The vaccine is frozen by introducing the vials in an ultracold deep freezer (–70°C) and then transferred to liquid nitrogen after 24 hours. Alternatively vials can be introduced in liquid nitrogen vapours for 3 hours and then immersed in the liquid nitrogen for storage (30). Vaccine is transported to the field in liquid nitrogen, and diluted 1/10 in isotonic buffered saline in a screw-cap bottle with a rubber or silicone septum for aseptic withdrawal. For dilution of vaccine frozen with glycerol, isotonic buffered saline should also contain 10% glycerol in order to avoid osmotic damage to the schizonts. The vaccine is administered subcutaneously within 30 minutes of thawing (28).

Cultured cells of two stocks are used in Iran. The second stock is administered 30–60 days after the inoculation of vaccine prepared from the first stock (18).

A fresh culture vaccine is used in Morocco, usually at a tenfold lower dose. However, there are problems with quality control of vaccines of this kind.

3. Batch control

In Israel before release the schizont vaccines are tested by the following procedure (29).

The frozen vaccine has a practically unlimited shelf life in the frozen condition, but is usually produced in small individual batches (3–5 thousand doses), which makes the full testing of each batch impracticable for economic reasons. It is recommended therefore that the master seed be tested for safety and efficacy, while each 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: Despite the fact that the master seed is prepared from attenuated schizonts, it is recommended for legal, as well as for practical reasons, to test the safety of a sample of actual vaccine that is produced from the particular master seed. For this purpose, 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 and clinical signs result from the vaccine in field cattle, the batch that has been involved, or another parallel one 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 6 weeks later; the vaccinated calves and the same number of unvaccinated calves are then 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 stabilate prepared from macerated infected ticks (for techniques see Section C2.1.) Experience shows that inoculation of stabilate (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 stabilate appear to be more reproducible than those obtained with different batches of live ticks, each infected under different conditions.

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 male unfed infected *Hyalomma* ticks have been used for infection of cattle. Alternatively, stabilate 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 smears from lymph node or liver biopsy, rate of piroplasm infection in thin 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 of the field isolate used for challenge, the species of infected ticks used to produce sporozoites, and the parasite dose used for challenge. Literature data (30) show that calves vaccinated with schizont vaccine may exhibit an apparently near total protection or may show a low level parasitaemia, accompanied by mild fever and insignificant alteration of the remaining parameters from their prevaccination values. A lesser degree of protection was exhibited when cattle vaccinated with schizont vaccine were challenged with tick-derived parasites from a geographically remote area. In contrast, in most of the trials the nonvaccinated control calves exhibited high a level of parasitaemia and pancytopenia accompanied by severe clinical manifestations. In the absence of specific medication, a considerable portion of control animals succumbed to the infection.

Field observations have also been used for evaluation of the efficacy of anti-theilerial vaccines (29, 35). 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 theilerial stage in vaccinated cattle showing no clinical signs is considered to be the result of inapparent tick-induced infection.

c) **Potency**

*Viability of schizont-infected cells:* Frozen vaccine remains essentially 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 (36). 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 (36), 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 x 10, 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 I.1.5.

e) **Method of use**

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 (18). 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 (29). 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 (28), it is generally considered that there is sufficient cross-protection among strains to provide adequate protection against field challenge throughout countries, such as Israel. In the vast infected areas of central Asia, a single stock has proved immunologically effective in 1.5 million cattle (11, 35). However, as described previously, two stocks are used routinely in Iran (18).
C2. Infection and treatment vaccination for *Theileria parva*

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 (32). 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* stabilates 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 consistent results in field immunisations, it is essential that tick-derived sporozoite stabilates are prepared from a fully characterised ‘working seed stabilate’. The ‘working seed stabilate’ should be derived directly from the reference ‘master seed stabilate’, with characteristics of the ‘master seed stabilate’ and available in suitable quantity for future preparation of immunising stabilates. More recent immunisation stabilates have been prepared according to a proposed set of standards (26).

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 moulting, are applied in ear-bags to healthy rabbits. About 600 ticks are applied to each ear and any 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 again rinsed 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 (emulsor 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 prelabelled plastic straws. This latter system is ideal for large volume stabilates, and colour coding and labelling provide extra security by identifying each straw. 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 infectivity of the stabilate is determined by inoculation of a standard dose of 1.0 ml into susceptible cattle. The stabilate is then titrated in cattle, and its infectivity and lethality at different dilutions are established for use in immunisation. 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 be a very mild or unapparent infection (4), 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 stabilate is examined or two treatments of tetracycline (on days 0 and 4) may be used. More recently 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 (4).

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 transmission and pick-up before leaving the isolation facilities. 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 I.1.6. Human safety in the veterinary microbiology laboratory).

d) Future standards for quality assurance and safety

Although a committee was set up in Kampala (5) under the aegis of the Organization of African Unity (OAU), the Food and Agriculture Organization of the United Nations (FAO), and the International Laboratory for Research on Animal Diseases (ILRAD) to determine standards for live vaccines for tick-borne diseases, no formal recommendations have yet been developed. The areas that need particular attention are standard operating procedures for various stages of stabilate preparation, standardised parasite characterisation methods, and safety and quality testing. This will ensure production of standard ECF vaccines that are safer and efficacious.

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 immunising stabilates. 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 safety by inoculation into BHK and Vero cells, followed by three passages in these systems (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-theilerial drugs, and cross-immunity studies.
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 MAb profile may not cross-protect, whereas stocks showing similar profiles give cross-protection (19). 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* (19). Currently there are *no vitro* assays that correlate with protection *in vivo*. Recently a 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 (33).

5. Vaccination strategy

Unlike *T. annulata*, where a considerable cross-protection is observed among different strains in the field, a very much more complex situation exists for *T. parva*. Two strategies are used to try to overcome this antigenic complexity. A combination of three stocks, which provides a broad spectrum of protection, has been tested in a number of countries. The combination was prepared in Malawi in an FAO project for distribution within the *T. parva*-endemic region. Subsequently, a similar trivalent stabilate was prepared for the FAO by ILRI. This latter stabilate was prepared to the latest proposed standards and is used safely and effectively in Tanzania. 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 break through 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


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SUMMARY

Haemorrhagic septicaemia (HS) is an acute, highly fatal septicaemic disease of cattle and buffaloes caused by certain serotypes of Pasteurella multocida. It is a primary pasteurellosis reproducible in susceptible animals with pure cultures of the causative organism alone.

The diagnosis of HS depends on the isolation of the causative organism, P. multocida, from the blood or bone marrow of a dead animal by cultural and biological methods, and the identification of the organism and type by biochemical and serological methods.

Identification of the agent: Pure cultures of P. multocida can be obtained by streaking material on to artificial media or by the inoculation of suspect infective material into mice, and the subsequent culture of their blood after death in a suitable medium. Identification is made on the basis of the morphological, cultural, and biochemical characteristics of P. multocida.

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.

Serological tests: Serological tests for detecting specific antibodies are not normally used for diagnostic purposes.

Requirements for vaccines and diagnostic biologicals: Vaccines against haemorrhagic septicaemia are simple 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. Potency tests are most conveniently carried out in mice and/or rabbits.

A. INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute, highly fatal, septicaemic disease of cattle and buffaloes caused by certain serotypes of Pasteurella multocida (2, 9, 12, 13). Presently, two serotypes are recognised – the Asian serotype and the African serotype. Four serotyping methods are now available – the ‘capsular’ serotyping method using an indirect haemagglutination (IHA) test (6), ‘somatic’ serotyping using agglutination, and the agar gel immunodiffusion (AGID) test (14, 20, 21), and a multiplex capsular polymerase chain reaction (PCR) typing system recently developed as a rapid, genetic alternative to the conventional ‘capsular’ serotyping method (26). The Asian strains belong to capsular type B only while the African strains are types B and E (15). Using the somatic typing methods, all strains belong to type 6 by the agglutination tests and type 2 by the AGID test.

The clinical syndrome of HS consists of an initial phase of temperature elevation (often unnoticed), a phase of respiratory involvement, and a terminal phase of septicaemia and recumbency leading to death. The incubation period is usually 1–3 days, and the course of the disease may range from sudden death, with no observable clinical signs, to a protracted course extending up to 5 days (9, 13).

Buffaloes are generally believed to be more susceptible to HS than cattle, and in this species, the disease course is shorter. In endemic areas, most deaths are confined to older calves and young adults. In nonendemic areas, massive epizootics may occur. Case fatality approaches 100% if treatment is not carried out sufficiently early (in the pyrexic stage) (9, 13).
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A clinical diagnosis can be made on the basis of characteristic signs, gross pathological lesions, herd history, morbidity and mortality patterns, species susceptibility, and age group affected. A tentative clinical diagnosis is generally useful in order to initiate control measures.

At post-mortem examination, the first obvious lesion is subcutaneous oedema, particularly in the submandibular and brisket region. Other lesions include petechial to ecchymotic haemorrhages, congestion and/or consolidation of lungs, fibrinous pneumonia, pleurisy and pericarditis (9, 13).

Laboratory confirmation of diagnosis is by isolation in culture or in mice, followed by identification of the causative agent by biochemical and serological methods as type B or E. Exceptionally, when in-vitro culture is not successful, a mouse may be inoculated with the clinical sample. Blood from the mouse is then used for the preparation of blood films on slides and for culture of the organism, which can be used subsequently in rapid slide agglutination tests. Further confirmation is carried out using biochemical and conventional serological tests.

B. Diagnostic Techniques

1. Isolation and identification of the agent

- Cultural and biochemical methods

The septicaemia in HS occurs at the terminal stage of the disease. Therefore, blood samples taken from sick animals before death may not always contain *P. multocida* organisms. Also, they are not consistently present in the nasal secretions of sick animals.

A blood sample or swab collected from the heart is satisfactory if it is taken within a few hours of death. If the animal has been dead for a long time, a long bone, free of tissue, can be taken. 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.

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 swabbed with alcohol, flame sterilised 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 cultures 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 medium for the growth of *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 (31).

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 cocccoid forms. 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 sulhide 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 (7). 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, hyaluronidic-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 reading of the test.

### Immunological methods

Several immunological tests are used for the identification of the HS-causing serotypes of *P. multocida*. These consist of a rapid slide agglutination test (20), an IHA test for capsular typing (6), an agglutination test using hydrochloric-acid-treated cells for somatic typing (21), the AGID test (1, 14, 32), and the counter immunoelectrophoresis test (CIEP) (8).

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 hemagglutination test (capsular typing)**

This was originally performed using antigen-sensitised human type ‘O’ red blood cells (RBCs) (6), but more recently sheep RBCs have been used (24, 31). 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, 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 Lucite plates.

i) The capsular extract of the unknown strain is prepared as described above and used to sensitise 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 dilution 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 for 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 quantitative tests 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 (1, 32). Antigens and antisera are the same as for capsular typing by the IHA method (6). 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 (14) are prepared in chickens. Oil-emulsified bacterin¹ (1 ml) is injected subcutaneously into the mid-portions 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.

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.

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 antisera:** 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.

¹ Preparation of a stable oil emulsion is a delicate process. 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 lanolin the higher the stability of the emulsion. However, a high percentage of lanolin will make the emulsion very viscous, which will greatly hinder the vaccination process in the field.
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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 (19, 21). 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 6:B (Asian HS), 6:E (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 (6) or by AGID tests (1, 32). The other is ‘somatic’ typing by the method of Namioka & Murata (19, 21, 22), and by the method of Heddleston et al. (14). 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. (16). AST methods are described in Chapter I.1.10. 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* (3). 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 categorisation. 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, ceftiofur, cefquinome, streptomycin, gentamicin, spectinomycin, florfenicol, tetracycline, sulfonamides, trimethoprim/sulfamethoxazole, erythromycin, tilmicosin, enrofloxacin (or other florquinolones).

• **Nucleic acid recognition methods**

PCR amplification of specific DNA sequences allows rapid detection and presumptive identification of organisms directly from either clinical specimens or from small amounts of mixed or pure bacterial cultures. Complex DNA extraction and purification procedures are not required unless blood, urine or faecal specimens are used. PCR-inhibitory compounds present in these materials need to be removed or inactivated prior to amplification.

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a) **Pasteurella-multocida-specific PCR assay**

PCR technology can be applied for rapid, sensitive and specific detection of *P. multocida* (17, 23, 29). While some disadvantages are evident with each published method, the rapidity and high specificity of the *P.*-multocida-specific PCR assay (29) provides optimal efficiency without the need for additional hybridisation. Although the use of hybridisation can confirm specificity, this approach is usually possible only in specialised laboratories. The *P.*-multocida-specific PCR identifies all subspecies of *P. multocida*: subsp. *multocida*, subsp. *gallicida*, and subsp. *septica* through specific amplification of a ~460 bp DNA fragment within the KMT1 gene. While DNA is also amplified from *P. canis* biovar 2, these species are readily distinguished by indole patterns.

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 (29), 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 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 (29):

- *P.*-multocida-specific PCR: KMT17 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. Cycling parameters for a Corbett FTS-320 Thermocycler (or similar) 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. The reaction is held at 4°C until required for electrophoresis; 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 (11) and B:2 (4) polysaccharide capsules provided the required information to determine the biosynthetic region of the remaining three serogroups (D, E, and F) (4). With this knowledge, serogroup-specific sequences were identified for use as primers in a multiplex capsular PCR-typing system (4). The *P.*-multocida-specific primers are included as an internal control for species identification.

Primer sequences (4):

- Multiplex capsular PCR: CAPA-FWD 5'-TGC-CAA-AAT-CGC-ACT-GAG-3'
- CAPA-REV 5'-TTC-CCA-TCA-ATG-CCA-TAT-G3'
- CAPB-FWD 5'-CAT-TTA-TCC-AAG-CTC-ACC-C3'
- CAPB-REV 5'-GCC-CGA-GAG-TTT-CAA-TCC-3'
- CAPD-FWD 5'-TAA-CAA-AAG-AAA-GAC-AGA-CC-3'
- CAPD-REV 5'-CAT-CTA-CCT-ACC-CTT-GAA-CTA-3'
- CAPE-FWD 5'-TCC-GCA-GAA-AAT-TAT-TAG-CTC-3'
- CAPE-REV 5'-GCT-TGC-TGC-TGC-ATT-TTG-TC-3'
- CAPF-FWD 5'-ATA-AGA-CAG-AAC-GAG-AAA-TCA-G-3'
- CAPF-REV 5'-TTC-CGC-CGT-CAA-TTA-CTC-G3'
- KMT1T7 5'-ATC-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 × 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 (4) it is suggested to use a standard cycling programme as per *P. multocida*-specific PCR assay. However, validation of the multiplex PCR system indicates that the following optimised cycling programme should be used for the Perkin Elmer GeneAmp PCR System 2440: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 90 seconds; with a final extension at 72°C for 5 minutes. 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 (5, 29). Comparative analysis with the *Haemophilus influenzae* Rd genome indicates that DNA regions amplified in both assays reside in close proximity, yet slight differences in specificity are evident. To date, the HS-causing type-B-specific PCR (29) remains 100% specific for HS-causing type B serotypes of isolated *P. multocida*. Type B cultures with the predominant somatic antigen being either type 2 or 5 are identified by the amplification of an ~620 bp fragment with the KTSP61 and KTT72 primers.

Primer sequences (29):

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS-causing type-B-specific</td>
<td>KTSP61  5'-ATC-CGC-TAA-CAC-ACT-CTC-3'</td>
</tr>
<tr>
<td></td>
<td>KTT72   5'-AGG-CTC-GTT-TGG-ATT-ATG-AAG-3'</td>
</tr>
</tbody>
</table>

Conditions for HS-causing type-B-specific PCR are as described for *P. multocida*-specific PCR.

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) 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 *Hha*I has proved useful for characterisation of type B HS-serotypes, with 13 unique fingerprint profiles among 54 isolates resembling the profile of the somatic serotype 2 reference strain (33). In contrast, while a single *Hha*I profile was observed among 13 serogroup E isolates, differentiation of these strains was possible following *Hpa*II digestion. Ribotyping and large DNA separation by pulsed-field gel electrophoresis also provide useful discrimination of serogroup B and E *P. multocida* isolates (27). However, these techniques are largely used for research purposes and require specialised equipment.

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 (10) and for differentiating post-vaccination isolates of *P. multocida* obtained from turkeys (15). However, RAPD and AP-PCR analysis of HS-causing *P. multocida* isolates have not been previously described. Repetitive sequence PCR analysis of *P. multocida* has provided useful discrimination of avian and swine isolates, although all HS-causing strains analysed demonstrated similar profiles (28, 30).

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 AND DIAGNOSTIC BIOLOGICALS

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.

Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

A local isolate of *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.

b) Method of culture

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.

Seed lots must be shown to be:

i) **Pure:** Free from adventitious agents.

ii) **Safe:** Produce no adverse reaction in the target species when given as recommended.

iii) **Efficacious:** Stimulate effective immunity as indicated by potency tests.

The necessary tests are described in Section C.4, below.

2. Method of manufacture

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 formalised 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 *P. multocida*. A suitable sterilised medium 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 121°C, and transferred aseptically into the tank that has previously been heat-sterilised with the rest of the ingredients.

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 (25). 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.

3. In-process control

Proper concentration of bacterial growth, the capsulation of the bacteria, purity of culture and efficient inactivation all need to be checked.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter I.1.5.

b) 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*.

c) 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 are 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.

d) Duration of immunity

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.

e) 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.

f) Method of use
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.

g) Precautions (hazards)
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.

5. Tests on the final product
a) Safety
See Section C.4.b.

b) Potency
See Section C.4.c.

REFERENCES


* * *
CHAPTER 2.3.13.

BOVINE SPONGIFORM ENCEPHALOPATHY

SUMMARY

Bovine spongiform encephalopathy (BSE) is a fatal neurological disease of adult cattle that was first recognised in Great Britain (GB) in 1986. The pathological changes, the epidemiological pattern, and the transmissibility of the disease indicate that BSE is one of the spongiform encephalopathies caused by unconventional transmissible agents or prions. The archetype for this group of diseases is scrapie of sheep and goats (see Chapter 2.4.8. Scrapie).

The current 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 proprietary concentrates or feed supplements. Initial cases of BSE in some other countries are considered to be the result of exports from GB of infected cattle or contaminated meat-and-bone meal, although exportations from other countries are now implicated. In others, initial cases are clearly indigenous, with no clear link with imported meat-and-bone meal, suggesting that earlier, undetected, cases may have occurred. A ban on the feeding of ruminant-derived protein to ruminants was first implemented in GB in July 1988. Since then, the feeding of mammalian-derived protein to ruminants has, with certain exemptions, been prohibited throughout the European Union and some other countries. From April 1996, this ban, with respect to mammalian meat-and-bone meal, was extended in the United Kingdom (UK) to all farmed food animals. Commission Decision 2000/766/EC of December 2000 included a temporary ban on the feeding of processed animal proteins to farmed animals kept for the production of food. Experimental transmissibility of BSE to cattle has been demonstrated following parenteral and oral exposure to brain tissue from affected cattle. As a result of control measures, the epizootics in some European countries, including the UK and Switzerland, are already in decline. Cases of BSE currently occur throughout most of Europe and have now also been detected in Asia and North America.

The BSE agent is also believed to be the common source of transmissible spongiform encephalopathies (TSEs) in several other species of bovidae and in species of felidae. There is evidence of a causal link between the BSE agent and a new variant form of the human TSE, Creutzfeldt-Jakob disease (CJD).

BSE, as it occurs in GB, 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 sufficiently distinctive to lead to suspicion of disease, particularly if differential diagnoses are eliminated. Early clinical signs may be subtle, 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 slaughtered, the brain examined and the carcass destroyed. Confirmation of the diagnosis is based primarily on immunohistochemical (IHC) examination of the brain. Lesions have been described only in the central nervous system (CNS). Recommendations for safety precautions for handling BSE-infected material now assume that BSE is a zoonosis and a containment category 3 (with derogation) has been ascribed.

Identification of the agent: No diagnostic test for the BSE agent in the live animal is presently available. The nature of the agents causing the TSE is unresolved. A disease-specific partially protease-resistant isof orm of a membrane protein PrP\(^1\) (PrP\(^{res}\)) has a critical importance in the pathogenesis of these diseases and according to the prion hypothesis is the sole component of the infectious agent.

1 PrP: Prion protein
To confirm the diagnosis of spongiform encephalopathy, histological and immunohistochemical examination of the brain is necessary. The correlation between the clinical diagnosis and the neurohistological diagnosis in BSE can, with appropriate experience of both clinical signs and pathological changes, be greater than 90%. The histopathological examination may also provide a differential diagnosis in clinically suspect cases in which lesions of BSE are not detected. The pathognomonic lesion is a combination of both spongiform change in grey matter neuropil and neuronal vacuolation of certain brainstem nuclei. This change is usually, but not invariably, bilaterally symmetrical. Detection of accumulations of abnormal PrP (PrPres) in the CNS of affected cattle by immunohistochemical methods offers a disease-specific diagnostic approach. PrPres can be detected in unfixed brain extracts by immunoblotting and other enzyme immunoassay methods. Characteristic patterns of accumulations of disease-specific PrP in formalin-fixed affected brain can be demonstrated by immunohistochemical methods. Both approaches are now widely used as confirmatory diagnostic methods and are recommended as adjuncts to histological examination. A negative diagnosis should not be made solely on the basis of the absence of detectable vacuolation. IHC and other PrP detection methods can provide a BSE diagnosis in animals that appear clinically normal with minimal (or no) spongiform lesions in the brain. Characteristic fibrils, homologous with scrapie-associated fibrils and composed of PrPres, can be visualised by electron microscopic examination of detergent-treated extracts of unfixed (or formalin-fixed) BSE-affected brains and have also been used to confirm the diagnosis, but lack the diagnostic sensitivity of the standard immunochemical methods.

BSE can be transmitted from brain tissue of terminally affected cattle to mice by intracerebral/intraperitoneal inoculation or by feeding, but incubation periods of several months precludes bioassay from routine use. This is the only practical method currently available for detection of infectivity.

**Serological tests:** Specific immune responses have not been detected in TSEs.

**Requirements for vaccines and diagnostic biologicals:** There are no biological products available currently. Commercial diagnostic kits for BSE are available and used for diagnosis of BSE in many countries.

### A. INTRODUCTION

A detailed account of bovine spongiform encephalopathy (BSE), its experimental transmissibility (20, 39), occurrence, epidemiology, clinical signs, pathology, diagnosis, prevention, and control has been given previously in English, French and Spanish (55). More recent reviews have provided updated information (2, 16, 17, 26, 28, 36, 46, 62, 80, 81, 96). BSE is an invariably fatal disease of domestic cattle, cases of which were first recognised in Great Britain (GB) in November 1986 (92). BSE has been shown to belong to that group of disorders known as the transmissible spongiform encephalopathies (TSEs) or prion diseases, typified in animal species by scrapie of sheep. These diseases are defined by the pathological accumulation, principally in the central nervous system (CNS) but also in lymphoreticular and peripheral nervous tissues, of an abnormal partially protease-resistant isoform of a host-encoded protein (PrP\textsubscript{C}), designated PrPres. Retrospective studies have indicated that the first cases of BSE presented around April 1985. The initial epidemiological studies established that the occurrence of BSE was in the form of an extended common source epizootic, due to feedborne infection with a scrapie-like agent in meat-and-bone meal used as a dietary protein supplement.

BSE has occurred in several countries other than the United Kingdom (UK) involving imported and/or indigenous cattle. The origin of such cases is most likely to have resulted directly or indirectly from the export of infected cattle or infected meat-and-bone meal from countries with occurrences of BSE, including historically the UK. It is clear that infection has subsequently been propagated within countries in which cases have occurred (24, 25, 31). Indeed, in some countries, the only cases detected reflect indigenous exposure rather than direct linkage with imported contaminated feed. Cases of BSE within indigenous cattle populations outside the UK have now been recorded in most European and some other countries where efficient surveillance or monitoring systems have been applied. These include Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Ireland, Israel, Italy, Japan, Liechtenstein, Luxembourg, the Netherlands, Poland, Portugal, Slovakia, Slovenia, Spain and Switzerland. For current statistics on BSE around the world, readers should consult the OIE Web site.

From July 1988 in GB and from January 1989 in Northern Ireland, the feeding of ruminant-derived protein to ruminants was prohibited. With some exceptions, a ban on the feeding of mammalian-derived protein to ruminant
animals has since been introduced elsewhere: in Switzerland (December 1990) (86) and throughout the European Union (June 1994) (62). From April 1996, this European Union ban, at least when protein was presented in the form of meat-and-bone meal, was extended in the UK to all farmed food animals, including horses and fish. From 1 January 2001 the use of mammalian meat-and-bone meal and fishmeal was prohibited throughout the European Union (32).

Experimentally it has been demonstrated that BSE can be transmitted to cattle following parenteral and oral exposure to brain tissue from affected cattle (20, 90). Epidemiological studies in Britain have revealed an increased risk for the offspring of clinical cases of BSE developing the disease themselves (27, 29, 30, 38, 98). Whether or not this is due to true maternal transmission has not been established. It is considered that this enhanced risk will not maintain endemic infection in the national (UK) cattle population, and indeed the estimates of increased risk for offspring of clinical cases have subsequently been revised downwards (26). There is no evidence of horizontal transmission of BSE between cattle. Epidemiological and transmission studies have not revealed evidence of a risk from semen (100) or milk (58, 84) or through embryos (100, 101). As a result of control measures, the epizootics in the UK and Switzerland (48) are in decline, others are showing the early effects of controls in the form of changes in age-specific incidence (24, 31, 65). In some countries the controls have not been in place long enough for the effects to be recognised. Interpretation of the status of epizootics in Europe has been enhanced by the introduction of active surveillance using rapid diagnostic tests, which have confirmed infection in cattle with clinical signs and also detected infected animals that have not been recognised as having reached clinical onset. Retrospective investigation at farms of origin frequently confirms that some signs have been presented before slaughter, but had not triggered consideration of a diagnosis of BSE.

The novel occurrence of TSEs in several species of captive exotic bovidae and felidae and in domestic cats during the course of the BSE epizootic is suspected or, in specific instances, known to have been caused by the BSE agent or an agent indistinguishable from BSE by current available methods. Exposure is presumed to have been via feed.

In the past, epidemiological studies have found no connection between the exposure of humans to agents causing animal spongiform encephalopathies and the occurrence of the human TSE – Creutzfeldt-Jakob disease (CJD). In particular no enhanced occupational or dietary risk from exposure to sheep products has been established, thus suggesting that scrapie agents are not a human health hazard under natural conditions of exposure. However, monitoring of cases of CJD in the UK resulted, in March 1996, in the announcement of the recognition of ten cases of an apparently new variant of sporadic CJD (v-CJD) in the UK (99). Strain-typing studies of the causal agent of v-CJD in mice (11, 76) have provided strong evidence that the same strain of agent derived from BSE is also found in v-CJD. Studies showing similarities between the Western-blot banding and glycosylation patterns of the disease-related isoform of PrP^res from patients with v-CJD and from certain animal species with naturally acquired or experimentally transmitted BSE, also support this conclusion (18, 49). Therefore, on present evidence, the most likely explanation of cases of v-CJD is exposure to the BSE agent, although the involvement of an agent indistinguishable from BSE by current methods cannot yet be excluded. The current incidence of the disease must still be viewed with caution. It is therefore now recommended that safety precautions for handling the BSE agent be based on the assumption that BSE is transmissible to humans. Because the incubation period of v-CJD is unknown, it is too early to predict the course of the epidemic; attempts to do so have produced widely varying predictions, although as cases continue to appear, the confidence limits are narrowing (19, 37, 41–43, 51, 59, 85).

Clinical BSE can be seen in adult cattle, and most cases occur in animals 4–5 years of age. There is no breed predilection, but the incidence of affected herds by functional type is much greater for dairy than beef, as, in the UK, it was mainly calves from dairy herds that were fed on concentrate rations containing meat-and-bone meal. Onset of clinical signs is not associated with season or stage of breeding cycle.

BSE has an insidious onset and usually a slowly progressive course (9, 55, 92, 97). Occasionally, a case will present with acute signs and then deteriorate rapidly (97), although frequency of observation is a significant factor in determining whether early clinical signs are missed. Presenting signs, though variable, usually include behavioural changes, apprehension, and hyper-reactivity. For example, affected cows may be reluctant to enter the milking parlour or may kick vigorously during milking. In dry cows especially, pelvic limb incoordination and weakness can be the first clinical features to be noticed. Neurological signs predominate throughout the clinical course and may include many aspects of altered mental status and behaviour, abnormalities of posture and movement, and aberrant sensation, but the most commonly reported nervous signs are apprehension, pelvic limb ataxia, and hyperaesthesia to touch and sound. The intense pruritus characteristic of some sheep with scrapie is not prominent in cattle with BSE; though in a proportion of cases there is rubbing and scratching activity. Affected cows will sometimes stand with low head carriage, the neck extended and the ears directed caudally (87). Abnormalities of gait include swaying of the pelvic quarters and pelvic limb hypermetria; features that are most readily appreciated when cattle are observed at pasture. Gait ataxia may also involve the pectoral limbs and, with advancing severity of locomotor signs, generalised weakness, resulting in falling and recumbency, can dominate the clinical picture. Reports of reduced rumination (3, 5), also bradycardia and altered heart rhythm (4), though not specific signs, suggest that autonomic disturbance is a feature of BSE. General
clinical features of loss of bodily condition, decreasing live weight, 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 (95). Clinical signs are essentially similar in other countries where BSE has occurred (9). 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 the complete destruction of the carcasses of affected cattle (63). 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.

Because of the link established between BSE and v-CJD, BSE and related TSE agents are now categorised, in respect to biohazard, with the human TSE (1). Consequently, veterinarians and laboratory workers conducting necropsies on BSE-suspect animals or handling tissues derived from such animals, must conduct the work under containment level 3, (see Chapter I.1.6), sometimes with derogations. There is no evidence that the pathogen is airborne. Therefore, HEPA filtration of laboratory air is not required. Additionally, TSE agents are not inactivated by conventional fumigants, so there is no need for the facility to be sealable to enable fumigation. These derogations are contained in codes of practice issued by the UK Advisory Committee on Dangerous Pathogens (ACDP) under the auspices of the UK Health and Safety Executive in support of British and European legislation to protect individuals at their place of work. Local risk assessment should always be carried out to allow for the nature of the work to be taken into consideration. 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. 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 18 minutes at 30 lb/in². 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 (82). Disinfection 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 (82, 83).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The nature of the agents causing animal or human TSEs remains unresolved (71). The disease-specific modified form (PrP<sup>res</sup>) of a host-coded, highly conserved, membrane protein (PrP<sup>C</sup>) of unknown function, is the only disease-specific macromolecule identified in the scrapie-like diseases. PrP<sup>res</sup> is also frequently referred to as PrP<sup>Sc</sup> and PrP<sup>d</sup> in scrapie and PrP<sup>bse</sup> in BSE. A substantial scientific view is that the agent is composed entirely of the disease-specific isoform of PrP and that the altered form is capable of inducing conversion of the normal form: the protein only or ‘prion’ hypothesis. The opposing view is that the agent is a virus or is virus-like and contains nucleic acid. The identification of multiple ‘strains’ or isolates of scrapie agents, with characteristic incubation periods and patterns of neuropathological change when transmitted to mice, is considered to be more in keeping with this latter hypothesis. Previous studies to determine the resistance of the agent to degeneration have been used to suggest that the TSE agents do not contain nucleic acids. However, critical analyses of the denaturation characteristics of TSE agents, including effects of ultraviolet and ionising irradiation, extremes of temperatures, autoclaving and a large range of chemical disinfectants, suggest that the data obtained are compatible with that obtained for small viruses (15). The molecular basis for strain variation is still unclear, although proponents of the prion hypothesis argue that it is not incompatible with the existence of distinct strains (66).

Characterisation of isolates by transmission of BSE to mice has shown that BSE is caused by a single major strain of agent that differs from characterised strains of the scrapie agent in sheep (10). Uniformity of the pathology among affected cattle has also supported the notion of a single BSE strain and enabled the definition of a particular disease phenotype for BSE (91). This specific pattern of neuropathology in the host species is an important feature of the case definition of BSE. It is difficult however to totally exclude the possibility that other strains of the agent may exist at low frequency. In the absence of in-vitro methods for isolation of the causative agent, the conventional basis of confirmation of the diagnosis in this group of diseases has been the demonstration of the morphological features of spongiform encephalopathy by histopathological examination. This remains necessarily, by definition, the only method by which this characteristic vacuolar pathology can be diagnosed. However, given the essential role of the PrP<sup>res</sup> molecule and increases in technical capabilities in this
area, it is now important that diagnostic approaches use one or more methods for the detection of the abnormal form of the protein. The demonstration by electron microscopy on CNS extracts of characteristic fibrils, termed scrapie-associated fibrils (SAF), which are composed largely of PrP\textsubscript{res}, is a further morphological diagnostic method. Methods of disease-specific PrP detection include immunohistochemical (IHC) demonstration, Western blotting/SAF-immunoblotting and, most recently, a number of rapid immunoassays for screening. The use of a particular method will depend on the purpose to which the diagnosis is to be applied in the epidemiological context, and its validation for that purpose. This range of purposes will extend from confirmation of the clinical diagnosis in the control of epizootic disease to the screening of healthy populations for evidence of covert or preclinical disease. The pathological case definition adopted will also differ according to whether the method is to be applied for confirmation of a case or for screening of a population. For the former it is important to use approaches that can monitor the pathological phenotype of BSE. It is also clear that the performance of individual methods will be crucial to this process of selection of a single method or a portfolio approach. It must be stressed that the development of methods, particularly rapid screening immunoassays for the detection of PrP\textsubscript{res}, is a rapidly evolving field. Care should be taken in the interpretation of data using methodologies that do not enable careful cross-referencing with the gold standards defined here. This is particularly important with respect to the definition of strain. Differences in methodology can, without appropriate comparison with previously published criteria, generate differences in results that do not justify notification of the identification of a new strain. Precipitate announcements of the detection of new strains can have serious repercussions, while on the other hand substantiated identification is important for policy and control purposes.

Quality control (QC) and quality assessment (QA) should form an essential part of the testing procedures. The OIE Reference Laboratories can be contacted to provide assistance in this area and to help develop inter-laboratory comparisons at the international level.

a) Sample preparation

For the preparation of material for diagnostic examination, cattle suspected of having the disease should be killed with an intravenous injection of a concentrated barbiturate solution following sedation, if necessary. The technical procedures concerned with collection, fixation, and histological processing have been described (8, 73) and are revised and summarised below.

In all circumstances of 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 important that a standard neuropathological approach be followed in which representative areas of the whole brain are examined. Departure from this is dependent on local national circumstances, including whether or not a differential diagnosis is required. Additionally, where rapid immunoassays are selected as the primary method of choice, care will be needed in ensuring that sampling and tissue preparation for one test do not compromise the ability to confirm pathological phenotype by histopathological means.

Brain tissue should be removed as soon as possible after death. Fresh material for potential use in tests to detect disease-specific PrP should be taken ideally as a complete coronal section (2–4 g) from the medulla, caudal to the obex, specifically avoiding damage to the obex region. The cervical spinal cord and the lateral hemisphere of cerebellum also offer optimal sampling areas that will not encroach on histopathological requirements. This tissue is stored frozen prior to testing; precautions must be taken to insure that the tissues for histological or IHC examination are not frozen as this will provide artefactual lesions that may compromise the identification of vacuolation, and/or target site location. It is possible (but not desirable) to undertake immunohistochemistry for PrP on material that has been frozen prior to fixation (21). However, it is still important to be sure that target sites have been identified and checked before a negative result can be recorded. If the remaining whole brain is sampled for the histopathological examination, it should be placed in approximately 4–6 litres of 10% formol saline fixative, which should be changed twice weekly. After fixation for 2 weeks, the brain is cut into coronal slices. The fixation time may be shortened by cutting the fresh brainstem into smaller coronal pieces, leaving intact the diagnostically important areas at the obex, the cerebellar peduncles and the rostral colliculi. Depending on some other factors (temperature, agitation, use of microwave) the fixation time for these small pieces of brainstem may be reduced to 2–5 days. The other formol-fixed parts of the brain may be used for differential diagnosis after completing the standard 2 weeks’ fixation. Initially, a single block cut at the obex of the medulla oblongata (Fig. 1) should be selected for histological processing by conventional paraffin wax embedding methods for neural tissue. Sections, cut at 5 μm thickness and stained with haematoxylin and eosin, are examined for characteristic spongiform change and neuronal vacuolation. If results are inconclusive because of minimal lesions, or the material is histologically uninterpretable due to autolysis or damage, or if there are no histological lesions present, it is necessary to carry out additional tests, including IHC or immunoblotting.
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 is consistent with that seen in the brains of cattle from the UK epizootic, it is adequate for monitoring purposes to remove the hind brain alone (Fig. 1). This can be achieved via the foramen magnum without removal of the calvarium. This will reduce the amount of fixative required, thereby lowering costs and improving safety, while maintaining representation of the major target areas for histological examination. The diagnosis may be confirmed if completely typical changes are present in the medulla at the level of the obex. When lesions are not obvious in the medulla (obex), immunohistochemistry should be performed. However, given the constant lesion pattern, this is unlikely to contribute additional confirmation in more than 0.5% of cases of BSE where lesions are absent in the medulla (obex) section (88). Clearly this abridged protocol does not allow a full neuropathological examination for differential diagnoses to be established, nor does it represent a comprehensive phenotypic characterisation of any TSE.

Where the index case is identified through active surveillance, the necessary brain areas for full phenotypic characterisation may not be available. In most countries, hind brain alone is collected (see below), even before the first confirmation of BSE. Ideally, 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 much more comprehensive sampling of the brain of positive animals and enable this recommended approach to the characterisation of cases. This is particularly important if un-validated tests are used, and where in the absence of direct comparison with the methods described here results in claims that new strains have been identified.

The processing of the brain tissue for use in the 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 for the variant methodology. The preferred sample for immunoassay should be at, or within 1.5 cm anterior to, the obex. The choice of target site should take into account the preferred method of confirmation, where the inability to examine brainstem histologically at the obex may prevent the detection of bilateral vacuolation. Sampling the rostral medulla for rapid test does not compromise examination by histological or immunohistochemical means. Hemisection of the brainstem at the level of the obex will result in loss of the ability to assess the symmetry of lesions, but the need for such assessment is less if immunohistochemistry is used. If this approach is adopted however, it becomes critical to ensure that the target site is not compromised. Both the dorsal nucleus of the vagus nerve (the target area for scrapie) and the nucleus of the solitary tract (the target area in cattle) are small, and lie close to midline (Fig. 2).
Figure 2. Cross section of the brainstem at the level of the obex identifying the key target sites for diagnosis by histopathology and immunohistochemistry in BSE (nucleus of the solitary tract [1] and the nucleus of the trigeminal tract V [2]) and scrapie (dorsal nucleus of the vagus [3]).

Inaccurate hemisecting could easily result in the complete loss of target area for confirmatory testing, and significantly reduce the effectiveness of the surveillance programme. Such an approach needs to be implemented with a very clear policy and monitoring programme for training and QA of sampling procedures. Because of the uneven distribution of PrP\textsuperscript{res}, sample size should be as specified in the diagnostic kit or if not specified should be at least 0.5 g. Performance characteristics of all of the tests may be compromised by autolytic changes. In order to reduce hazard to the operators collecting large numbers of samples for an active surveillance programme, bovine brains should be sampled without opening the cranium. This is readily achieved, even at abattoirs, following training of operators in the use of a specially designed spoon, which can be inserted through the foramen magnum of the severed head. The following is a protocol that has been drafted by the OIE Reference Laboratory, Bern, Switzerland. Some rapid test manufacturers also sell disposable spoons for brain removal.

- **Removal of the brainstem**

After the head has been separated from the body between the atlas and foramen magnum, the head is put on a support with the frontal bone down; the caudal end of the brainstem is visible through the foramen magnum. The brainstem is dissected through the foramen magnum without opening the skull by means of a specially designed spoon with sharp edges and a long handle (Fig. 3). The spoon is inserted into the foramen magnum between the brainstem and the bone and moved along the wall of the skull moving to the left and the right to sever the cranial nerves on both sides, while avoiding damage to the brain tissue by keeping close to the bone. The spoon is advanced for a distance of approximately 7 cm in this fashion and then bent downwards cutting and separating the caudal medulla oblongata (with some fragments of cerebellum) from the rest of the brain. The spoon — remaining in a bent downward position — is then pulled towards the operator. In this way the severed brainstem slips out of the skull through the foramen magnum.
Fig. 3. The head is separated from the body and placed on a support upside down; the brainstem (bs) is separated from the bone with cutting movements left and right (curved arrows) by means of a long-handled specially designed spoon with sharp edges, inserted in the foramen magnum between bone and brain tissue. The preferred sample for immunoassay should be at, or within 1.5 cm anterior to the obex.

b) Histological examination

The initial pathological case definition of BSE was based on the histopathological changes in the CNS, and this has provided the usual basis for confirmation of the clinical diagnosis of BSE (88, 92). The histopathological examination also allows confirmation of the characteristic neuropathological phenotype of BSE (78, 94). The histopathological changes are neurodegenerative and closely resemble those of scrapie in sheep. The most prominent features are vacuolar and comprise a spongiform change in the neuropil of grey matter in specific neuroanatomical areas, and single or multiple vacuoles within neuronal perikarya. The precise appearance of the spongiform change in TSEs, as observed by light microscopy, has been defined previously (56). In BSE, spongiform change is the predominant form of vacuolar change. Both forms of vacuolation are bilaterally distributed and usually symmetrical with a consistent pattern of severity relative to distribution throughout the brain (89, 94). The high frequency of occurrence of neuroparenchymal vacuolation in certain anatomic nuclei of the medulla oblongata at the level of the obex has, in the BSE epizootic in the UK, provided a satisfactory means of establishing a diagnosis on a single section of the medulla (88). However, observation of equivocal lesions in the medulla at this level requires examination of other brain areas to detect cases of BSE with minimal or potentially atypical lesions and, when necessary, to establish pathological differential diagnoses. Neurodegenerative changes other than vacuolation are not prominent in BSE. A gliosis (astrocytosis), as seen in scrapie, is another feature, particularly in sites of vacuolar change. Detection of gliosis is assisted by the use of special stains and immunohistochemistry. For example, astrocytosis can be demonstrated by the immunohistochemical detection of increased glial fibrillary acidic protein (GFAP).

The interpretation of observed vacuolar changes in the bovine brain must be approached with caution. Vacuoles within the perikarya, indistinguishable from those of BSE, have been reported in neurones of the red and oculomotor nuclei of the midbrain and other brainstem nuclei as an incidental finding in cattle (34, 40, 57, 92). Thus, like the diagnosis of scrapie, which may be confounded by the occurrence of such neuronal vacuolation scattered in the medullae of healthy sheep (see Chapter 2.4.8. Scrapie) (86, 87), histopathological diagnosis of BSE must not rely on the presence of occasional solitary vacuolated neurones. Even relatively numerous vacuolated neurones in the red nucleus and in the habenular nuclei must be disregarded. The presence of spongiform change in the neuropil in specific neuroanatomical locations in BSE provides the most confidence of minimising false-positive diagnoses.

As with scrapie of sheep, the possibility of BSE cases occurring in which brain lesions are minimal or undetectable by light microscopy, is a potential problem that can be resolved only by diagnostic criteria independent of histopathology (73, 93). (See also the next section.)
c) Detection of disease-specific forms of PrP

In the past in many countries histopathological examination confined to the medulla oblongata is the laboratory investigational method that has been used to handle large numbers of suspect cases. Demonstration of typical changes provides a definitive diagnosis. Many laboratories have now supplemented or replaced histopathological examination by IHC and other PrP-detection methods. When the results of the histopathological examination are inconclusive or negative, or the brain material is unsuitable for histological examination because of autolysis or damage, detection of abnormal accumulation of PrP becomes the method of choice. Applying these PrP tests becomes increasingly important in the decaying phase of the epizootic and in surveillance programmes where critical monitoring of disease is required.

In conjunction with, or even as an alternative to, the histopathological evaluation of medulla sections is the use of IHC to detect PrP\textsuperscript{res} accumulation in formalin-fixed, paraffin-embedded material (47, 94). Several protocols have been applied successfully to the IHC detection of PrP for the diagnosis of BSE (44, 47, 54, 94). Harmonisation toward a fully validated standardised routine diagnostic IHC method is desirable. However, it is likely that only the general principles can be prescribed, with precise methods being determined by each individual laboratory. A European Commission (EC) funded collaboration among European laboratories, which addressed the need for harmonisation of diagnostic methods, concluded that the total standardisation of methods was difficult and possibly unnecessary. Local conditions will always dictate a degree of inter-laboratory variation, and each method should be optimised for use with the standard tissues and common reagents (such as water) used locally. Historically there has also been a dependence on 'in-house' polyclonal antibodies, but the increase in commercially available monoclonal antibodies has reduced this variation significantly. It is much more important to achieve a standardised output, as monitored by participation in QA exercises, and by comparison with the results of a standardised model method.

The technique is more sensitive than routine histopathology as it can detect cases in the last months of incubation before the occurrence of vacuolar changes, at least in experimentally induced cases of BSE (62) and possibly also in the natural disease (23). BSE can therefore be diagnosed by IHC in animals with equivocal (or no) morphological changes. The technique does not necessarily require lengthy tissue fixation, although for accuracy the guidelines established for histopathology still apply and, providing the tissue can be adequately processed histologically, it works well in autolysed tissues in which morphological evaluation is no longer possible. IHC detection of abnormal PrP accumulations is as sensitive as the Western blotting method for detection of PrP\textsuperscript{res} (69). In combination with good histological preparations, IHC allows detection of abnormal PrP accumulations and, as this abnormal PrP accumulation, like the vacuolar pathology, exhibits a typical distribution pattern and appearance, it provides simultaneous evaluation or confirmation of the disease phenotype.

Those laboratories without previous immunohistochemical experience may wish to adopt the model method shown below. As with other techniques described in this text, it will be insufficient in itself to enable immediate replication in a laboratory. Nevertheless, in consultation with the authors and following specific modifications that are required to account for variations in reagent qualities or concentrations, it can be used for comparison with locally preferred methods. The method described below is that used by the OIE Reference Laboratory in the United Kingdom for the diagnosis of BSE, and for the preparation of reference materials for other laboratories.

- Disease-specific PrP – immunohistochemical labelling
  - Reagents
    Store commercial preparations according to the manufacturer’s instructions.

    Tris buffered saline Tween 20 (0.5%), pH 7.6 (TBST)
    Formic acid (96%)
    Citrate buffer solution (0.2%), pH 6.4
    Hydrogen peroxide (30% w/v) (Sigma)
    Methanol
    Vector Labs, Vectastain Elite RAT IgG avidin–biotin complex (ABC) kit
    Copper sulphate solution (0.5%)
    Di-amino-benzidine (DAB) (Sigma)
    Mayer’s haematoxylin
    0.5% conc. HCl in absolute ethanol
Absolute ethanol
Xylene
Ammonia water

Primary antibody (rat anti-bovine PrP monoclonal R145 antibody, available from Veterinary Laboratories Agency, Weybridge, UK)

• **Tissues**

Formalin-fixed tissues of maximum thickness of 3 mm. Immerse in 96% formic acid for 1 hour. Rinse in running water for 10 minutes. Immerse in neutral buffered formalin for 1 hour and routinely process to wax.

• **Method**

Following preparation of paraffin wax embedded blocks (according to routine methods), cut sections at 3 µm thickness and mount on polysine-coated slides or equivalent. Air dry and place in 60°C oven overnight.

  i) Deparaffinise sections in xylene for 10 minutes.
  ii) Wash in absolute ethanol twice for 30 seconds (agitate).
  iii) Wash in running tap water for 10 minutes.
  iv) Immerse in 96% formic acid for 5 minutes. (Formic acid treatment is used for preliminary antigen retrieval, which also maintains good tissue morphology during subsequent autoclaving. It also increases operator safety by reducing infectivity levels in the tissue being handled.)
  v) Wash in running tap water for 10 minutes.
  vi) Immerse in citrate buffer solution in an open plastic trough and autoclave for 5 minutes at 121°C.
  vii) Cool at room temperature for 10 minutes then rinse in running tap water for 10 minutes.
  viii) Immerse in 3% hydrogen peroxide in methanol for 20 minutes.
  ix) Rinse in running water for 10 minutes.
  x) Dry sections and isolate with wax pen.
  xi) Sections should not be allowed to dry from now on.
  xii) Rinse sections in TBST twice.
  xiii) Apply normal rabbit serum (NRS)* for 60 minutes at room temperature. (*Dilute NRS according to manufacturer’s instructions.)
  xiv) Drain off NRS and apply primary antibody (rat anti-bovine PrP monoclonal antibody R145 at 1/3000) for 16–18 hours at room temperature.
  xv) Rinse sections in TBST twice for 2–3 minutes each time.
  xvi) Apply secondary antibody* (rabbit anti-rat IgG) for 60 minutes at room temperature. (*Dilute secondary antibody according to manufacturer’s instructions.)
  xvii) Prepare avidin–biotin complex (ABC) reagent according to manufacturer’s instructions and leave at room temperature for 30 minutes before use.
  xviii) Rinse sections in TBST twice for 3 minutes each time.
  xix) Apply ABC for 30 minutes at room temperature.
  xx) Rinse sections in TBST twice for 3 minutes each time.
  xxi) Apply DAB for 10 minutes.
  xxi) Rinse in tap water twice for 2 minutes each time.
  xxiii) Immerse in 0.5% aqueous copper sulphate for 5 minutes.
  xxiv) Rinse in tap water for 1 minute.
  xxv) Immerse sections in Mayers haematoxylin for 5 minutes.
  xxvi) Rinse in tap water until water runs clear.
  xxvii) Differentiate in 1% acid alcohol (1% HCl in ethanol) for 2–3 seconds.
  xxviii) Rinse sections in tap water for 1 minute.
Abnormal accumulations of PrP as shown by IHC are considered to have potential for the preclinical diagnosis of scrapie in sheep (in some, but not all, genotypes) using tonsillar (72) or nictitating membrane (64) lymphoid tissue biopsies. However, BSE infectivity has not been detected by mouse bioassay or IHC in lymphoid tissues at any time during the incubation period or clinical disease course (90), other than in distal ileum containing Peyer’s patches in experimentally infected cattle. Currently, this suggests that these tissues are unlikely to be of use diagnostically. One unpublished result of the detection of infectivity in the tonsil of an experimentally infected animal 10 months after oral exposure, remains incomplete. The finding is in contrast to negative results in tonsils collected at earlier and later time points in the study, including clinically affected animals, and does not indicate a breakthrough in the scope for in-vivo testing of cattle.

Detection of PrPres by SAF-purification followed by immunoblotting techniques (35, 50, 79), is carried out on fresh (unfixed) or frozen brain or spinal cord material. Improvements in purification methods for extracting PrP (7, 22) have contributed to increased sensitivity of this method. Where sufficient sample size remains, this methodology frequently provides a sensitive method of confirming diagnosis following initial suspicion of disease using more recent rapid tests (see below). For that reason, and in the absence of a full and accessible published method elsewhere, the method for SAF-immunoblotting is reproduced below.

**Protocol for the SAF-Immunoblot for TSE diagnosis**

The following is an example of a protocol for the purification and detection of the disease specific isoform of the prion protein (PrPres) from unfixed brainstem material. Purification of the scrapie-associated fibrils (SAF) is first achieved by ultracentrifugation followed by a proteinase K treatment to digest residual cellular PrP C. Fibrils are then spun through a sucrose cushion for further purification prior to immunoblot detection of PrPres.

### Sample preparation

Material must be taken from the brainstem and, if available, from the obex region (this is the region with the highest PrPres content). Samples should be taken as follows:

- **Negative control:** 4 g
- **Positive control:** 4 g
- **Suspect sample with strong rapid test signal (e.g. optical density [OD] >2.5 in Bio-Rad Platelia):** 1 g, this material will be completely treated with Proteinase K (PK)*.
- **Suspect sample with weak rapid test signal (e.g. OD <2.5 in Bio-Rad Platelia):** 2 g +PK and 2 g –PK treatment

*NOTE: Depending on the rapid test result, the amount of suspect brain stem material that is taken for confirmatory purposes may be either 2 g or 1 g. However, if a negative result occurs in a sample where only 1 g has been examined, this sample must be repeated with at least 2 g +PK treatment.

i) Take appropriate amount of brainstem material (obex region).

ii) Cut into small pieces (removing dura mater) and add 5 ml of brain lysis buffer (BLB) (10 g N-lauroyl-sarcosine, sodium salt [SIGMA #L5125] in 100 ml 0.01 M sodium phosphate buffer, pH 7.4, plus protease inhibitors – 10 µl of 100 mM phenylmethylsulfonylfluoride [PMSF] and 10 µl of 100 mM N-ethyl-maleimide [NEM]).

iii) Homogenise thoroughly in a glass homogeniser (douncer).

iv) Carefully transfer into a 50 ml plastic tube.

v) Add another 2–5 ml of BLB into the douncer, rinse well and also add this into the plastic tube, bringing the end volume of the homogenate to 10 ml. Sonicate for 1 minute.

vi) Transfer sample into quickseal tube, balance weight by adding BLB, and close tube with tube topper.

vii) Centrifuge at 20,000 g (17,000 rpm) for 30 minutes at 10°C in a 70 Ti Beckmann ultracentrifuge rotor.
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viii) Carefully remove the supernatant with a syringe and add into a clean centrifuge tube. Then fill with BLB to the neck of the tube. Seal tubes as above and centrifuge at 177,000 g (46,000 rpm) for 135 minutes at 10°C in a 70 Ti Beckmann ultracentrifuge rotor.

ix) Discard the supernatant and suspend the pellet in 3 ml distilled water with 50 µl 1 M Tris/HCl, pH 7.4 (0.0167 M) by gentle aspiration with a pipette. For less than 2 g brain material, only 1.5 ml distilled water with 25 µl 1 M Tris/HCl, pH 7.4 (0.0167 M) is used.

x) Incubate in a water bath at 37°C for 15 minutes stirring regularly.

xi) For the KI-HSB solution, make up 1.5 g sodium thiosulphate, 1.0 g N-lauroyl-sarcosine in 1 ml 1 M Tris/HCl and add 10 g of potassium iodide for a 10% solution or 15 g for 15% solution. Dilute to 100 ml with distilled water. Add 6 ml of the 15% KI-HSB solution to the sample and incubate for a further 30 minutes as in step x. For less than 2 g brain material, 3 ml 15% KI-HSB is added.

xii) Divide the solution into two aliquots of 4.5 ml (only for samples that are divided into plus and minus PK treatment).

xiii) To one aliquot add 1 mg/ml PK and incubate for 1 hour as in step x. Amount of PK solution to be added – for 2 g and less: + 45 µl PK.

xiv) To the other aliquot, add 4.5 ml of 10% KI-HSB and then carefully transfer into an ultracentrifuge tube. Carefully add 2 ml 20% sucrose with a long syringe to the bottom of the tube and fill tube to neck with 10% KI-HSB and centrifuge at 189,000 g (51,000 rpm) for 1 hour at 10°C.

xv) Carry out step xiii on the PK-treated sample.

xvi) Carefully tip off supernatants and drain tubes well, always paying attention to the pellet.

xvii) Resuspend samples in 40 µl 1 x sample buffer (2 ml of 20% sodium dodecyl sulphate [SDS], 1 ml Tris/HCl [1 M, pH 7.4] 1 ml mercaptoethanol, 0.6g sucrose, 1–3 drops bromphenol blue, 15 ml distilled water).

xviii) Sonicate samples for 30 seconds.

xix) Centrifuge briefly to concentrate the sample in the bottom of the tube.

• SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and Western blot

i) Assemble a large and a small glass plate and spacers for a minigel after cleaning glass plates with 70% ethanol and drying them off completely.

ii) Prepare separation gel and pour or pipette the gel between the plates up to 2 cm from the top of the small glass plate. Carefully pipette a layer of isopropanol on the top of the gel and let set for approx. 60 minutes.

iii) Carefully rinse the gel surface with deionised water. Prepare the stacking gel and pour or pipette it on top of the separation gel. Insert a comb within 30 seconds and let the gel set for 5 minutes.

iv) Pull the comb and assemble the gel apparatus.

v) Fill 1 x electrophorese gel chamber with electrophoresis buffer (25 mM Tris [3.03 g/litre], 192 mM glycine [14.4 g/litre], 20% methanol [200 ml/litre], 0.2% SDS [2 g/litre]). Gel must be well below buffer level. Carefully clean the wells from gel rests with a pipette.

vi) Load 30 µl of each sample on 13% SDS mini-gel after incubating samples for 5 minutes at 95°C.

vii) Run gel at 100 V until samples have moved well into the separation gel, then continue at 200 V (will take another 30–40 minutes). Stop the gel run when blue colour front has just run out at the bottom of the gel.

viii) Set transfer: on the semidry chamber, assemble in the following order:

- three layers of Whatman paper, slightly larger than the membranes in size, soaked well in blotting buffer;
- immobilon-P-Transfer-membrane (this membrane must have been equilibrated first in methanol for 5 minutes and then in blotting buffer beforehand);
- gel;
- three layers of Whatman paper soaked well in blotting buffer.

Close semi-dry chamber and run the transfer at 15 V for 50 minutes.

ix) Take membrane out of the transfer cell and block it in 20 ml 1-Block (5% non-fat dried milk in phosphate buffered saline [PBS] + 0.1% Tween 20) for 30 minutes at room temperature on a rocker.
x) Incubate for 1.5 hours at room temperature or over night at 4°C with the primary antibody in 1-Block.

xi) Wash three times for 10 minutes each in PBS + 0.1 % Tween 20 (PBS/Tween) on a rocker.

xii) Incubate for 1 hour with the conjugate (GAM-AP) diluted 1/000 in PBS/Tween at room temperature on a rocker.

xiii) Wash three times for 10 minutes each in PBS/Tween at room temperature on a rocker.

xiv) Incubate in 1 x assay buffer for 2 minutes at room temperature on a rocker.

xv) Visualise bound antibodies by adding 1.5 ml CDP Star detection reagent (Tropix) on each blot, incubate for 5 minutes, then wrap membrane in plastic foil and detect signals in a camera or by exposing a film.

• Interpretation of results

As this protocol aims at the concentration of the insoluble PrP res by ultracentrifugation, no signal would be expected to be seen from the digested negative control sample and the undigested aliquot, containing only soluble PrPC, is likely to give only a low signal. Prion protein is glycosylated twice. As a consequence after PK digestion PrPres is yielding bands at m.m. 30–27 kDa, 26–24 kDa and 21–19 kDa in the immunoblot. For BSE PrPres the upper band is most prominent. This is also true for most scrapie PrPres signals, but banding intensities can also be different.

Negative control:
- Not treated with PK: no or only weak PrPC (33–35 kDa) signal.
- Treated with PK: no PrP specific signal.

Positive control:
- Not treated with PK: very strong signal, often too strong to differentiate single PrP bands, highest signal intensity at 33–35 kDa.
- Treated with PK: three PrPres bands visible.

A shift in molecular weight should be visible between the undigested and the digested fraction of the positive control to verify the PK activity.

Diagnostic sample:
- The PK-treated diagnostic sample is diagnosed as positive if PrPres signals are clearly visible. Diagnostic samples should preferentially be loaded on the same blot as the PK-treated positive control. They must always be loaded on the same gel as an undigested PrPC control sample to be able to judge the shift in molecular weight.
- The PK-treated diagnostic sample is diagnosed as negative if there is no detectable PrPres-specific signal.
- Testing must be repeated if the results of the positive or negative control are untypical or if the result of the diagnostic sample is inconclusive, e.g. as signals are:
  - very faint (repeat SAF preparation with a higher amount of brain material)
  - banding pattern does not match the positive control (repeat procedure, use other diagnostic methods in addition).

• Rapid tests for the detection of the disease-specific forms of PRP

Automated Western blot and enzyme-linked immunosorbent assay (ELISA) techniques have been developed that allow screening of large numbers of brain samples (60, 61, 69, 70) and are now commercially available. Such techniques can be performed rapidly and are potentially more sensitive than the histopathological evaluation and may compare with that of SAF-immunoblot. Evaluation in comparison with the sensitivity of immunohistochemistry remains to be determined. In a trial conducted on behalf of the EC (60, 61), it was demonstrated that one such Western immunoblot method, or either of two specific ELISA methods, when evaluated on brain tissue, were suitable for diagnostic use in specifically targeted populations. This evaluation was 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. These tests are now approved and used in European and some other countries for large-scale screening and surveillance programmes. They provide a means of initial screening for animals in the late stages (the last few months) of the incubation period, 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 recently developed 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. In some countries, given the speed with which results can be obtained, the rapid tests are the preferred primary test, but confirmation of a diagnosis of BSE requires examination of fixed brain by histopathology and/or IHC.

A further generation of rapid diagnostic tests was evaluated by the EC in 2001 (33, 70). The process has highlighted the need for such an evaluation process, and identified the dangers of using research tools prematurely for active surveillance. Although the evaluation programme is in support of European legislation on surveillance for BSE, the consequences are of relevance to other countries as well. 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. The studies initiated by the EC represent evaluations of the tests: test validation is currently ongoing. Caution must be exercised in the comparative interpretation of tests applied to apparently healthy animals as different tests may vary in their sensitivities relative to stage of incubation and pathogenesis of the disease.

Brief details of the five tests now approved for use in the European Union are provided below. See Section B.1. regarding the interchangeability of terminology for the abnormal form of PrP.

- The Enfer Test\textsuperscript{2} is a qualitative microplate-based chemiluminescent immunassay for the detection of resistant prion protein (PrP\textsuperscript{res}). PrP\textsuperscript{res} in extracted samples is bound to prepared wells in microtitre plates and detected with an anti-PrP polyclonal primary antibody, a horseradish peroxidase-conjugated secondary antibody and a chemiluminescent substrate.

- The Platelia\textsuperscript{3} or TeSeE\textsuperscript{3} test is a sandwich immunoassay which uses two monoclonals for the detection of abnormal prion protein, which is resistant to proteinase K. The presence of abnormal PrP is indicated by a coloured substrate. The test has been approved in a manual and automated format.

- The Prionics\textsuperscript{4} Check Western Blot is a Western blot based method for the detection of the disease specific form of the prion protein. Samples are homogenised and treated with protease to remove normal PrP (PrP\textsuperscript{c}) and to convert PrP\textsuperscript{res} to the PrP27-30 fragment. Samples are separated by gel electrophoresis, transferred to a membrane and PrP27-30 is identified by its immuno-reactivity with anti-PrP antibodies and by the molecular weights of stained bands.

- The CDI test\textsuperscript{5} is an automated conformational dependent immunoassay for the detection of disease causing PrP\textsuperscript{res}. The test is based on a sandwich ELISA using a europium labelled antibody to detect any PrP present in the sample. The comparison of normal (PrP\textsuperscript{c}) to total (PrP\textsuperscript{c} and PrP\textsuperscript{res}) allows an assessment to be made of the level of abnormal PrP\textsuperscript{res} in the sample.

- The Prionics\textsuperscript{6} Check LIA is a microplate-based chemiluminescence immunoassay. Samples are treated with proteinase K to degrade PrP\textsuperscript{c}, while PrP\textsuperscript{res} is reduced to the 27–30 kd fragment. The proteolytic reaction is stopped and PrP\textsuperscript{res} contained in the sample is detected in a sandwich immunoassay with a chemiluminescent enzyme substrate reaction.

\textbf{d) Other diagnostic tests}

The demonstration of characteristic fibrils, the bovine counterpart of SAF (see Chapter 2.4.8. Scrapie), by negative-stain electron microscopy in detergent extracts of fresh or frozen brain or spinal cord tissue (75, 79, 92) has been used as an additional diagnostic method for BSE and may be particularly useful when histopathological approaches are precluded by the occurrence of post-mortem decomposition (74). Recent work on scrapie indicates that, with modification, the method may be applied successfully to formalin-fixed tissue (13). Detection of fibrils has been shown to correlate well with the histopathological diagnosis of BSE (77), but does not offer the specificity or sensitivity available from IHC or immunoblotting methods. Some of

\textsuperscript{2} Enfer test distributed by: Abbott Diagnostics, Abbott Laboratories, 100 Abbott Park Road, Abbott Park, IL 60034-3500, USA.

\textsuperscript{3} Platelia Bio-Rad (A subsequent version of this test the TeSeE test has been approved by the EU and is now available): Bio-Rad, 3 Boulevard Raymond Poincar, 92430 Marnes la Coquette, Paris, France.

\textsuperscript{4} Prionics Check Western Blot and Prionics LIA: Prionics, Wagistrasse 27a, CH-8952 Schlieren, Switzerland, Distributor: Roche Diagnostics GmbH, 68298 Mannheim, Germany.

\textsuperscript{5} CDI test: InPro Biotechnology, 870 Dubuque Avenue, South San Francisco, CA 94080, USA.

\textsuperscript{6} Prionics LIA, Wagistrasse 27a, CH-8952 Schlieren, Switzerland (see footnote 4), www.prionics.com
the rapid immunological tests are also effective in the presence of autolysis (14) and given their greater sensitivity than SAF detection, may be the preferred tests in such circumstances.

BSE infection can be shown by intracerebral/intraperitoneal inoculation (39) or by feeding mice with brain tissue from terminally affected cattle (6), but bioassay is impractical for routine diagnosis because of the long incubation period (≥292 days). Further development of transgenic mice overexpressing the bovine PrP gene may potentially offer bioassays with reduced incubation periods for BSE. However, data obtained from one such study did not derive incubation periods substantially shorter than that of conventional mouse strains (12).

There remains the need for a test for BSE that can be applied to the live animal and has sensitivity capable of detecting PrP\textsuperscript{res} at the low levels, such as may occur in the early stages of incubation of the disease. Potential approaches to diagnosis are published from time to time, often of a preliminary nature and based on limited amounts of data. None has progressed to the point of peer review and evaluation by others, and claims should be interpreted with care.

Certain protein markers, notably apolipoprotein E (Apo E), can be detected by two-dimensional gel electrophoresis in cerebrospinal fluid of clinically suspected, histopathologically confirmed cases of BSE (53). Apo E is, however, a nonspecific marker for neurodegeneration and has not been shown to be useful for diagnosis of preclinical cases of BSE. Assay of cerebrospinal fluid for the 14-3-3 protein is not of diagnostic use in BSE (68). Similarly, studies of S-100 proteins in cerebrospinal fluid (45) and serum (67) did not give results that would provide diagnostically useful tests for BSE. The electrochemical detection of metabolites in urine (52) has a final performance validation that gave specificity and sensitivity values below that required for it to have a possible independent role in the diagnosis of BSE. Preliminary data indicating that a derivative of the PrP molecule may be detected in the urine of infected cattle (77) is leading to further investigations with a view to the marketing of a diagnostic test kit, but further evaluation and peer review of the data are required.

2. Serological tests

Similarities with scrapie, in which no immune response in the host has been detected, suggest that there is not likely to be an immune response in BSE.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available currently. As discussed previously, diagnostic kits have been licensed for use in many countries.

REFERENCES


Chapter 2.3.13. — Bovine spongiform encephalopathy


*   *

**NB:** There are OIE Reference Laboratories for Bovine spongiform encephalopathy (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.3.14.
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, and can be caused by either of two gammaherpesviruses. The alcelaphine herpesvirus-1 (AIHV-1), the natural host of which – the wildebeest – is infected inapparently, 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 was formerly referred to 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 viruses 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 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.

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, immunofluorescence, and immunocytochemistry. However, the antibody response of clinically affected animals is limited, with no neutralising antibody developing, so that detection relies on the use of immunofluorescence 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 or immunoblotting. While antibody often can be detected by immunofluorescence in cattle with MCF, in more acutely affected animals, such as deer, antibody is not always present. The more recently developed 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.

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. Disease caused by AIHV-1 is restricted to those areas of Africa where wildebeest are present and to zoological collections elsewhere, and formerly has been referred to as wildebeest-derived 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.

• 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 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 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 (10). 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 (11).

• 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 perportal 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 caused by either agent are essentially similar. However, apart from histological examination, the methods available for diagnosing AIHV-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 (13). The recent publication of the full nucleotide sequence of the virulent low passage virus (C500) will form the basis of further studies of this virus (4).

1. **Identification of the agent**

   - **Clinically affected animals**

   a) **Isolation**

   The striking feature of AIHV-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 × 10⁶ cells/ml, and inoculated into preformed cell monolayer cultures. Bovine thyroid cells have been used extensively, but most primary and low passage cell monolayer 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 (>40) for evidence of cytopathic effects (CPE). These appear characteristically 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).

A restriction map of the standard laboratory strain (WC11) using the restriction enzymes HindIII, EcoRI, BamHI and SmaI has been published and most of the genome has been cloned (2). Such clones can be used to identify and characterise other bovid gammaherpesvirus isolates. Sequence data on the WC11 isolate of AIHV-1 have been generated and primers suitable for use in the PCR have been identified (7). None, however, has been established as appropriate for use as diagnostic aids. In addition, the complete genome of the virulent C500 isolate has been published (4).

- **Natural hosts**

It is almost certain that all free-living wildebeest are infected with AIHV-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 blue wildebeest calves in South Africa (12).

Virus isolation will seldom be contemplated. 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.

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. Although other methods of antibody detection have been described, none has proved to be satisfactory. A competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) was first developed for detecting antibody to OvHV-2 (9) using an MAb (15-A) that targets an epitope that appears to be conserved among all MCF viruses.

- **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 AIHV-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 validation of the CI-ELISA is ongoing.

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 AIHV-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,
reduces 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 AlHV-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, 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 UV 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 multwell 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 TCID₅₀ (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 1/10⁴ 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 AlHV-1 containing approximately 10³ TCID₅₀ 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.

vii) 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 antibodies 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 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 very limited success. High-titre VN antibodies can be induced, but these are evidently not protective.

AIHV-1 stock (strain WC11) is grown in primary or secondary cell cultures of bovine kidney, bovine thyroid, low passage bovine testis, or other permissive cell type. The virus is stored in aliquots at –70°C. The stock is titrated to determine the dilution that will give 100 TCID₅₀ 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 TCID₅₀ 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 × 10⁵ cells/ml.

  vii) Incubate the plates in a humidified CO₂ 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 TCID₅₀ with a permissible range 30–300) and the control sera. The standard positive serum should give a titre within 0.3 log₁₀ 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 (9) using an 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 AIHV-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: AIHV-1, AIHV-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 (8). 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 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) (8). 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 (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 antibodies-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 sulfuric 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:

      \[
      \text{% Inhibition} = \left(\frac{\text{Sample OD (Average)}}{\text{Mean negative control OD}}\right) \times 100
      \]
  
  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 more readily to red deer (*Cervus elaphus*) and other deer
species, water buffalo (*Bubalus bubalis*) and even more readily to Père 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 is rapidly becoming the method of choice.

It must be emphasised that the viral cause of SA-MCF has not been 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 (5), but not in neutralisation assays; (b) the development of antibody that cross-reacts with AIHV-1 in the IFA test in a proportion of 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, AIHV-1 DNA.

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 disease following inoculation into experimental animals (14). Such cell lines contain viral sequences that hybridise with clones of AIHV-1 DNA (3). Several viral fragments have been cloned from a genomic library of one such line and have been the subject of further characterisation. A subclone that did not hybridise with AIHV-1 was chosen for sequencing and found to code for a protein very similar to the tegument protein of Epstein–Barr virus. 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 (1). 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 described (6) and is likely to have valuable future application.

   • **Polymerase chain reaction**

     Extraction of DNA from clinical material if performed according to the protocol defined in an appropriate extraction kit (e.g. Quiagen DN easy 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 sulphide, 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.

   • **Natural hosts**

     The domestic sheep is the natural host of OvHV-2 and all animals tested have been shown to be seropositive for AIHV-1. Furthermore, it would appear that some lambs become infected in utero, rendering it very difficult to identify uninfected animals. Neonatal lambs are often the source of infection, though sheep of all ages can transmit disease. Virus has never been recovered from sheep, but the PCR developed for detecting OvHV-2 DNA in clinical cases of MCF has also been employed to detect virus in the peripheral blood leukocytes of normal adult sheep and in nasal secretions and the oropharynx of lambs during the first months of life. Thus it would appear that, as with AIHV-1 infection in wildebeest, sheep become infected with OvHV-2 at a young age and thereafter remain latently infected for life.

     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 is not known.

2. Serological tests

Antibody to OvHV-2 has only been detected by 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 generally is not 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 (9) using an 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. However, there was poor correlation between the development of antibody detected in serum of lambs and the acquisition of infection with OvHV-2 as indicated by the presence of viral DNA detected by PCR, and only a proportion of sheep were found to be positive. This is in marked contrast to other studies employing IFA, which indicate that most if not all domestic sheep are serologically positive. 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. Thus it is likely that some of the negative results obtained by CI-ELISA are due to the extreme epitope specificity of such an MAb-based test that fails to detect antibody in a proportion of sera. Results from such a test should therefore be interpreted with caution. A reformatted test has, however, recently been reported, which may overcome the problem of sensitivity (8) and provide a test of greater of utility.

The CI-ELISA, as described in Section B1.2.d has been used successfully to detect antibody against OvHV-2.

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 farmed deer every reasonable effort must be taken to segregate the management of sheep and deer, 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.

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

No successful vaccination regimen has been identified. However, most investigations have employed isolates of AIHV-1 that are adapted to tissue culture and that are generally avirulent. Recent studies of the molecular changes in the virus have shown that in the course of adaptation to in-vitro culture, a specific deletion occurs in the viral DNA that appears to be consistent among different isolates during the process of attenuation. Thus, it is proposed that this deleted segment codes for a virulence factor(s). The omission of this product from previously tested vaccines may have contributed to the poor success with these experiments. A sequence homologous to
this deletion is also present in OvHV-2, suggesting that it is conserved and thus provides further weight to the hypothesis that it may have an important role in the pathobiology of MCF (4).

Future vaccine strategies should therefore consider incorporating these viral components that may protect against both forms of MCF.

REFERENCES


* * *
chapter 2.3.15.

trypanosomosis
(tsetse-transmitted)

summary

Tsetse-transmitted trypanosomosis\(^1\) is a disease complex caused by several species of protozoan parasites of the genus Trypanosoma, transmitted cyclically by the genus Glossina (tsetse flies). 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.

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 sensitive test, used on a more experimental basis, is the polymerase chain reaction.

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.

Requirements for vaccines in diagnostic biologicals: No biological products are in use at the present time.

A. INTRODUCTION

Tsetse-transmitted trypanosomosis\(^1\) is a disease complex caused by several species of protozoan parasites of the genus Trypanosoma, transmitted cyclically by the genus Glossina (tsetse flies). Tsetse infest 10 million square kilometres and affect 37 countries, mostly in Africa. The disease infects various species of mammals but, from an economic point of view, tsetse-transmitted trypanosomosis is particularly important in cattle (in southern Africa referred to as nagana or tsetse-fly disease). It is mainly caused by Trypanosoma congolense, T. vivax and, to a lesser extent, T. brucei brucei. Trypanosoma uniforme, T. simiae and T. suis are other, less common tsetse-transmitted species. Trypanosoma vivax is also transmitted mechanically by biting flies, as exemplified by its presence in South and Central America. Tsetse-transmitted trypanosomosis also affects humans, causing sleeping sickness, through infection with either T. brucei gambiense or T. brucei rhodesiense.

Clinical signs of tsetse-transmitted trypanosomosis may include intermittent fever, oedema, abortion, and emaciation. Anaemia usually develops in affected animals and this 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

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\(^1\) 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., Hiepe 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’.
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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).

B. DIAGNOSTIC TECHNIQUES

A variety of diagnostic tests are available (25) 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 (23). 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. Due to this low sensitivity, the apparent parasitological prevalence of trypanosomosis is generally 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 drop 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. A 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
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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. 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 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 (2). 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 (16, 20), 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. theileri* 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 (28), 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 ciresaseal 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.

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.

The microhaematocrit centrifugation technique is more sensitive than the direct examination techniques (15). 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 (8). 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) (1). The method has been used for the diagnosis of T. b. gambiense infections. The method is probably too expensive for the routine large-scale use in animal trypanosomosis surveys.

b) Dark-ground/phase-contrast buffy coat technique

The buffy coat technique or Murray method (21) 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 and covered with a coverslip (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.

Trypanosoma congolense: Small, sluggish, adheres to RBCs by anterior end.

Trypanosoma theileri: More than twice the size of pathogenic trypanosomes, tends to rotate.

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 (15). 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. 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 haematocrit 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 (18). 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.

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 (26). 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 (14).

• 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. 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
An antigen-detection enzyme-linked immunosorbent assay (ELISA) for trypanosomosis has been described (22). Field evaluations of the test have given inconsistent results (5). Therefore, additional work is needed to discover and overcome the cause of those inconsistencies before the test can be used in the routine diagnosis of trypanosomosis.

• 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 (4, 6, 19); 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: T. brucei 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. Recently PCR restriction fragment length polymorphism (RFLP) assays have been developed that allow the identification of all Trypanosoma species as single or mixed infections using one single test (3, 7, 9).

Standard 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.

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 coat spotted on to filter paper (9, 12). 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) (13) and the trypanosomal antibody-detection ELISA (11, 17). The identification of major antigens of trypanosomes, and their production as recombinant molecules or synthetic peptides, is leading to the current development and validation of new tests based on the use defined molecules. Thus, in the near 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 (27) has been replaced by a new technique for the preparation of trypanosomal antigens (13), 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 (17) has recently been further developed for use in large-scale surveys of bovine trypanosomosis (11). ELISAs using T. congolense or T. vivax precoated microtitre plates have been developed that have the advantage that a standardised denatured antigen is used, and which can be stored for long periods at room temperature (24).

The standard antigen for trypanosomosis antibody tests is derived from bloodstream-form trypanosomes. Antigens are prepared as a soluble fraction of trypanosomes purified by DEAE anion-exchange
chromatography of parasites from whole blood of infected rats, with lysis 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 (10).

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 (29). Results are, therefore, quantifiable. The cut-off value is determined using known positive and negative field samples (5).

Both antibody-detection tests have high sensitivity and specificity. Their species specificity is generally low. They detect immune responses to current and past infections and can, therefore, only provide a presumptive diagnosis of active infection.

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 biological products are in use at the present time.

REFERENCES


Chapter 2.3.15. — Trypanosomosis (tsetse-transmitted)


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NB: There is an OIE Reference Laboratory for Trypanosomosis (tsetse-transmitted) (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).