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.

* *
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>2.9.3.</td>
<td>European foulbrood</td>
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<td>Nosemosis of bees</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

<table>
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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>
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<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>Full Description</th>
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<tr>
<td>Agent id.</td>
<td>Agent identification</td>
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<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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<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>ATCC¹</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>BBAT</td>
<td>Buffered <em>Brucella</em> antigen test</td>
</tr>
<tr>
<td>BFK</td>
<td>Bovine fetal kidney (cells)</td>
</tr>
<tr>
<td>BGPS</td>
<td>Beef extract-glucose-peptone-serum (medium)</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby hamster kidney (cell line)</td>
</tr>
<tr>
<td>BLP</td>
<td>Buffered lactose peptone</td>
</tr>
<tr>
<td>BPAT</td>
<td>Buffered plate antigen test</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSF</td>
<td>Bovine serum factors</td>
</tr>
<tr>
<td>CAM</td>
<td>Chorioallantoic membrane</td>
</tr>
<tr>
<td>CEF</td>
<td>Chicken embryo fibroblast</td>
</tr>
<tr>
<td>CF</td>
<td>Complement fixation (test)</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming unit</td>
</tr>
<tr>
<td>CIEP</td>
<td>Counter immunoelectrophoresis</td>
</tr>
<tr>
<td>CK</td>
<td>Calf kidney (cells)</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>CPLM</td>
<td>Cysteine-peptone-liver infusion maltose (medium)</td>
</tr>
<tr>
<td>CSY</td>
<td>Casein-sucrose-yeast (agar)</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>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 immunosorbert assay</td>
</tr>
<tr>
<td>EMTM</td>
<td>Evans’ modified Tobie’s medium</td>
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<tr>
<td>EYL</td>
<td>Earle’s modified Tobie’s medium</td>
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<tr>
<td>FAT</td>
<td>Fluorescent antibody test</td>
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<tr>
<td>FAVN</td>
<td>Fluorescent antibody virus neutralisation</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<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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<td>GIT</td>
<td>Growth inhibition test</td>
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<td>HA</td>
<td>Haemagglutination</td>
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<td>HAD</td>
<td>Haemadsorption</td>
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<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
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<td>HEP</td>
<td>High-egg-passage (virus)</td>
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<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid [buffer]</td>
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<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>ID₉₀</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>
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<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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<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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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<td>PAP</td>
<td>Peroxidase–antiperoxidase (staining procedure)</td>
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<td>PAS</td>
<td>Periodic acid-Schiff (reaction)</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<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>
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<tr>
<td>PPD</td>
<td>Purified protein derivative</td>
</tr>
<tr>
<td>PPLO</td>
<td>Pleuropneumonia-like organisms</td>
</tr>
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<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>Description</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>
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<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>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
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<td>SPF</td>
<td>Specific pathogen free</td>
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<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:

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

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:

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

• 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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2.10.10. Hendra and Nipah virus diseases
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2.10.13. Verocytotoxigenic Escherichia coli
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2.10.14. Listeria monocytogenes
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SECTION 2.4.

SHEEP AND GOAT DISEASES IN LIST B

CHAPTER 2.4.1.

OVINE EPIDIDYMITIS

(\textit{Brucella ovis})

SUMMARY

\textit{Brucella ovis} produces a clinical or subclinical disease in sheep that is characterised by genital lesions in rams, and placentitis in ewes. Accordingly, the main consequences of the disease are reduced fertility in rams, infrequent abortions in ewes, and an increased perinatal mortality. The disease has been reported in Argentina, Australia, Brazil, Canada, Chile, France, Germany, Hungary, Mexico, New Zealand, Peru, Romania, Russia, the Slovak Republic, South Africa, Spain, the United States of America and Uruguay, but probably occurs in most sheep-raising countries.

\textbf{Identification of the agent:} The existence of clinical lesions (unilateral or, occasionally, bilateral epididymitis) in rams may be indicative of the existence of infection, but laboratory examinations are necessary to confirm the disease. Laboratory confirmation may be based on direct or indirect methods. Direct diagnosis is made by means of bacteriological isolation of \textit{B. ovis} from semen samples or tissues of rams, or vaginal discharges and milk of ewes, on adequate selective media. Molecular biological methods, such as polymerase chain reaction and pulse-field gel electrophoresis, are being developed. However, indirect diagnosis based on serological tests is preferred for routine diagnosis.

\textbf{Serological tests:} The complement fixation test (CFT), agar gel immunodiffusion (AGID) test and enzyme-linked immunosorbent assay (ELISA) using soluble surface antigens obtained from \textit{B. ovis}, can be used. Some ELISAs using recombinant proteins and monoclonal antibodies are being tested in field trials. The sensitivities of the AGID test and ELISA are similar and sometimes the ELISA has higher sensitivity than the CFT. A combination of the AGID test and ELISA seems to give the best results in terms of sensitivity. However, with regard to simplicity and cost, the AGID test is the most practicable test for diagnosis of \textit{B. ovis}. However, the prescribed test for international trade remains the CFT.

\textbf{Requirements for vaccines and diagnostic biologicals:} Seed cultures for antigen or vaccine production should be obtained from internationally recognised laboratories. A single standard dose (10^9 colony-forming units) of the live \textit{B. melitensis} Rev.1 vaccine, administered subcutaneously or conjunctivally, can be used safely and effectively in rams, for the prevention of \textit{B. ovis} infection. This vaccine strain should meet minimal quality standards: adequate concentration, absence of dissociation, adequate residual virulence and immunogenicity and free of extraneous agents (see Chapter 2.4.2. Caprine and ovine brucellosis [excluding \textit{B. ovis} infection]).

A. INTRODUCTION

\textit{Brucella ovis} causes a genital infection of ovine livestock manifested by epididymitis, infrequent abortions, and increased lamb mortality. Passive venereal transmission via the ewe appears to be a frequent route of infection,
but ram-to-ram transmission is also common1 (2). Infected ewes may excrete \textit{B. ovis} in vaginal discharges and milk and, accordingly, ewe-to-ram and lactating ewe-to-lamb transmission could also be determinant mechanisms of infection.

The demonstration of the existence of genital lesions (unilateral or, occasionally, bilateral epididymitis) by palpating the testicles of rams may be indicative of the presence of this infection in a given flock. However, this clinical diagnosis is not sensitive enough because only about 50% of rams infected with \textit{B. ovis} present epididymitis (2). Moreover, the clinical diagnosis is extremely unspecific due to the existence of many other bacteria causing clinical epididymitis. The most frequently reported isolates causing epididymitis in rams include \textit{Actinobacillus seminis}, \textit{A. actinomycetemcomitans}, \textit{Histophilus ovis}, \textit{Haemophilus spp.}, \textit{Corynebacterium pseudotuberculosis ovis}, \textit{B. melitensis} and \textit{Chlamydophila abortus} (formerly \textit{Chlamydia psittaci}) (4, 5, 8, 10, 12, 22, 28, 31). It must be emphasised that many palpable epididymal lesions in rams are sterile, trauma-induced spermatic granulomas.

Although cattle, goats and deer have been proved susceptible to \textit{B. ovis} in artificial transmission experiments, natural cases have been reported only in deer (19). To date, no human cases have been reported, and \textit{B. ovis} is considered to be non-zoonotic. However, in areas where \textit{B. melitensis} infection co-exists with \textit{B. ovis}, special care is required when handling samples, which should be transported to the laboratory in leak-proof containers (for further details see Chapter 2.3.1.).

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

   a) **Collection of samples**
   
   The most valuable samples for the isolation of \textit{B. ovis} from live animals are semen, vaginal swabs and milk. For the collection of vaginal swabs and milk, see the instructions given in Chapter 2.4.2. Caprine and ovine brucellosis (excluding \textit{B. ovis} infection). Semen (genital fluids) can be collected easily in swabs taken from the preputial cavity after electro-ejaculation. If an electro-ejaculator is not available, swabs can be taken from the vagina of brucellosis-free ewes immediately after natural mating.

   For the isolation of \textit{B. ovis} after necropsy, the preferred organs in terms of probability of isolation are the epididymides, seminal vesicles, and ampullae and inguinal lymph nodes in rams, and the uterus, iliac and supra-mammary lymph nodes in ewes. However, to obtain maximum sensitivity, a complete search that includes other organs and lymph nodes (spleen, cranial, scapular, prefemoral and testicular lymph nodes) should be performed. Dead lambs and placentas should also be examined. The preferred culture sites in aborted or stillborn lambs are abomasal content and lung.

   Samples for culture should be refrigerated and transported to the laboratory to be cultured as soon as possible after collection. The organism remains viable for at least 72 hours at room temperature and survival is enhanced at 4°C or, preferably, by freezing the tissue samples.

   b) **Staining methods**
   
   Semen or vaginal smears can be examined following staining by Stamp’s method (1, 7) (see Chapter 2.4.2.), and characteristic coccobacilli should be demonstrated in many infected animals (29). Examination of Stamp-stained smears of suspect tissues (ram genital tract, inguinal lymph nodes, placentas, and abomasal content and lung of fetuses) may also allow a rapid presumptive diagnosis.

   However, other bacteria with similar morphology or staining characteristics (\textit{B. melitensis}, \textit{Coxiella burnetii}, and \textit{Chlamydophila abortus}) can also be present in such samples, making the diagnosis difficult for inexperienced personnel. Microscopy results should always be confirmed by culture of the microorganism.

   c) **Culture**
   
   The best direct method of diagnosis is bacteriological isolation on adequate culture media. Semen, vaginal swabs, or milk samples can be smeared directly on to plates with adequate culture media and incubated at

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1 Under the semi-extensive production systems (most common in European Mediterranean countries) rams are usually housed together. Direct ram-to-ram transmission during nonbreeding periods is quite frequent and has been suggested to take place by several routes, including the rectal mucosa. Most ram-to-ram infections, however, are produced through the oral route. Housed rams establish hierarchies (head-to-head combats), and it is frequent that ‘dominated’ rams, after being ‘mated’ by the dominant rams, lick the prepuce of these dominant rams as an act of submission. If these dominant rams are infected, the probability of having \textit{B. ovis} in the prepuce (excretion in the semen) is very high.
37°C in an atmosphere of 5–10% CO₂. Tissues should be macerated and ground in a small amount of sterile saline or phosphate buffered saline (PBS) with a stomacher or blender, before plating.

Growth normally appears after 3–4 days, but cultures should not be discarded as negative until 7 days have elapsed. Colonies of *B. ovis* become visible (0.5–2.5 mm) after 3–4 days of incubation, and are rough phase, round, shiny and convex.

*Brucella ovis* can be isolated in nonselective media, such as blood agar base enriched with 10% sterile ovine or bovine sera, or in blood agar medium with 5–10% sterile ovine blood. However, the inoculum frequently contains other bacteria, which often overgrow *B. ovis*. Accordingly, the use of selective media may be preferred. Various *B. ovis* selective media have been described. The modified Thayer–Martin's medium (3, 13) is recommended. Briefly, it can be prepared with GC medium base (38 g/litre; Biolife Laboratories, Milan, Italy) supplemented with haemoglobin (10 g/litre; Difco) and colistin methanesulphonate (7.5 mg/litre), vancomycin (3 mg/litre), nitrofurantoin (10 mg/litre), nystatin (100,000 International Units [IU]/litre = 17.7 mg) and amphotericin B (2.5 mg/litre) (all products from Sigma Chemical, St Louis, United States of America [USA]). Working solutions are prepared as follows:

**Solution A:** Add 500 ml of distilled water to the GC medium base, heat the paste carefully while stirring continuously and autoclave at 120°C for 20 minutes.

**Solution B:** Suspend the haemoglobin in 500 ml of distilled water, adding the water slowly to avoid lumps. Once dissolved, add a magnetic stirrer and autoclave at 120°C for 20 minutes.

**Antibiotic solution (prepared daily):** colistin, nystatin and vancomycin are suspended in a mixture of methanol/water (1/1); nitrofurantoin is suspended in 1 ml of a 0.1 M NaOH sterile solution. For amphotericin B, it is recommended to prepare a stock solution of 10 mg/ml amphotericin B with 10 mg dissolved first in 1 ml sterile dimethyl sulphoxide (C₂H₆OS, for analysis; ACS) and then added to 9 ml of PBS (10 mM, pH 7.2). Any stock solution remaining can be stored some days at 4°C. All antibiotic solutions must be filtrated through 0.22 µm filters before addition to the culture medium.

Once autoclaved, stabilise the temperature (45–50°C) of both solutions A and B with continuous stirring. Mix both solutions (adding A to B), avoiding bubble formation. Add the antibiotic solutions while stirring continuously and carefully. Dispense into sterile plates.

Once prepared, the plates should not be stored for long periods, and freshly prepared medium is always recommended. This medium is also suitable for the isolation of *B. melitensis* (see Chapter 2.4.2.).

All culture media should be subjected to quality control with the reference strain, to show that it supports growth.

Another suitable, but less effective, antibiotic combination is: vancomycin (3 mg/litre); colistin (7.5 mg/litre); nystatin (12,500 IU/litre); and nitrofurantoin (10 mg/litre).

d) **Identification and typing**

*Brucella ovis* colonies are not haemolytic. They are circular, convex, have unbroken edges, are always of the rough type when examined by oblique illumination, and test positive in the acriflavine test (1, 7). *Brucella ovis* lacks urease activity, fails to reduce nitrate to nitrite, is catalase positive and oxidase negative, does not produce H₂S and, although it does not grow in the presence of methyl violet, it usually grows in the presence of standard concentrations of basic fuchsin and thionin. The cultures are not lysed by *Brucella*-phages of the Tbilissi (Tb), Weybridge (Wb) and Izatnagar (Iz) groups at the routine test dilution (RTD) or 10⁴ RTD, while they are lysed by phage R/C (1, 7). Most laboratories are not equipped for a complete identification, and a practical schedule for presumptive identification is needed. Most *B. ovis* isolates can be correctly identified on the basis of growth characteristics, direct observation using obliquely reflected light, Gram or Stamp's staining, catalase, oxidase, urease and acriflavine tests. However, definitive identification should be carried out by reference laboratories with experience in identification and typing of *Brucella*. A method of pulse-field gel electrophoresis can differentiate *B. ovis* from other *Brucella* species (17). Moreover, *B. ovis* can be differentiated from the other *Brucella* species through its specific polymerase chain reaction restriction fragment length polymorphism (PCR RFLP) patterns for genes omp2a, omp2b, omp25 and omp31, coding for the major outer membrane proteins of all *Brucella* species (27).

## 2. Serological tests

The most efficient and widely used tests are the complement fixation test (CFT), the double agar gel immunodiffusion (AGID) test and the indirect enzyme-linked immunosorbent assay (ELISA). Several countries have adopted various standard diagnostic techniques for *B. ovis*, but the only test prescribed by the OIE and the...
Chapter 2.4.1. – Ovine epididymitis (Brucella ovis)

European Union (EU) for international trade is the CFT. However, it has been demonstrated that the AGID test shows similar sensitivity to the CFT, and it is a simpler test to perform. Although standardisation is lacking, numerous independent studies have shown that the ELISA is more sensitive and specific than either the CFT or AGID test, and with further validation studies the ELISA could become a suitable candidate for future designation as a prescribed test for B. ovis diagnosis.

- Antigens

When rough Brucella cells are heat-extracted with saline (hot-saline method, HS), they yield water-soluble antigenic extracts, the major component of which precipitates with sera to rough Brucella (9, 18). For this reason, the HS extracts have been referred to as the ‘rough-specific antigen’ or, when obtained from B. ovis, as the ‘B.-ovis-specific antigen’. However, the chemical characterisation of the HS extracts from B. ovis has shown that they are enriched in rough lipopolysaccharide (R-LPS), group 3 outer membrane proteins and other outer membrane components (20). Thus, HS extracts contain LPS determinants specific for B. ovis, but also additional antigenic components, some of them shared with rough and smooth B. melitensis and other Brucella (23). Such components account for the cross-reactivity that is sometimes observed with the HS method and sera of sheep infected with B. melitensis or vaccinated with Rev.1 (20). The HS extract, due to its water solubility and high content of relevant cell-surface epitopes, is the best diagnostic antigen and has been widely used for the serological diagnosis of B. ovis infection.

Brucella ovis REO 198, a CO₂- and serum-independent strain, is recommended as a source of the HS antigens to be used in serological tests. This strain can be obtained from INRA². Solid media described in Section B.1.c. are satisfactory for the growth of B. ovis REO 198. HS antigen is prepared as follows:

i) Exponentially grow a suitable strain of B. ovis, preferably aerobic and nonserum dependent, e.g. REO 198, in one of the following ways: for 48 hours in trypticase–soy broth flasks in an orbital incubator at 37°C and 150 rpm; or in Roux bottles of trypticase–soy agar, or other suitable medium, with 5% serum added (not necessary when using the REO 198 strain); or in a batch-type fermenter as described for B. abortus, but with the addition of 5% serum to the medium (not necessary when using the REO 198 strain).

ii) Cells are washed twice and then resuspended in 0.85% saline (12 g of dried cells or 30 g of wet packed cells in 150 ml).

iii) The cell suspension is then autoclaved at 120°C for 15–30 minutes.

iv) After cooling, the suspension is centrifuged (15,000 g, 4°C, 15 minutes) and the supernatant fluid is filtered and dialysed against distilled water (3 ‰ 100 volumes) at 4°C for at least 2 days.

v) The dialysed fluid can be ultracentrifuged (100,000 g, 4°C, 6–8 hours), and the sediment is resuspended in a small amount of distilled water and freeze-dried.

HS is then resuspended either in distilled water (for use in the AGID test), veronal buffered saline (for use in the CFT), or carbonate/bicarbonate buffer or PBS (for use in the ELISA) and titrated against a set of adequate positive and negative sera.

The resuspended HS is kept at 4°C with 0.5% phenol as preservative (only for use in the AGID test) or freeze-dried. Freezing and thawing should be avoided (9). The CFT antigen should be standardised against the International anti-B. ovis Standard Serum³ to give 50% fixation at a 1/100 serum dilution.

a) Complement fixation test (the prescribed test for international trade)

There is no standardised method for the CFT, but the test is most conveniently carried out using the microtitration method. Some evidence shows that cold fixation is more sensitive than warm fixation (6, 21, 24), but that it is less specific. Anticomplementary reactions, common with sheep serum, are, however, more frequent with cold fixation.

Several methods have been proposed for the CFT using different concentrations of fresh sheep red blood cells (SRBCs) (a 2–3% 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

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2 Institut national de la recherche agronomique (INRA) Laboratoire de Pathologie Infectieuse et Immunologie, 37380 Nouzilly, France.

3 Obtainable from the OIE Reference Laboratory for Brucellosis, VLA Weybridge, Addlestone, Surrey KT15 3NB, United Kingdom.
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Barbital (veronal) buffered saline (VBS) is the standard diluent for the CFT. This is prepared from tablets available commercially, otherwise it may be prepared according to the formula described elsewhere (see Chapter 2.3.1. Bovine brucellosis). The test sera should be inactivated for 30 minutes in a water bath at 60–63°C, and then diluted (doubling dilutions) in VBS. The stock solution of HS antigen (2.5–20 mg/ml in VBS) is diluted in VBS as previously determined by titration (checkerboard titration). Usually, only one serum dilution is tested (generally 1/10).

Using standard 96-well microtitre plates with round (U) bottom, 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 and second rows. Volumes of 25 µl of CFT buffer are added to all wells except those of the first row. Serial doubling dilutions are then made by transferring 25 µl volumes of serum from the second 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.

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 techniques) of sensitised SRBCs is added to each well. The plates are reincubated 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 titre of the serum under test is the highest dilution in which there is 50% or less haemolysis.

**Standardisation of the results of the complement fixation test**

There is a unit system that is based on the International Standard for anti-Brucella ovis Serum (International Standard 1985 [see footnote 2]). This serum contains 1000 IU/ml. If this serum is tested in a given method and gives a titre of, for example 200, then the factor for an unknown serum tested by that method can be found from the formula: 1000/200 = titre of test serum = number of ICFTU (International CFT units) of antibody in the test serum per ml. It is recommended that any country using the CFT on a national scale should obtain agreement among the different laboratories performing the test by the same method, to allow the same level of sensitivity and specificity to be obtained against an adequate panel of sera from B. ovis culture positive and Brucella-free sheep. Results should always be expressed in ICFTU, calculated in relation to those obtained in a parallel titration with a standard serum, which itself may be calibrated against the International Standard.

**Interpretation of the results:** Generally, sera giving a titre equivalent to 50 ICFTU/ml or more are considered to be positive in the EU.

b) **Agar gel immunodiffusion test**

The AGID test (2) uses the following reagents: Good grade Noble agar or agarose, sodium chloride (NaCl), and borate buffer (prepared with boric acid [12.4 g]; potassium chloride [14.5 g]; distilled water [1600 ml]; adjusted to pH 8.3 with 0.2 M NaOH solution and made up to 2000 ml with distilled water).

To prepare the gels, dissolve 1 g of agarose (or Noble agar), 10 g of NaCl and 100 ml of borate buffer by boiling while stirring continuously. On a flat surface, cover clean glass slides with the necessary amount of molten gel to form a bed of 2.5 mm depth (3.5 ml approximately for standard microslides). After the gel has solidified (15–20 minutes), wells are cut in it using a gel puncher. The wells should be 3 mm in diameter and 3 mm apart, and should be arranged in a hexagonal pattern around a central well that is also 3 mm in diameter. The test can be adapted to Petri dishes and other patterns.

Sera to be examined are placed in alternate wells separated by a control positive serum (infection proved by bacteriology), with the antigen at its optimum concentration in the central well. The results are read after incubation for 24 and 48 hours at room temperature in a humid chamber. A positive reaction is a clearly defined precipitin line between the central well and the wells of the test sera that gives total or partial
identity with that of the positive controls. Precipitin lines not giving total identity may also appear and correspond to minor antigenic components of HS extracts (antibodies to these components can also be common in infections due to *B. melitensis*). These reactions should also be considered to be positive. Before a definitive reading, it is important to wash the slides for 1 hour in a 5% sodium citrate water solution to clean unspecific precipitin lines.

The HS (2.5–20 mg/ml) diluted in distilled water and containing 0.5% phenol as a preservative is the antigen used in the AGID test (this preserved antigen can be stored at 4°C for at least 1 month). Dilutions of antigen are tested with a panel of 20–30 sera from rams naturally infected with *B. ovis* and with a panel of *Brucella*-free sheep. The optimum concentration of antigen is that giving the clearest precipitation line with all the sera from *B.*-ovis-infected rams being negative with the sera from *Brucella*-free sheep.

c) Enzyme-linked immunosorbent assay

Several variations of this assay have been proposed. The assay described here is an indirect ELISA using ABTS (2.2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) as chromogen, but other procedures are also suitable. Tests are performed on 96-well flat-bottomed ELISA plates. Reagent and serum dilutions are made in PBS, pH 7.2, with the addition of 0.05% Tween 20 (PBST). Antigen dilutions are made in a carbonate/bicarbonate buffer, pH 9.6, or, alternatively, in PBS, pH 7.2. Plates are washed after antigen coating and between incubations, where appropriate, usually with PBST. The antigen (HS) and conjugate are checkerboard titrated, and dilutions are selected to give the best discriminating ratio between negative and positive standard sera. Secondary antibodies (anti-ovine IgG [H+L chains]) antibodies are usually conjugated to horseradish peroxidase (HRPO), although other enzymes or conjugates (such as protein G) can be used. A monoclonal antibody to bovine IgG,–HRPO conjugate has been found to be suitable for use in the ELISA (26). If a peroxidase conjugate is used, the chromogen, usually ABTS, is diluted in a substrate buffer (composed of citric acid trisodium and citric acid; pH 4). The substrate, hydrogen peroxide (H₂O₂), is added to this, and the plates are incubated for 15–60 minutes at room temperature. The reaction may be stopped with 1 mM sodium azide, and the colour change is read at 405–414 nm (for further details see Chapter 2.3.1.).

The antigen used in the ELISA is the HS in stock solution at 1 mg/ml in coating buffer, titrated in a checkerboard titration, with different dilutions of antigen, conjugate and substrate, against a standard serum or against serial dilutions of a panel of sera from *B. ovis* culture positive and *Brucella*-free sheep to determine the most sensitive and specific dilution (usually 5–10 µg/ml).

- **Test procedure**
  
  i) **Cell culture quality microtitre plates** are coated by the addition of 100 µl of a predetermined antigen dilution in carbonate buffer, pH 9.6, to each well. Plates are incubated for 2 hours at 37°C. Alternatively, the coating can be made overnight at 4°C with 100 µl/well of the predetermined antigen dilution in PBS, pH 7.2. Plates are then washed four times to remove unbound antigen and dried by tapping firmly upside down on an absorbent paper. The coated plates can be used immediately or dried and stored at 4°C (the stability in these conditions is adequate for at least 1 month).
  
  ii) **Sera:** Dilute test and positive and negative control serum samples 1/200 by the addition of a minimum of 10 µl of serum to 2 ml PBST. This serum dilution is usually the optimal when using both polyclonal and monoclonal conjugates. However, dilutions of 1/50 are the optimal when using the protein G-peroxidase conjugate (14) Add 100 µl/well volumes of samples in duplicate to the microtitre plates. The plates are covered, incubated at 37°C for 1 hour, and washed three times with the PBST washing buffer.
  
  iii) **Conjugate:** The titrated conjugate is diluted in PBST, added (100 µl) to the wells, and the plate is covered and incubated for 1 hour at 37°C. After incubation, the plates are washed again three times with PBST.
  
  iv) **Substrate:** The solution of ABTS in substrate buffer is added (100 µl/well) and the plates are incubated for 15–60 minutes at room temperature with continuous shaking.
  
  v) **Reading and interpreting the results:** Absorbance is read automatically in a spectrophotometer at 405–414 nm. Absorbance values may be expressed as percentages of the mean absorbance of the positive control or, preferably, transformed into ELISA units calculated either manually or by using a computer and a curve-fitting program from a standard curve constructed with the series of positive control dilution results. The threshold should be calculated by testing a sufficiently large collection of *Brucella*-free sheep sera, the sensitivity of the test being assessed on an adequate collection of sera from *B. ovis*-infected animals.

Comparative studies have shown that the ELISA has better sensitivity than either the AGID test or the CFT (16, 21, 25, 32, 33). Due to the existence of some ELISA-negative and AGID-positive sera, the combination of the AGID test and ELISA gives optimal sensitivity (16). However, the combination of CF test and ELISA
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or CF and AGID tests does not improve the sensitivity of ELISA alone (16). Moreover, the CFT has other important disadvantages such as complexity, obligatory serum inactivation, anticomplementary activity of some sera, the difficulty of performing it with haemolysed sera, and prozone phenomena. Because of its sensitivity, simplicity and easy interpretation, the AGID test is very practicable for routine diagnosis in nonspecialised laboratories.

Little is known about the existence of false positive results in B. ovis serological tests as a consequence of infections due to bacteria showing cross-reacting epitopes with B. ovis. The foot rot agent (Dichelobacter nodosus) has been described as showing cross-reactions with B. ovis (30), but the extent and practical consequences of this cross-reactivity in B. ovis diagnostic tests is unknown.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Vaccination is probably the most economical and practical means for medium-term control of B. ovis in areas with a high incidence of infection. For long-term control, consideration should be given to the effect of vaccination on serological testing, and B.-ovis-free accreditation programmes have to be implemented. The live B. melitensis strain Rev.1 (see Chapter 2.4.2.) is probably the best available vaccine for the prophylaxis of B. ovis infection (2). A single standard dose (10⁹ colony-forming units) of Rev.1 administered, subcutaneously (in a 1 ml volume) or conjunctivally (in a 25–30 µl volume), to 3–5 month-old rams confers adequate immunity against B. ovis. Conjunctival vaccination has the advantage of minimising the intense and long-lasting serological response evoked by subcutaneous vaccination, thereby improving the specificity of serological tests (2). When used in young animals, the Rev.1 vaccine is safe enough and side-effects appear to be rare. However, there is limited information concerning the safety of Rev.1 vaccine when used in adult rams. One study found that the subcutaneous or conjunctival vaccination of 13 month-old rams did not produce undesirable side-effects and protected rams against B. ovis, although the number of animals was limited in this study (15). This finding should be reinvestigated using a larger number of adult rams as, in countries with extensive management and high levels of incidence, it would be advisable to vaccinate both young and healthy adult rams. However, B. melitensis Rev.1 vaccine should not be used in countries affected by B. ovis but free of B. melitensis and other vaccines that do not cause a serological response should be used. The B. abortus RB51 live vaccine has not proven successful against B. ovis in sheep (11) and no alternative vaccines are currently available.

REFERENCES


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**NB:** There are OIE Reference Laboratories for Ovine epididymitis (*Brucella ovis*) (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 2.4.2.

CAPRINE AND OVINE BRUCELLOSIS
(excluding Brucella ovis)

SUMMARY

Brucella melitensis (biovars 1, 2 or 3) is the main causative agent of caprine and ovine brucellosis. Sporadic cases caused by B. abortus have been observed, but clinical disease is rare. Brucella melitensis appears to be endemic in the Mediterranean region, but infection is widespread worldwide. North America (except Mexico) is believed to be free from the agent, as are Northern Europe, South-East Asia, Australia and New Zealand.

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 melitensis is highly pathogenic for humans, causing one of the most serious zoonoses in the world, and all infected tissues, cultures and potentially contaminated materials should be handled at a high biohazard containment level.

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.

Serological and allergic skin tests: The rose bengal plate agglutination and complement fixation tests are usually recommended for screening flocks and individual animals. The serum agglutination test is not considered to be reliable for use in small ruminants. For pooled samples, there are no useful tests such as the milk ring test in cattle. The brucellin allergic skin test can be used as a screening or complementary test in unvaccinated flocks, provided that a purified, lipopolysaccharide (LPS)-free, standardised antigen preparation is used. Results must then be interpreted in relation to the clinical signs, history, and results of serological or cultural examination.

Requirements for vaccines and diagnostic biologicals: Brucella melitensis strain Rev.1 remains the reference vaccine to immunise sheep and goats at risk of infection from B. melitensis and to which any other vaccines should be compared. Production of Brucella antigens or Rev.1 vaccine is based on a seed-lot system. Seed cultures to be used for antigens for serological and allergic skin tests and for vaccines should originate from reference centres. They must conform to minimal standards for viability, smoothness, residual infectivity and immunogenicity, if applicable. Brucellin preparations for the intradermal test must be free of smooth lipopolysaccharide and must not produce nonspecific inflammatory reactions or interfere with serological tests. Antigens for serological diagnosis 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 sheep and goats (excluding Brucella ovis infection) is primarily caused by one of the three biovars of \(B. \text{melitensis}\). Sporadic cases caused by \(B. \text{abortus}\) or \(B. \text{suis}\) have been observed in sheep and goats, but clinical signs are rare. Pathologically and epidemiologically, \(B. \text{melitensis}\) infection in sheep and goats is very similar to \(B. \text{abortus}\) infection in cattle (see Chapter 2.3.1. Bovine brucellosis). In most circumstances, the primary route of transmission of \(Brucella\) is the placenta, fetal fluids and vaginal discharges expelled by infected ewes and goats when they abort or have a full-term parturition. Shedding of \(Brucella\) is also common in udder secretions and semen, and \(Brucella\) may be isolated from various tissues, such as lymph nodes from the head and those associated with reproduction, and from arthritic lesions (2).

Brucellosis has also been reported in the one-humped camel (\(Camelus \text{dromedarius}\)) and in the two-humped camel (\(C. \text{bactrianus}\)), related to contact with large and small ruminants infected with \(B. \text{abortus}\) or \(B. \text{melitensis}\). In addition, brucellosis due to \(B. \text{abortus}\) has been observed in the domestic buffalo (\(Bubalus \text{bubalus}\)), American and European bison (\(Bison \text{bison; Bison bonasus}\)), yak (\(Bos \text{grunniens}\), elk/wapiti (\(Cervus \text{elaphus}\)) and also occurs in the African buffalo (\(Syncerus \text{caffer}\)) and various African antelope species. The manifestations of brucellosis in these animals are similar to those in cattle or sheep and goats.

The World Health Organization (WHO) laboratory biosafety manual classifies \(Brucella\) (and particularly \(B. \text{melitensis}\)) 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 Chapter I.1.6. Human safety in the veterinary microbiology laboratory and refs 1, 28, 67 and 68 of Chapter 2.3.1. Bovine brucellosis). Laboratory manipulation of live cultures or contaminated material from infected animals is hazardous, as is handling large volumes of \(Brucella\), and must be done under containment level 3 or higher conditions, as outlined in Chapter I.1.6., to minimise occupational exposure.

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 (\(B. \text{abortus}\), \(B. \text{melitensis}\), etc.) would be mere biovars (for a review see Chapter 2.3.1., 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 nomenspecies: \(Brucella \text{abortus}\), \(B. \text{melitensis}\), \(B. \text{suis}\), \(B. \text{neotomae}\), \(B. \text{ovis}\) and \(B. \text{canis}\). The first three of these are subdivided into biovars based on cultural and serological properties (see Tables 1 and 2 in Chapter 2.3.1.). 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, \(B. \text{cetaceae}\) and \(B. \text{pinnipediae}\) (see Chapter 2.3.1. refs 10 and 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

1. Identification of the agent

Please refer to Chapter 2.3.1. Bovine brucellosis.

2. Serological tests

In situations where bacteriological examination is not practicable, diagnosis of \(Brucella\) infection must often be based on serological methods (2, 18). In routine tests, anti-\(Brucella\) antibodies are detected in serum. The most widely used serum-testing procedures for the diagnosis of smooth \(Brucella\) infections in sheep and goats are the buffered \(Brucella\) antigen tests (BBAT), i.e. the card and rose bengal (RB) plate agglutination tests, which are essentially the same, and the complement fixation test (CFT). The bulk milk ring test, which has been very useful in cattle, is ineffective in small ruminants.
Chapter 2.4.2. – Caprine and ovine brucellosis (excluding Brucella ovis)

In small ruminants, the RB test (RBT) and the CFT are the most widely used methods (17) and are the only prescribed tests. The RBT is not completely specific, but appears to be adequate as a screening test for detecting infected flocks or for guaranteeing the absence of infection in brucellosis-free flocks. However, due to the relative lack of sensitivity of both tests, discrepancies between results obtained using the RBT and the CFT are not rare in infected sheep and goats (6). The results of the two tests should therefore be considered simultaneously to increase the likelihood of detecting infected individuals and to improve control of the disease in areas where it has not been completely eradicated (1, 4, 6). When, for practical or economic reasons, the CFT cannot be used simultaneously with the RBT in eradication programmes, it is recommended to improve the sensitivity of the RBT by using 75 µl of serum and 25 µl of antigen in place of an equal volume of each. This simple modification increases RBT sensitivity and minimises the discrepancies between RBT and CFT results (6). Because Rev.1-induced antibodies cannot be discriminated from wild type, brucellosis serological tests should be interpreted in the context of the vaccination status in the flock. In addition these tests are not specific enough to discriminate serological reactions due to *B. melitensis* from the false-positive reactions (FPSR) due to cross-reacting bacteria such as *Yersinia enterocolitica* O:9.

Good diagnostic results have been obtained in sheep and goats with indirect or competitive enzyme-linked immunosorbent assays (ELISAs) using various antigens, but generally those with a high content of smooth lipopolysaccharide (LPS) are the most reliable. These ELISAs provide similar or better sensitivity than both RBT and CFT, but like the classical tests, ELISAs are unable to differentiate infected animals from animals recently vaccinated with the Rev.1 vaccine (19) or animals infected with cross-reacting bacteria. A highly immunogenic periplasmic protein from *B. abortus* (20) and *B. melitensis* (11) has been applied to brucellosis diagnosis in different host species. Indirect and competitive ELISAs with this antigen could be sensitive and specific tests for diagnosing *B. melitensis* infection in sheep and have been reported to be useful in differentiating Rev.1 vaccinated from infected animals (10, 13). All these ELISAs have potential advantages in sensitivity and specificity with respect to both RBT and CFT, but a great deal of work is still required on standardisation of reagents to be used for diagnosing *B. melitensis* infection in sheep and goats (15).

- **Reference sera**

  The primary reference serum for standardising RBT and CFT in sheep and goats is the OIE International Standard Serum (OIEISS; see Chapter 2.3.1. Bovine brucellosis).

- **Production of cells**

  Please refer to Chapter 2.3.1. Bovine brucellosis. *Brucella abortus* biovar 1 strains 99 or 1119 are the only strains recommended for the preparation of RBT and CFT in sheep and goats.

  a) **Buffered Brucella antigen test (a prescribed test for international trade)**

    - **Rose bengal plate agglutination test**
      
      Please refer to Chapter 2.3.1. Bovine brucellosis.

    - **Antigen production**
      
      Please refer to Chapter 2.3.1. Bovine brucellosis. Note that RB antigen made with *B. abortus* is usually used to test for *B. melitensis*.

    - **Test procedure**
      
      Please refer to Chapter 2.3.1. Bovine brucellosis.

  b) **Complement fixation test (a prescribed test for international trade)**

    - **Antigen production**
      
      Please refer to Chapter 2.3.1. Bovine brucellosis. Note that CF antigen made with *B. abortus* is usually used to test for *B. melitensis*.

    - **Test procedure**
      
      Please refer to Chapter 2.3.1. Bovine brucellosis.
3. Other tests

a) Brucellin skin test

An alternative diagnostic test is the brucellin skin test, which can be used for screening unvaccinated flocks, provided that a purified (free of sLPS) and standardised antigen preparation (e.g. brucellin INRA) is used.

The brucellin skin test has a high sensitivity for the diagnosis of *B. melitensis* infection in small ruminants and, in absence of vaccination, is considered one of the most specific diagnostic tests (2, 4, 15, 17). This test is of particular value for the interpretation of FPSR due to infection with cross-reacting bacteria (FPSR affected animals are always negative in the skin test), especially in brucellosis-free areas.

Despite its high sensitivity not all infected animals show positive skin test responses to brucellin and, moreover, Rev.1 vaccinated animals can react in this test for years (15). Therefore this test cannot be recommended either as the sole diagnostic test or for the purposes of international trade.

To obtain suitable results it is essential to use standardised brucellin preparations that do not contain sLPS, as this antigen may provoke antibody-mediated inflammatory reactions or induce antibodies that interfere with subsequent serological screening. One such preparation is brucellin INRA, which is prepared from a rough strain of *B. melitensis* that is commercially available1.

- **Test procedure**
  i) A volume of 0.1 ml of brucellin is injected intradermally into the lower eyelid.
  ii) The test is read after 48 hours.
  iii) Any visible or palpable reaction of hypersensitivity, such as an oedematous reaction leading to an elevation of the skin or thickening of the eyelid (>2 mm), should be interpreted as a positive reaction.

Although in the absence of vaccination the brucellin intradermal test is one of the most specific tests in brucellosis, diagnosis should not be made exclusively on the basis of positive intradermal reactions and should be supported by adequate serological tests. The intradermal inoculation of brucellin might induce a temporary anergy in the cellular immune response. Therefore an interval of 6 weeks is generally recommended between two tests repeated on the same animal.

b) Native hapten tests

The native hapten-based gel precipitation tests2 (see Chapter 2.3.1.) are also of interest in sheep and goats as they are very specific for discriminating the serological responses of infected animals (positive) from those induced in Rev.1 vaccinated animals (usually negative).

The optimal diagnostic sensitivity (around 90%) is obtained in the double gel diffusion (DGD) or reverse radial immunodiffusion tests for sheep and goats, respectively (14, 19).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

C1. Brucellin

Please refer to Chapter 2.3.1. Bovine brucellosis.

C2. Vaccines

*Brucella melitensis* strain Rev.1 vaccine

The most widely used vaccine for the prevention of brucellosis in sheep and goats is the *Brucella melitensis* Rev.1 vaccine, which remains the reference vaccine with which any other vaccines should be compared. The Rev.1 vaccine is used as a freeze-dried suspension of live *B. melitensis* biovar 1 Rev.1 strain for the immunisation of sheep and goats. It is normally given to lambs and kids aged between 3 and 6 months as a single subcutaneous injection. The standard dose is between 0.5 × 10⁹ and 2.0 × 10⁹ viable organisms. The

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1 Brucellergène OCB®, Synbiotics Europe, 2 rue Alexander Fleming, 69007 Lyon, France.
2 The detailed procedure could be obtained from the Departamento de Sanidad Animal, Servicio de Investigacion Agraria/DGA, Apartado 727, 50080 Zaragoza, Spain.
subcutaneous vaccination induces strong interferences in serological tests and should not be recommended in combined eradication programmes (14, 19). However, when this vaccine is administered conjunctivally to lambs and kids aged between 3 and 6 months it produces a similar protection without inducing a persistent antibody response, thus facilitating the application of eradication programmes combined with vaccination (14, 19). Care must be taken when using Rev.1 vaccine to avoid the risk of contaminating the environment or causing human infection. In many developing countries and endemic areas, vaccination of the whole population has to be considered as the best option for the control of the disease (5). However, Rev.1 vaccine is known to often cause abortion and excretion in milk when animals are vaccinated during pregnancy, either with a full or reduced dose (5). These side-effects are considerably reduced when adult animals are vaccinated conjunctivally (full dose) before mating or during the last month of pregnancy. Therefore, when mass vaccination is the only means of controlling the disease, a vaccination campaign should be recommended using the standard dose of Rev.1 administered by the conjunctival route when the animals are not pregnant or during the lambing season (5).

The subcutaneous vaccination of young animals and the vaccination of adult animals, even at reduced doses, may lead to long-term persistence of vaccinal antibodies in a significant proportion of vaccinated animals that creates serious interferences in the serological diagnosis of brucellosis. As indicated above, conjunctival vaccination minimises these problems. Therefore, the serological diagnosis of brucellosis should take into account the vaccinal state of the herd and the overall frequency distribution of antibody titres detected in the group of animals tested.

1. Seed management

a) Characteristics of the seed

*Brucella melitensis* biovar 1 strain Rev.1 original seed for vaccine production can be obtained commercially\(^3\). A European reference Rev.1 strain that possesses the characteristics of the Rev.1 original seed has been recently produced by the European Pharmacopoeia\(^4\).

Production of *Brucella* live vaccines is based on the seed-lot system described above (Section B.2.) for RBT and CFT antigens. Strains should be cultured in a suitable medium. Strain Rev.1 must conform to the characteristics of *B. melitensis* biovar 1, except that it should grow more slowly. Additionally, when incubated in air (atmospheres containing CO\(_2\) alter the results) at 37°C, it should grow on agar containing streptomycin (2.5 µg/ml), and it should be inhibited by the addition to a suitable culture medium of sodium benzylpenicillin (3 µg [5 International Units (IU)]/ml), thionin (20 µg/ml) or basic fuchsin (20 µg/ml). Recently, polymerase chain reaction amplification and molecular techniques have been used to further characterise the vaccine (3, 12). It must also conform to the characteristics of residual virulence and immunogenicity in mice of the original seed.

b) Method of culture

Serum–dextrose agar, and trypticase–soy agar, to which 5% serum or 0.1% yeast extract may be added, are among the solid media that have been found to be satisfactory for propagating the Rev.1 strain (2, 21). Rev.1 strain does not grow well on potato agar.

For vaccine production, Rev.1 may be grown under conditions similar to those described for S99 and S1119-3 (see Chapter 2.3.1.), except that Rev.1 generally needs 3–5 days to grow, the phenol saline is replaced by a freeze-drying stabiliser, and the organisms are not killed but are stored at 4°C while quality control examinations are carried out as described below. Moreover, the specific requirements for Rev.1 vaccine production recommend that: each seed lot (i.e. the culture used to inoculate medium for vaccine production) should be no more than three passages removed from an original seed culture and that the harvest of a vaccine lot should be no more than three passages from a seed lot or an original seed. The original seed culture should always be checked for the absence of dissociation before use. The recommended method for preparing seed material is given in ref. 2. The following freeze-drying stabiliser (sterilised by filtration) is of proven value: enzymatic digest of casein (2.5 g); sucrose (5 g); sodium glutamate (1 g); distilled water (100 ml).

c) Validation as a vaccine

Numerous independent studies have confirmed the value of *B. melitensis* strain Rev.1 as a vaccine for protecting sheep and goats from brucellosis. Its virulence is unchanged after passage through pregnant sheep and goats. Abortions may result when the Rev.1 vaccine is inoculated into pregnant ewes or goats. The vaccine-induced abortions are not avoided using reduced doses, and doses as low as 10\(^6\), used either

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3 Obtainable from Institut National de la Recherche Agronomique (INRA), Laboratoire de Pathologie Infectieuse et Immunologie, 37380 Nouzilly, France.
4 Obtainable from the European Pharmacopoeia, BP 907, 67029 Strasbourg Cedex 1, France.
subcutaneously or conjunctivally, have been demonstrated to induce abortions and milk excretion of the vaccine virus (5).

A Rev.1 vaccine is efficient if it possesses the characteristics of the Rev.1 original strain, i.e. those of *B. melitensis* biovar 1 reference strain 16M (ATCC No. 23456), except those specific for the strain Rev.1 (2, 17), and if it proves to be satisfactory with respect to immunogenicity and residual virulence (8) (see below).

2. Method of manufacture (2, 21)

For production of *B. melitensis* strain Rev.1 vaccine, the procedures described above for antigens (2) can be used except that the cells are collected in a freeze-drying stabiliser and deposited by centrifugation. The yield from one fermenter run or the pooled cells from a batch of Roux flask cultures inoculated on the same occasion from the same seed lot constitutes a single harvest. More than one single harvest may be pooled to form the final bulk that 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. The volume of the final bulk is adjusted by adding sufficient stabiliser so that a dose contains an appropriate number of viable organisms. After adjusting the cell concentration of the final bulk, tests for identity, dissociation and absence of contaminating organisms are conducted (see below).

3. In-process control

In-process checks should be made on the growth of Rev.1 vaccine from either solid or liquid medium to verify identity and to ensure purity and freedom from dissociation to rough forms during preparation of seed lots, single harvests, final bulks and the final (filling) lots. At least 99% of cells in seed lots and 95% of cells in final lots should be in the smooth phase.

Cell concentration should be estimated on the bulks and precisely determined on final lots. Immunogenicity and the residual virulence (50% persistence time or 50% recovery time) should also be determined on seed lots and final lots.

4. Batch control

With freeze-dried vaccine, the control tests should be conducted on the vaccine reconstituted in the form in which it will be used.

a) Sterility (or absence of extraneous microorganisms)

The Rev.1 vaccine should be checked for bacterial and fungal contamination as prescribed in Chapter I.1.5.

b) Safety

The Rev.1 vaccine is a virulent product *per se*, and it should keep a minimal virulence to be efficient (see Section C2.4.c. in Chapter 2.3.1.). A safety test may be performed on sheep and goats when a new manufacturing process is started and when a modification in the innocuousness of the vaccine preparation is expected. This control should be done as follows: the test uses 12 ewe lambs and 12 goat kids, aged 4–6 months. Six young females of each species 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.

c) Potency

A Rev.1 vaccine is efficient if it possesses the characteristics of the Rev.1 original strain, i.e. if it is satisfactory with respect to identity, smoothness, immunogenicity, and residual virulence (9). Batches should also be checked for the number of viable organisms.

- **Identity**

  The reconstituted Rev.1 vaccine should not contain extraneous microorganisms. *Brucella melitensis* present in the vaccine is identified by suitable morphological, serological and biochemical tests and by culture: when incubated in air at 37°C, Rev.1 strain is inhibited by addition to the suitable culture medium of 3 μg (5 IU) per ml of sodium benzyl-penicillin, thionin (20 μg/ml) or basic fuchs in (20 μg/ml); the strain grows on agar containing 2.5 μg per ml of streptomycin.
• **Smoothness (determination of dissociation phase)**

Please refer to Chapter 2.3.1. Bovine brucellosis.

Sometimes slight and difficult to observe differences, can be seen in the size of Rev 1 colonies. The small colonies (1–1.2 mm in diameter) are typical for Rev.1, but larger Rev. 1 colonies can appear depending on the medium used, the amount of residual moisture in the incubator atmosphere, and the presence or absence of CO₂. The frequency of variation in colony size occurs normally at a ratio of 1 large to 10³ small colonies. Both Rev.1 variants are of the S (smooth) type. To avoid an increase in this colony size variation along successive passages, it is important to always select small colonies for preparation of seed lots.

• **Enumeration of live bacteria**

Please refer to Chapter 2.3.1. Bovine brucellosis.

• **Residual virulence (50% persistence time or 50% recovery time) (7, 16)**

The same technical procedures indicated for 50% recovery time (RT₅₀) calculation of S19 vaccine (see Chapter 2.3.1.) have to be applied for Rev.1, except that *B. abortus* S19 seed lot or batch to be tested (test vaccine) and the S19 original seed culture (used as a reference strain), respectively, are replaced by the corresponding *B. melitensis* Rev.1 seed lot or batch to be tested (test vaccine) and the *B. melitensis* Rev.1 original seed culture as the reference strain. For the reference original Rev.1 strain, RT₅₀ and confidence limits are around 7.9 ± 1.2 weeks. A given Rev.1 vaccine seed lot or batch should keep similar residual virulence to be efficient.

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.

• **Immunogenicity in mice**

The same technical procedures indicated for immunogenicity calculation of S19 vaccine (see Chapter 2.3.1.) have to be applied for Rev.1, except that *B. abortus* S19 seed lot or batch to be tested (test vaccine) and the *B. abortus* S19 original seed culture (used as a reference strain), respectively, are replaced by the corresponding *B. melitensis* Rev.1 seed lot or batch to be tested (test vaccine) and the *B. melitensis* Rev.1 original seed culture as the reference strain.

Conditions of the control experiment are satisfactory when: i) the response in unvaccinated mice (mean of Y) is at least of 4.5; ii) the response in mice vaccinated with the reference Rev.1 vaccine is lower than 2.5; and iii) the standard deviation calculated on each lot of six mice is lower than 0.8.

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.

• **d) Duration of immunity**

It is accepted that subcutaneous or conjunctival vaccination with standard doses of Rev.1 confers a solid and durable immunity in sheep and goats. However, growing field evidence shows that the immunity conferred declines with time, and revaccination could be advisable in endemic areas.

The use of reduced doses of Rev.1 produces a less efficient immunity, while side-effects, such as antibody responses or induction of abortion, are not fully avoided.

• **e) Stability**

Strain Rev.1 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.

• **f) Preservatives**

Antimicrobial preservatives must not be used in live Rev.1 vaccine. For preparation of the freeze-dried vaccine, a stabiliser as described in Section C2.4.f. of Chapter 2.3.1. is recommended.
Chapter 2.4.2. – Caprine and ovine brucellosis (excluding Brucella ovis)

**g) Precautions (hazards)**

*Brucella melitensis* Rev.1, although an attenuated strain, is still capable of causing disease in humans. The cell cultures and suspensions must be handled under appropriate conditions of biohazard containment (see Chapter I.1.6.). 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 a recommended concentration. Medical advice should be sought in the event of accidental exposure. The efficacy of the antibiotic treatment of infections caused by Rev.1 in humans has not been adequately established. If Rev.1 contamination occurs, a combined treatment with doxycycline plus rifampicin could be recommended.

**5. Tests on the final product**

a) **Safety**

See Section C2.4.b. of Chapter 2.3.1.

b) **Potency**

For the freeze-dried vaccine, the potency must be determined on the final product. The tests are as described in Section C2.4.c. of Chapter 2.3.1.

**REFERENCES**


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**NB:** There are OIE Reference Laboratories for Caprine and ovine brucellosis (excluding *Brucella ovis*) (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 2.4.3.

CONTAGIOUS AGALACTIA

SUMMARY

Contagious agalactia is a serious disease syndrome of sheep and goats that is characterised by mastitis, arthritis, keratoconjunctivitis and, occasionally, abortion. Mycoplasma agalactiae is the main cause of the disease in sheep and goats, but *M. capricolum* subsp. *capricolum* (Mcc), *M. mycoides* subsp. *mycoides* LC (MmmLC; LC = large colonies) and *M. putrefaciens* produce a clinically similar disease, more often in goats, which may be accompanied by pneumonia. Antibodies to MmmLC and Mcc have been detected in South American camelids (alpacas, llamas and vicunas), but no mycoplasmas have yet been isolated.

Identification of the agent: Definitive diagnosis requires the isolation of the causative mycoplasmas from the affected animals, which are then identified by biochemical, serological and, increasingly, molecular tests such as the polymerase chain reaction. Samples of choice include milk, eye and ear swabs, and joint fluid. All four mycoplasmas grow relatively well in most mycoplasma media.

Serological tests: Detection of antibodies in serum by complement fixation test or enzyme-linked immunosorbent assay (ELISA) provides rapid diagnosis of disease, but may not be very sensitive in chronically affected herds and flocks. Indirect ELISAs have been used routinely in control programmes for screening herds for *M. agalactiae*. Confirmation of infection by isolation and identification is usually necessary in areas believed to be free of contagious agalactia. Serological tests are not widely available for *M. putrefaciens*.

Requirements for vaccines and diagnostic biologicals: Commercial vaccines for *M. agalactiae*, inactivated with formalin, are widely used in southern Europe, but are not considered to be very efficacious. Under experimental conditions, *M. agalactiae* vaccines inactivated with saponin or phenol have been shown to be more protective than formalised preparations. Live vaccines for *M. agalactiae* are used in Turkey, where they are reported to be more protective than inactivated vaccines. Autogenous vaccines for MmmLC and, occasionally, for Mcc are believed to be used in some countries. No vaccines exist for *M. putrefaciens*, as the disease it causes is not considered to be sufficiently serious or widespread.

A. INTRODUCTION

Contagious agalactia is a disease of sheep and goats that is characterised by mastitis, arthritis and keratoconjunctivitis, and has been known for nearly 200 years. It occurs in Europe, western Asia, the United States of America (USA) and North Africa, and is mainly caused by *Mycoplasma agalactiae* (5). In recent years, *M. capricolum* subsp. *capricolum* (Mcc) and *M. mycoides* subsp. *mycoides* LC (MmmLC; LC = large colonies) have also been isolated in many countries from sheep and goats with mastitis and arthritis. The clinical signs in these infections were sufficiently similar to those of contagious agalactia to lead Perreau (23) to suggest that these infections could also be considered to be contagious agalactia. In addition, *M. putrefaciens* also causes mastitis and arthritis in goats, which is indistinguishable from that caused by *M. agalactiae*, MmmLC and Mcc (10). The consensus of the working group on contagious agalactia of the EC COST¹ Action 826 on ruminant mycoplasmoses, which met in Toulouse, France, in 1999, was that all four mycoplasmas should be considered as causal agents of contagious agalactia.

Clinically, the disease caused by *M. agalactiae* may be recognised by elevated temperature, inappetence, alteration in consistency of the milk in lactating ewes with decline and subsequent failure of milk production,

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¹ European Cooperation in the field of Scientific and Technical Research.
lameness and, in some animals, keratoconjunctivitis (5). Pregnant animals may abort. *Mycoplasma agalactiae* may occasionally be found in lung lesions (17), but pneumonia is not a consistent finding. Bacteraemia is common, particularly for *MmmLC* and *Mcc* and could account for the isolation of the organism from sites where it is only transiently present.

Mastitis, arthritis, pleurisy, pneumonia, and keratoconjunctivitis may all result from infection with *MmmLC*. *MmmLC* has one of the widest geographical distribution of ruminant mycoplasmas, being found on all continents, including South America, where small ruminants are kept and wherever contagious agalactia and caprine pleuropneumonia are reported (5, 19); however the lack of diagnostic facilities for mycoplasma diseases in many countries means that it is probably under reported. *MmmLC* is mostly confined to goats but has occasionally been isolated from sheep with balanoposthitis and vulvovaginitis (35) and cattle (24). Cases usually occur sporadically, but the disease may persist and spread slowly within a herd. After parturition, the opportunity for spread in milking animals increases, and kids ingesting infected colostrum and milk become infected. The resulting septicaemia, with arthritis and pneumonia, gives rise to high mortality in kids (9, 30).

*Mcc* is widely distributed and highly pathogenic, particularly in North Africa (4), but the frequency of occurrence is low (5). Goats are more commonly affected than sheep, and clinical signs of fever, septicaemia, mastitis, and severe arthritis may be followed rapidly by death (5, 6). Pneumonia may be seen at necropsy. The severe joint lesions seen in experimental infections with this organism are accompanied by intense periarticular subcutaneous oedema affecting tissues to some distance from the joint (6). In the United Kingdom genital lesions were seen in sheep as a result of a sporadic outbreak of *Mcc* infection (13).

*Mycoplasma putrefaciens* is common in milking goat herds in Western Europe where it can be isolated from animals with and without clinical signs (18). It has also been associated with a large outbreak of mastitis and agalactia leading to severe arthritis in goats accompanied by abortion and death (but not pyrexia) in California, USA (10). *Mycoplasma putrefaciens* was the major finding in an outbreak of polyarthritis in kids in Spain (29).

Antibodies to *MmmLC* and *Mcc*, but not *M. agalactiae*, have been detected in South American cameldids, including llamas, alpacas and vicunas, but as yet no mycoplasmas have been isolated (20). These cameldids are affected by a range of mycoplasma-like diseases, including polyarthritis and pneumonia, so it is likely that mycoplasmas including *MmmLC* and *Mcc* may be found in the future.

## B. DIAGNOSTIC TECHNIQUES

### 1. Identification of the agents

#### a) Selection of samples

Preferred samples from living animals include: nasal swabs and secretions; milk from mastitic females or from apparently healthy females where there is a high rate of mortality/morbidity in kids; joint fluid from arthritic cases; eye swabs from cases of ocular disease; blood for antibody detection from affected and non-affected animals (21). The ear canal has also been shown to be rich source of pathogenic mycoplasmas, although in practice the presence of nonpathogenic mycoplasmas at this site may make confirmation difficult (8). Mycoplasmas may be isolated from the blood during the acute stage of the disease when there is mycoplasmaemia. From dead animals, samples should include: udder and associated lymph nodes, joint fluid, lung tissue (at the interface between diseased and healthy tissue) and pleural/pericardial fluid. Samples should be dispatched quickly to a diagnostic laboratory in a moist and cool condition. All four causative mycoplasmas are relatively easy to isolate from internal organs, joints and milk and grow well in most mycoplasma media, producing medium to large colonies in 3–4 days.

#### b) Mycoplasma medium

The usual techniques used in the isolation of mycoplasmas apply to all four causative organisms (21). Many media have been reported to grow the causative mycoplasmas. The formulation of an Eaton’s general purpose medium is as follows:

Dissolve 21 g of Becton Dickinson (Oxford, UK) PPLO (pleuropneumonia-like organisms) broth base (without crystal violet) in 700 ml of distilled water. To PPLO broth base add 100 ml of freshly prepared yeast extract, 200 ml of unheated horse serum, 1 g of glucose, 0.5 ml of ampicillin (200,000 International Units [IU/ml], and 12.5 ml of 0.2% phenol red. Adjust pH to between 7.6 and 7.8 and sterilise by filtration. Prepare solid medium by adding 10 g of LabM agar No. 1 (Bury, UK) or agar of equivalent quality and dispense into sterile Petri dishes.

Thallium acetate (250 mg/litre), which is toxic and inhibitory to some mycoplasmas but not those causing contagious agalactia, may be a necessary component of the transport medium to reduce bacterial contamination.
contamination from clinical samples, but should be omitted once the mycoplasmas begin to grow in vitro. A satisfactory alternative to thallium acetate may be colistin sulphate (37.5 mg/litre).

- **Test procedure**
  i) Make tenfold dilutions (10⁻¹–10⁶) of the liquid sample (milk, synovial fluid, eye and ear swabs) or tissue homogenate in appropriate broth medium.
  ii) Spread a few drops of each sample on the agar medium and dispense a 10% (v/v) inoculum into broth medium.
  iii) Streak swabs directly on to agar medium.
  iv) Incubate inoculated broths (optimally with gentle shaking) and agar media at 37°C in humidified atmosphere with 5% carbon dioxide.
  v) Examine broths daily for signs of growth (indicated by a fine cloudiness or opalescence) or changes in pH indicated by a colour change and examine agar media under ×35 magnification for typical ‘fried egg’ colonies.
  vi) If no mycoplasma growth is seen after 7 days, subculture a 10% (v/v) inoculum of broth into fresh broth and spread about 50 µl of this on to agar media.
  vii) Repeat as for step v. If no mycoplasmas are seen after 21 days’ incubation, consider the results to be negative.
  viii) If bacterial contamination results (seen as excessive turbidity), filter sterilise by passing 1 ml of contaminated broth through a 0.45 µm filter into fresh broth medium.

Clinical samples frequently contain more than one mycoplasma species so clone purification of colonies is often considered necessary before performing biochemical and serological identification, in particular the growth and film inhibition tests (GIT and FIT, respectively). However, cloning is a lengthy procedure taking at least 2 weeks. The immunofluorescence test (7), dot immunobinding tests (26) and, more recently, polymerase chain reaction (PCR) tests (see Section B.1.e.) do not require cloning as these tests can detect the pathogenic mycoplasmas in mixed cultures, saving a great deal of time.

c) **Biochemical tests**

The first test that should be performed on the cloned isolates is sensitivity to digitonin, which separates mycoplasmas from acholeplasmas; the latter are ubiquitous contaminants that can overgrow the mycoplasmas of interest. Growth in liquid medium containing glucose (1%), arginine (0.2%), and phenolphthalein diphosphate (0.01%), on solid medium containing horse serum or egg yolk for the demonstration of film and spots, and on casein agar or coagulated serum agar to test for proteolysis, are among the most useful tests for differentiating the four mycoplasmas (27). These biochemical characteristics, however have been increasingly found to be variable for the individual mycoplasmas and have little diagnostic value. The most impressive biochemical characteristic that differentiates *M. putrefaciens* from all other mycoplasmas is the odour of putrefaction it produces in broth culture. Other features that may be helpful include: film and spot production seen on the surface of the broth and solid media caused by *M. agalactiae* and to a lesser extent by *M. putrefaciens*; and the proteolytic activity of *Mcc* and *Mmm*LC on casein and coagulated serum.

A rapid and highly convenient biochemical test that exploits the C8-esterase activity of *M. agalactiae* was reported recently (14). The mycoplasma forms red colonies on agar media within 1 hour of adding the chromogenic substrate, SLPA-octanoate (a newly synthesised ester formed from a C8 fatty acid and a phenolic chromophore). This activity is shared with *M. bovis*, although this mycoplasma is rarely found in small ruminants. Isolates need not be cloned as *M. agalactiae* can be detected easily in mixed cultures. If necessary PCRs can be used to distinguish rapidly *M. agalactiae* from *M. bovis* (see Section B.1.e.).

d) **Serological identification**

Identification of isolates using specific antisera is usually carried out with the GIT, FIT (28) or the indirect fluorescent antibody (IFA) test (7). A recently developed dot immunobinding test, which is carried out in microtitre plates, offers many improvements (rapidity, higher throughputs) over the other tests such as rapidity and higher throughputs (26). For *M. agalactiae*, film inhibition may often be more reliable as growth inhibition is not seen with all isolates; it can also be used for serodiagnosis. Film production by the mycoplasma may be enhanced by the incorporation of 10% egg yolk suspension into the solid medium.

- **Test procedure**
  i) Inoculate at least two dilutions of 48-hour cloned broth cultures (10⁻¹ and 10⁻²) on to predried agar media by allowing 50 µl of the cultures to run down the tilted plates using the ‘running drop’ technique (21). Remove any excess liquid with a pipette.
ii) Allow the plates to dry. It is possible to apply two or three well separated running drops to each 90 mm diameter plate.

iii) Apply filter paper discs containing 30 µl of specific antiserum to the culture; ensure good separation of discs (at least 30 mm).

iv) Incubate the plates as for mycoplasma culture and examine daily by eye against a light background.

- **Interpretation of the results**

A zone of inhibition over 2 mm, measured from the paper disc to the edge of mycoplasma growth is considered to be significant. Partial inhibition can occur with weak antiserum or where there are mixed cultures. Stronger reactions can be obtained if about 60 µl of antiserum is added to 6 mm diameter wells made in the agar with a cork borer or similar device (28).

In the IFA test, specific antisera are applied to colonies on solid medium. Homologous antiserum remains attached after washing and is demonstrated by adding fluorescein-conjugated antiglobulin, washing, and viewing the colonies with an epifluorescence microscope (7). Controls should include known positive and known negative control organisms, and a negative control serum.

Antisera for these serological tests have traditionally been prepared against the type strains of the various *Mycoplasma* species, and most field isolates have been readily identified using these antisera. As more strains have been examined, however, some have been found to react poorly with these antisera, while reacting well with antisera to other representative strains of the species. Intraspecies variation in antigenic composition has not been reported for *M. putrefaciens*, but occurs to some degree with *M. agalactiae* and with *Mcc* strains. Thus, diagnostic laboratories may need to have several antisera to enable all strains of the species to be identified.

e) **Nucleic acid recognition methods**

PCR assays are routinely used in many laboratories and are extremely sensitive. They can provide a rapid early warning system when carried out on clinical samples, enabling a full investigation to take place when results are positive. However negative results should not be considered definitive. Several PCRs specific for *M. agalactiae* have been developed and show similar levels of sensitivity, although they are based on different gene sequences (11, 31, 32). They can be used directly on nasal, ocular, synovial and tissue samples; they have been used on milk samples where they have been reported to be more sensitive than culture (32), although occasionally undefined inhibitors may interfere with the test (5). PCRs can also be used, more reliably, on mycoplasmas growing in culture. A positive result, particularly in an area previously free of contagious agalactia, should be confirmed by isolation and identification of the mycoplasma using standard procedures.

PCRs have also been reported for *Mmm*LC and *Mcc* (3) and a PCR for *M. putrefaciens* is presently undergoing evaluation in the United Kingdom.

- **Test procedure**

The following primers based on the *uvrC* gene have been shown to be specific for *M. agalactiae* (31). PCRs may need to be optimised in each laboratory. Positive and negative control DNA should be run in each assay.

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MAGAUVRC1-L  CTC-AAA-AAT-ACA-TCA-ACA-AGC
MAGAUVRC1-R  CTT-CAA-CTG-ATG-CAT-CAT-AA
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i) Extract DNA from *Mycoplasma* isolates or clinical material using the appropriate method (3).

ii) Carry out PCR methods in 50 µl reaction mixtures containing: 1 µl of sample DNA, 20 pmol of each primer (see above), 1 mM each dNTP, 10 mM Tris/HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl and 1.25 mM U *Taq* DNA polymerase.

iii) Subject the mixture to 35 amplification cycles in a thermal cycler with the following parameters: 30 seconds at 94°C, 30 seconds at 50°C annealing temperature and 1 minute at 72°C.

iv) Analyse the PCR products by electrophoresis on a 0.7% agarose at 110 V for 2 hours and visualise by staining with ethidium bromide. A 1.7 kb fragment indicates the presence of *M. agalactiae*.
Chapter 2.4.3. – Contagious agalactia

2. Serological tests

a) Complement fixation

Perreau et al. (25) reported a standard complement fixation test (CFT) for *M. agalactiae* that has also been applied to other mycoplasmas involved in the contagious agalactia syndrome. Antigens are prepared from washed organisms, standardised by opacity, and lysed, either ultrasonically or by using sodium lauryl sulphate followed by dialysis. Sera are inactivated at 60°C for 1 hour, and the test is carried out in microtitre plates with overnight fixation in the cold or at 37°C for 3 hours. The haemolytic system is added, and the test is read after complete lysis is shown by the antigen control. A positive result is complete fixation at a serum dilution of 1/40 or greater for the following mycoplasmas: *M. agalactiae*, *Mcc*, and *Mmm* LC. The CFT is regarded as a herd test and at least ten sera are tested from each herd, preferably from acute and convalescent cases.

Some sera from healthy flocks react in the CFT using *M. agalactiae* up to a serum dilution of 1/20, but rarely react with the other two antigens. However, in flocks infected with *M. agalactiae*, sera giving a homologous reaction at 1/80 may cross-react at up to 1/40, the positive threshold, with the other two antigens. It is often difficult to perform the CFT if the quality of the test sera is poor; where possible, the enzyme-linked immunosorbent assay (ELISA) is preferred.

b) Enzyme-linked immunosorbent assay

ELISAs using sonicated or Tween-20-treated antigens have been reported to be more sensitive than the CFT for the detection of antibody to *M. agalactiae* (5). Problems of nonspecificity have been overcome by the use of monoclonal or protein G conjugates in the ELISA (15). The use of these conjugates enables the testing of sera from a wide range of mammalian species, including camelids. A number of commercial ELISA kits are now available and these are being used for large-scale surveys in France and the United Kingdom (5, 20). In a ring trial of serological tests for *M. agalactiae* organised in 1998 under the auspices of the EC COST Action 826 on ruminant mycoplasmoses, commercial ELISAs performed better than ‘home-made’ kits.

ELISAs are not widely available for the other three causative mycoplasmas although ‘home-made’ assays are carried out by some laboratories.

c) Immunoblotting test

Immunoblotting tests have been reported as the most sensitive and specific tests for *M. mycoides* subsp. *mycoides* SC, the cause of contagious bovine pleuropneumonia (see Chapter 2.1.6.). Immunoblotting tests have also been described for *M. agalactiae* (20, 33). Strong bands at approximately 80 and 55 kDa were seen with sera with antibodies to *M. agalactiae*, while sera from healthy flocks show no bands or very faint bands of different sizes. Diluting the sera to 1/50 improves the discrimination between positive and negative sera (20).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Vaccines for the prevention of contagious agalactia due to *M. agalactiae* are used widely in the Mediterranean countries of Europe and in western Asia. No single vaccine has been universally adopted, and no standard methods of preparation and evaluation have been applied.

1. Vaccines for *Mycoplasma agalactiae* infection

a) Inactivated vaccines for *Mycoplasma agalactiae* infection

In Europe, where live vaccines for *M. agalactiae* are not acceptable, attention has focused on the use of killed organisms, mostly using formalin and an adjuvant such as aluminium hydroxide in an oil emulsion. The titres of the preparations, before inactivation, are very high (10⁸–10¹⁰ colony-forming units per ml) and are derived from laboratory strains. Autogenous vaccines made from milk, brain and mammary gland homogenates from infected sheep material have been used for many years in parts of Italy, although their efficacy is far from proven. Their use, however, is likely to be discontinued because of their recent link to severe outbreaks of scrapie in sheep and goats (1).

A formalised vaccine has given some protection against experimental infection of goats with *M. agalactiae* in Spain but, despite three vaccinations per year for 6 years, it could not prevent clinical disease following the introduction of naturally infected animals (16). Furthermore formalised vaccines did not reduce the excretion of *M. agalactiae* in the milk compared with challenged controls (22). It is possible that in some
instances the apparent lack of protection given by vaccines could be the result of animals being infected with one of the other four mycoplasmas involved in the contagious agalactia syndrome (12).

More recently vaccines inactivated with phenol or with saponin have given superior protection against experimental infections compared with formalin, sodium hypochlorite or heat-inactivated vaccines (34).

b) **Live attenuated vaccines for *Mycoplasma agalactiae* infection**

Live attenuated vaccines against *M. agalactiae* have been used in Turkey for many years and have been reported to provide better protection in ewes and their lambs than inactivated vaccines (36). However they can produce a transient infection with shedding of mycoplasma.

Live vaccines should not be used in lactating animals and should be part of a regional plan in which all flocks from which animals are likely to come into contact be vaccinated at the same time.

2. **Vaccines for *Mycoplasma mycoides* subsp. *mycoides* LC infection**

There is little recent published information on the availability of vaccines for MmmLC although it is believed that inactivated vaccines are widely used in many Mediterranean countries, suggesting that their production and use is localised (5). In experimental trials in Israel, a preparation of MmmLC, inactivated with formalin and emulsified with mineral oil and sorbitol, gave some protection to 1-day old and 6-week old kids against a virulent challenge (2). Except for a few field trials, the vaccine was never used widely as the occurrence of the disease caused by MmmLC has declined markedly in the past two decades.

3. **Mycoplasma capricolum subsp. *capricolum* and *M. putrefaciens***

Although infections with *Mcc* and *M. putrefaciens* are severe, their prevalence is relatively low and, as might be expected, little or no work appears to have been carried out on preventive vaccination for these infections.

**REFERENCES**


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CHAPTER 2.4.4/5.

CAPRINE ARTHRITIS/ENCEPHALITIS
& MAEDI-VISNA

SUMMARY

Maedi-visna (MV), or ovine progressive pneumonia (OPP), of sheep and caprine arthritis/encephalitis (CAE) of goats are persistent virus infections caused by closely related lentiviruses. Phylogenetic analyses comparing nucleotide sequences of MV virus (MVV) and CAE virus (CAEV) has demonstrated that these are closely related lentiviruses. The primary route of transmission of MV/OPP virus to lambs or CAEV to kids is via colostrum or milk during nursing. Ovine lentiviruses have been identified in most of the sheep-rearing countries of the world, with the notable exceptions of Australia and New Zealand. The distribution of CAEV is highest in industrialised countries, and seems to have coincided with the international movement of European breeds of dairy goats. Clinical and subclinical MV and CAE are associated with progressive, mononuclear cell inflammatory lesions in the lungs, joints, udder and central nervous system. Indurative mastitis is common in both species, and its economic significance may be underestimated. Laboured breathing associated with emaciation caused by progressive pneumonitis is the predominant features in clinically affected sheep, whereas polyarthritis is the main disease in goats. However, most lentivirus-infected sheep and goats are largely asymptomatic, but remain persistent carriers of virus and are capable of transmitting infection via colostrum or milk and respiratory secretions. The most practical and reliable approach to confirming a diagnosis of MV or CAE is a combination of serology and clinical evaluation. Although serology represents the most cost-effective method of diagnosing persistently infected, clinically normal animals, it should be understood that testing errors occur, and the frequency of error depends on the performance data of the particular serological assay being used. Serological methods are of greatest value for screening entire populations rather than for the detection of single seropositive animals.

Identification of the agent: Virus isolation can be attempted from live clinical or subclinical cases by co-cultivating peripheral blood or milk leukocytes with appropriate ovine or caprine cell cultures, such as choroid plexus (MVV) or synovial membrane (CAEV) cells. However, this is time-consuming and not always successful. Following necropsy, virus isolation is most readily accomplished by establishing explant cultures of affected tissues, e.g. lung, choroid plexus, synovial membrane or udder. Also, alveolar macrophages may be obtained from the lung at post-mortem and co-cultivated with susceptible cells. The cytopathic effects are characteristic, consisting of the appearance of refractile stellate cells and syncytia. The presence of MVV or CAEV can be confirmed by immunolabelling methods and electron microscopy.

Nucleic acid recognition methods – polymerase chain reaction, Southern blotting and in situ hybridisation – have been described and are now being used routinely in many laboratories for the rapid detection of the agents.

Serological tests: Most infected sheep and goats possess detectable specific antibodies that can be assayed by a number of different serological tests. The two most commonly used are the agar gel immunodiffusion test and the enzyme-linked immunosorbent assay (ELISA). At the time of publication, the 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. Western immunoblotting and immunoprecipitation are also performed, but only in specialised laboratories. A milk antibody assay may be appropriate in dairy goat herds. The time required for seroconversion following infection can be relatively prolonged and unpredictable, being measured in months rather than in weeks. However, after seroconversion, the antibody response usually persists and antibody-positive sheep and goats are regarded as virus carriers.
**Requirements for vaccines and diagnostic biologicals:** There are no biological products available.

### A. INTRODUCTION

Maedi-visna (MV), or ovine progressive pneumonia (OPP), of sheep and caprine arthritis/encephalitis (CAE) of goats are persistent virus infections caused by closely related lentiviruses. Phylogenetic analyses comparing nucleotide sequences of MV virus (MVV) and CAE virus (CAEV) show clear indications of the existence and epidemiological importance of cross-species transmission between sheep and goats without demonstrating clearly that one virus has emerged from the other (16, 19, 27, 32). MV/OPP and CAE are characterised by lifelong persistence of the causal agent in host monocytes and macrophages, and a variable length of time between infection and induction of a serologically detectable antiviral antibody response. Most infected sheep and goats do not exhibit clinical disease, but remain persistently infected and are capable of transmitting virus (2–4).

Maedi-visna is an Icelandic name that describes two of the clinical syndromes recognised in MV virus (MVV)-infected sheep. ‘Maedi’ means ‘laboured breathing’ and describes the disease associated with a progressive interstitial pneumonitis, and ‘visna’ means ‘shrinkage’ or ‘wasting’, the signs associated with a paralysing meningoencephalitis. Whereas progressive lung disease is the primary finding with MVV infection, chronic polyarthritis, with synovitis and bursitis is the primary clinical outcome of CAEV infection. Encephalitis occurs primarily in kids aged between 2 and 6 months following CAEV infection. Indurative mastitis occurs in both syndromes. The lungs of sheep affected by MV do not collapse when removed from the thorax and often retain the impression of the ribs. The lungs and lymph nodes increase in weight (up to 2–3 times the normal weight). The lesions are uniformly distributed throughout the lungs, which are uniformly discoloured or mottled grey-brown in colour and of a firm texture. Udders affected by MV are diffusely indurated and associated lymph nodes may be enlarged.

When MV/OPP or CAE is the suspected cause of clinical disease, confirmation of the diagnosis can be achieved by a combination of clinical evaluation, serology and, when necessary, histological examination of appropriate tissues collected at necropsy. Important tissues to examine include lung for progressive interstitial pneumonitis, brain and spinal cord for meningoencephalitis, udder for indurative mastitis, affected joints and synovium for arthritis, and kidney for vasculitis (5, 7, 8, 21, 22). The nature of the inflammatory reaction in each site is similar, consisting of an interstitial, mononuclear cell reaction, sometimes with large aggregates of lymphoid cells and follicle formation.

### B. DIAGNOSTIC TECHNIQUES

#### 1. Identification of the agent

Isolation and characterisation of MVV or CAEV would not normally be attempted for routine diagnostic purposes. Due to the persistent nature of these infections, the establishment of a positive antibody status is sufficient for the identification of virus carriers. However, due to a late seroconversion after infection, negative serology may occur in recently infected animals.

There are two approaches to the isolation of MVV and CAEV: one for use with the live animal, and the second for use with necropsy tissues.

**a) Isolation from the live animal**

- **Maedi-visna virus**

  The MV provirus DNA is carried in circulating monocytes and tissue macrophages. Virus isolation from the live animal therefore requires the establishment of leukocyte preparations, with aseptic precautions, from peripheral blood or milk during lactation, culturing them together with indicator cells. Sheep choroid plexus (SCP) cells are commonly used for this purpose. These indicator cells can be prepared as primary explant cultures from fetal or newborn virus-free lambs, and their number can be multiplied over three to four passages for storage in liquid nitrogen. The recovered SCP cells are suitable for co-cultivation for up to 10 or 15 passages. Although the cells continue to grow well thereafter, their susceptibility to MVV may become reduced.

  Leukocyte preparations can be made from peripheral blood as buffy coats by the centrifugation at 1000 g of heparinised, ethylene diamine tetra-acetic acid (EDTA) or citrated samples for 15 minutes. The cells are aspirated, suspended in Hanks’ balanced salt solution (HBSS), and further purified by centrifugation at
400 g on to a suitable cushion (e.g. Ficoll Paque [Pharmacia]) for 40 minutes. The interface cells are spin-washed once or twice in HBSS at 100 g for 10 minutes, and the final cell pellet is resuspended in medium to a concentration of approximately 10^6 cells/ml; cells are generally cultured for 10–12 days in Teflon bags and are then added to a washed monolayer of slightly subconfluent SCP cells in a flask with an area of 25 cm².

Leukocytes can be similarly deposited from milk by centrifugation, when they are spin-washed, resuspended and finally added to SCP monolayer cultures.

These cultures are maintained at 37°C in a 5% CO₂ atmosphere, changing the medium and passaging as necessary. They are examined for evidence of a cytopathic effect (CPE), which is characterised by the appearance of refractile stellate cells with dendritic processes accompanied by the formation of syncytia. The cultures should be maintained for several weeks before being discarded as uninfected. Once a CPE is suspected, cover-slip cultures should be prepared. These are fixed, and evidence of viral antigen is sought by immunolabelling, usually by means of indirect fluorescent antibody or by the use of indirect immunoperoxidase methods. In addition, the cells of any suspect monolayers are deposited by centrifugation, and preparations are made for the identification of any characteristic lentivirus particles by transmission electron microscopy. Reverse transcriptase in the supernatant of the cell culture is indicative of the presence of retroviruses.

- **Caprine arthritis/encephalitis virus**

  The same principles that apply to the isolation of MVV also apply to the isolation of CAEV. CAEV was originally isolated by explantation of synovial membrane from an arthritic goat (5). With live CAEV-infected goats, peripheral blood, milk, and possibly joint fluid aspirate represent the most suitable specimens from which leukocyte preparations can be established. Goat synovial membrane (GSM) cells are suitable indicator cells. If a CPE is suspected, tests for detection of viral antigen should be carried out, as described above.

b) **Isolation from necropsy tissues**

- **Caprine arthritis/encephalitis virus and Maedi-visna virus**

  Samples of suspect tissues, collected as fresh as possible, such as lung, synovial membranes, udder, etc., are collected aseptically into sterile HBSS or cell culture medium and minced finely in a Petri dish using scalpel blades. Individual fragments are collected by Pasteur pipette and transferred to flasks of 25 cm², approximately 20–30 fragments per flask, and a drop of growth medium is placed carefully on each. The flasks are then incubated at 37°C in a humid 5% CO₂ atmosphere, and left undisturbed for a few days to allow the individual explants to adhere to the plastic. Fresh medium can be added with care, after which rafts of cells will gradually grow out from the fragments. When there is sufficient cell out-growth, the cultures are trypsin dispersed to allow the development of cell monolayers. These can be examined for CPE, and any suspected virus growth is confirmed in the same way as for the co-cultivations.

  Adherent macrophage cultures are easy to establish from lung-rinse material (post-mortem broncho-alveolar lavage) and can be tested for virus production by serology, electron microscopy, or reverse transcriptase assay within 1–2 weeks. Virus isolations can be done by co-cultivation of macrophages and SCP or GSM cells as described for leukocytes above.

c) **Nucleic acid recognition methods**

  Most virus disease diagnostic laboratories will be equipped for the basic cell culture procedures described above. Many laboratories can now also carry out nucleic acid recognition methods. In the case of MVV and CAEV, these methods, such as the polymerase chain reaction (PCR) followed by Southern blotting and in situ hybridisation (15), are all methods that can be applied to the detection and recognition of specific nucleic acid sequences within the proviral DNA of MVV and CAEV. PCR techniques for the detection of OPP and CAE nucleic acid have been described and are routinely used in many laboratories. These molecular techniques are playing a role in eradication programmes to supplement serology and to determine the infection status of those animals that cannot be definitively diagnosed by serology (17).

  An important issue in the use of PCR is specificity. Due to the possibility of amplifying unrelated sequences from the host's genomic DNA, the amplified product should be checked by hybridisation, digestion with restriction endonucleases known to be within the target sequence or sequencing of the amplicon. The use of one of these procedures will remove the possibility of false positives. The sensitivity can be improved by the use of nested PCR.

2. **Serological tests**

  Ovine and caprine lentivirus infections are persistent, so that antibody detection is a valuable serological tool for identifying virus carriers. MV and OPP viruses are antigenically indistinguishable by methods that employ
polyclonal antisera. However, the close antigenic relationship between MVV and CAEV does not extend to identical efficiencies in their use for detection of heterologous antibody (18).

The assays now commonly used are agar gel immunodiffusion (AGID) (6, 10, 31) and the enzyme-linked immunosorbent assay (ELISA) (13, 14, 33). The AGID is specific, reproducible and simple to perform, but experience is required for reading the results. The ELISA is economical, and stages of the process can be automated, thus making it useful for screening large numbers of sera. However, the sensitivity and specificity of the ELISA depends on the quality of the antigen. In the case of MV/OPP and CAE viruses, the production of satisfactory antigen preparations has limited its routine application. Modifications to the MV/OPP and CAE ELISAs are emerging, such as use of recombinant protein antigen (25, 26), double antibody sandwich methods, and monoclonal antibodies (14), which may ultimately result in its wider application. ELISA protocols have been used for several years in some European countries in control and eradication schemes of MVV in sheep (24) and CAEV in goats. In general, however, AGID remains the most frequently used test.

a) Agar gel immunodiffusion (the prescribed test for international trade)

There are two MV/OPP and CAE viral antigens of major importance in routine serology, a viral envelope glycoprotein commonly referred to as gp135, and a core protein, p28. These are both conserved in an antigen preparation consisting of medium harvested from infected cell cultures and concentrated approximately 50-fold by dialysis against polyethylene glycol. The WLC-1 strain of OPP virus is commonly used.

It is important to recognise that the sensitivity of the AGID test for detecting anti-CAEV antibody is dependent on the antigen used (1, 18). It was demonstrated that an AGID test with CAEV gp135 afforded greater sensitivity than an AGID test with CAEV p28 (1). Also, it was shown that when compared with immunoprecipitation, the sensitivity of the AGID test for anti-CAEV antibody was 35% greater with CAEV antigen than OPP virus antigen (18). The most likely explanation for this difference in sensitivity between the CAE and OPP virus antigen for the detection of anti-CAEV antibody is that although the immunoprecipitation assay requires only the binding of a single epitope by antibody to obtain a positive result, precipitation in an agar gel requires multiple epitope–antibody interactions. Although the OPP and CAE viruses are closely related antigenically, the degree of the antigenic relatedness is not known, and it is likely that there are fewer antibody/antigen interactions in the heterologous system. When the appropriate antigen is used, the AGID test performance is high. When compared with immunoprecipitation, the AGID for the detection of anti-CAEV antibody, if CAEV antigen was used, had 92% sensitivity and 100% specificity (18).

In MVV-infected sheep and CAEV-infected goats, the predominant precipitating antibody response detected routinely is directed against the gp135 antigen. An anti-p28 response is usually present at a lower titre than the anti-gp135 response.

In some CAEV-infected goats there is evidence to suggest that an anti-gp135 antibody response is produced, in the absence of an anti-p28 response and vice versa, in a proportion of individuals (9, 26). Hence, for validation of the test, standard sera producing both anti-gp135 and anti-p28 precipitin lines are required.

The gel medium is 0.7–1% agarose in 0.05 M Tris buffer, pH 7.2, with 8.0% NaCl. The test is conveniently performed in plastic Petri dishes, or in 10 cm² plastic trays. The pattern and size of the wells will determine the number of sera tested per plate. Various well patterns can be adopted, but a hexagonal arrangement with a central well is usual: for example, a pattern with alternating large (5 mm in diameter) and small (3 mm in diameter) peripheral wells, 2 mm apart and 2 mm from a central antigen well that is 3 mm in diameter. The large peripheral wells are used for test sera and the small ones for standard sera. A weak positive control must be included in each test. The plates are incubated overnight at ambient room temperature in a humid chamber, and then examined for precipitin lines. Plates may be incubated for a further 24 hours if needed.

An important consideration is the need for experienced personnel to interpret the AGID. Interpretation of AGID results is dependent on the antigen used. Examples of AGIDs with different antigen preparations and a guide for interpretation of the results can be found in ref. 1.

b) Enzyme-linked immunosorbent assay

Various ELISA protocols have been described. At present, an ELISA using purified whole virus as antigen appears to be the most practical option for routine diagnosis (12, 30, 33). Antigen was produced by

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1 This virus has been distributed by Dr Howard Lemkuhl, National Animal Disease Center, United States Department of Agriculture, P.O. Box 70, Ames, Iowa, USA.
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Differential centrifugation and detergent treatment of supernatant from infected cell cultures, and was used to coat microplates. Reagents were added sequentially as follows:

i) test serum,
ii) horseradish peroxidase-conjugated rabbit anti-sheep immunoglobulin,
iii) colour developer,

with specified incubation times, temperatures, and washing procedures between each stage. Positive and negative controls were included, and the test results were measured as absorbance, by photometer (30).

Recently a competitive ELISA (C-ELISA) for the detection of anti-gp135 (CAEV) antibodies in goats was described (11, 12). This C-ELISA was also used for the detection of serum antibodies to ovine progressive pneumonia virus in sheep (11). This C-ELISA uses a monoclonal antibody (MAb 74A) that binds to a conformational epitope of CAEV gp135 (23). Based on comparison with immunoprecipitation as a standard of comparison, the sensitivity and specificity of the C-ELISA was 100% and 96.4%, respectively.

The ELISA technique is also applicable to milk, but given that lentivirus antibody titres in milk are lower (possibly 10% of those in sera), a much reduced sensitivity would be expected (18). Because the primary route of CAEV transmission is via colostrum and milk, the testing of milk samples for anti-CAEV or anti-MVV antibody would not provide timely information for the prevention of transmission, especially to offspring from the immediate gestation (17).

The ELISA is easy to perform in laboratories that have the necessary equipment (a spectrophotometer) and reagents. It is convenient for large-scale screening and, particularly for veterinary diagnosis, as it is a reliable technique for demonstrating small ruminant lentiviruses (SRLVs) antibodies in sheep and goats. It requires a relatively pure antigen. At the time of publication, the 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.

The production of antigens for the use in ELISAs has been described. Antigenic preparation must contain at least one of the major antigens of SRLVs, i.e. the envelope (gp135 = surface protein [SU] and gp44 = transmembrane protein [TM]) and the capsid (p25) (33). These antigens may be present in a whole-virus preparation or produced as recombinant proteins or synthetic peptides (12, 17, 28, 29, 33). Thus, recombinant gag gene product fused with glutathione S-transferase fusion protein antigen has been produced in Escherichia coli. Recombinant antigens produced in E. coli provide a consistent source of antigen for international distribution and standardisation. Recombinant antigens or synthetic peptides have been used in indirect ELISAs (I-ELISAs).

For I-ELISA, wells of the microplate are coated with 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 (e.g. 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.

Specific MAbs, which define surface protein epitopes, have been used in a C-ELISA for SRLVs to capture surface envelope as antigen (24): C-ELISA overcomes the problem of antigen purity, as the specificity of this test depends only on the MAb used.

For C-ELISA, sample sera containing anti-SRLV antibodies inhibit binding of enzyme–labelled MAb to SRLV antigen coated on the plastic wells. Binding of the enzyme-labelled MAb conjugate is detected by the addition of enzyme substrate and quantified by subsequent colour product development. Strong colour development indicates little or no blockage of enzyme-labelled MAb binding and therefore the absence of SRLV antibodies in sample sera. In contrast, weak colour development due to the inhibition of the enzyme-labelled MAb binding to the antigen on the solid phase indicates the presence of SRLV antibodies in sample sera.

Materials and reagents

- Microtitre plates with 96 flat-bottomed wells, freshly coated or previously coated with SRLV antigen; microplate reader (spectrophotometer; 405, 450, 490 and 620 nm filters); 37°C humidified incubator; 1-8- and 12-channel pipettes with disposable plastic tips; microplate shaker (optional); fridge; freezer.

- Positive and negative control sera; conjugate (e.g. ruminant anti-immunoglobulin labelled with peroxidase); tenfold concentration of diluent (e.g. phosphate buffered saline/Tween); distilled water; 10× wash solution; substrate or chromogen (e.g. ABTS [2,2’-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid]) or TMB [3,3',5,5'-tetramethylbenzidine]); stop solution (e.g. detergent, sulfuric acid).
• **Indirect ELISA: test procedure**

i) Dilute the serum samples, including control sera, to the appropriate dilution (e.g. 1/20) and distribute 0.1–0.2 ml per well (in duplicate if biphasic ELISA). Control sera are positive and negative sera provided by the manufacturer and an internal positive reference serum from the laboratory in order to compare the titres between different tests.

ii) Cover the plate with a lid and incubate at room temperature or 37°C for 30–90 minutes. Empty the contents and wash three times in washing solution at room temperature.

iii) Add the appropriate dilution of freshly prepared conjugate to the wells (0.1 ml per well). Cover each plate and incubate as in step ii. Wash again three times.

iv) Add 0.1 ml of freshly prepared or ready-to-use chromogen substrate solution to each well (e.g. ABTS in citrate phosphate buffer, pH 5.0, and 30% H₂O₂ solution [0.1 µl/ml]).

v) Shake the plate; after incubation, stop the reaction by adding stopping solution to each well (e.g. 0.1 ml sulphuric acid).

vi) Read the absorbance of each well with the microplate reader at 405 nm (ABTS) or 450–620 nm (TMB). The absorbance values will be used to calculate the results.

vii) **Interpretation of the results**

For commercial kits, interpretations and validation criteria are provided with the kit.

For example: calculate the mean absorbance (Ab) of the sample serum and of the positive (Ab⁺) and negative (Ab⁻) control sera, and for each serum, calculate the percentage:

\[
\frac{Ab - Ab_{neg}}{Ab_{pos} - Ab_{neg}} \times 100
\]

Interpret the results as follows:
- Ab <30% negative serum
- Ab 30–40% doubtful serum
- Ab >40% positive serum

• **Competitive ELISA: test procedure**

i) Add 0.05 ml of undiluted serum and positive/negative controls to antigen-coated plate.

ii) Incubate for 1 hour at room temperature.

iii) Empty the plate and wash the plate three with diluted wash solution.

iv) Add 0.05 ml of diluted antibody-peroxidase conjugate to each well. Mix well and incubate for 30 minutes at room temperature.

v) After the 30-minute incubation, empty the plate and repeat the washing procedure described in step iii.

vi) Add 0.05 ml of substrate solution (ex: TMB) to each well. Mix and cover plate with aluminium foil. Incubate for 20 minutes at room temperature. Do not empty wells.

vii) Add 0.05 ml of stop solution to each well. Mix. Do not empty wells.

viii) Immediately after adding the stop solution, the plate should be read on a plate reader (620, 630 or 650 nm).

ix) **Interpretation of results**

Example: Calculation: 100 – [[Sample Ab x 100]/(Mean negative control Ab)] = % inhibition.

For goats, if a test sample causes >33.2% inhibition, it is positive; if a test sample causes <33.2% inhibition, it is negative. For sheep, if a test sample causes >20.9% inhibition, it is positive; if a test sample causes <20.9% inhibition, it is negative.

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

There are no biological products available. MAbs recognising conformational epitopes of the CAEV envelope glycoprotein, gp135 have been described (23).
REFERENCES


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NB: There are OIE Reference Laboratories for Caprine arthritis/encephalitis & Maedi-visna (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**

Contagious caprine pleuropneumonia (CCPP) is a severe disease of goats caused by Mycoplasma capricolum subspecies capripneumoniae (Mccp). This organism is closely related to three other mycoplasmas: M. mycoides subsp. mycoides large colonies (LC), M. mycoides subsp. capri, and M. capricolum subsp. capricolum. Unlike true CCPP, which is confined to the thoracic cavity, the disease caused by the latter three mycoplasmas is accompanied by prominent lesions in other organs and/or parts of the body besides the thoracic cavity.

Typical cases of CCPP are characterised by extreme fever (41–43°C), high morbidity and mortality rates in susceptible herds affecting all ages and both sexes, and abortions in pregnant goats. After approximately 2–3 days of high fever, respiratory signs become apparent: respiration is accelerated and painful, and in some cases is accompanied by a grunt. Coughing is frequent, violent and productive. In the terminal stages, animals are unable to move – they stand with their front legs wide apart, the neck is stiff and extended, and sometimes saliva continuously drips from the mouth. Post-mortem examination reveals fibrinous pleuropneumonia with massive lung hepatisation and pleurisy, accompanied by accumulation of straw-coloured pleural fluid.

**Identification of the agent:** Definitive diagnosis requires culture of the causative organism from lung tissue samples and/or pleural fluid taken at post-mortem. After cloning and purification, isolates can be identified by several biochemical, immunological and molecular tests. Recently a polymerase chain reaction based test has been described and shown to be specific, sensitive and can be applied directly to clinical material, such as lung and pleural fluid.

**Serological tests:** Serological tests have not been widely applied for identifying the cause of pleuropneumonia in goats or sheep. Such tests are best used on a herd basis rather than for diagnosis in individual animals. The complement fixation test remains the most widely used serological test for CCPP, except in Kenya where latex agglutination is used. Indirect hemagglutination is also used. A specific competitive enzyme-linked immunosorbent assay has been developed, but is not widely available. As with the other serological tests, it does not detect all reactors, but its specificity and suitability for large-scale testing make it an appropriate test for epidemiological investigations.

**Requirements for vaccines and diagnostic biologicals:** Vaccine against CCPP caused by Mccp is available commercially from the Kenya Veterinary Vaccines Production Institute.

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**A. INTRODUCTION**

Contagious caprine pleuropneumonia (CCPP) is a severe disease of goats occurring in many countries in Africa and Asia where the total goat population is more than 500 million (1). Classical, acute CCPP is caused by Mycoplasma capricolum subsp. capripneumoniae (Mccp) (17), originally known as the F38 biotype. This organism was first isolated and shown to cause CCPP in Kenya (18–21); it has subsequently been isolated in the Sudan, Tunisia, Oman, Turkey, Chad, Uganda, Ethiopia, Niger, Tanzania, Eritrea and the United Arab Emirates. Disease indistinguishable from naturally occurring CCPP has been experimentally reproduced with Mccp by several groups of workers.

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1 Kenya Veterinary Vaccines Production Institute, P.O. Box 53260, Nairobi, Kenya. E-mail: kehevapi@africaonline.co.ke
B. DIAGNOSTIC TECHNIQUES

The diagnosis of outbreaks of respiratory disease in goats, and of CCPP in particular, is complicated, especially where it is endemic. It must be differentiated from other similar clinico-pathological syndromes such as: peste des petits ruminants, to which sheep are also susceptible; pasteurellosis, which can be differentiated on the basis of distribution of gross lung lesions; and what has been called ‘mastitis, arthritis, keratitis, pneumonia and septicæmia syndrome or more often as contagious agalactia syndrome (38). As the longer name implies, the pneumonia is accompanied by prominent lesions in other organs. The disease caused by \textit{Mccp} is readily contagious and fatal to susceptible goats of all ages and both sexes, does not affect sheep or cattle, and is characterised histopathologically by an interstitial, intralobular oedema of the lung (13).

1. Identification of the agent

a) Microscopy of lung exudates, impression smears or sections

\textit{Mccp} shows a branching filamentous morphology \textit{in vivo} that can be observed by dark-field microscopy in exudates or tissue suspensions from lesions or pleural fluid. Alternatively, smears made from cut lung lesions can be stained by the method of May–Grünwald–Giesma and examined by light microscopy. The other caprine mycoplasmas show a short filamentous or coccobacillary morphology. Neither of these techniques provides a definitive diagnosis.

b) Nucleic acid recognition methods

The polymerase chain reaction (PCR) is used to amplify a conserved segment of the 16S rRNA gene of the mycoplasmas. The PCR product is then analysed by restriction enzyme cleavage for the identification of the \textit{Mccp} amplicon. The test is used directly on clinical materials such as lung tissue and pleural fluid (3). However, isolation of \textit{Mccp} remains the confirmatory test.

c) Gel precipitin tests to detect antigen in tissue specimens

\textit{Mccp} releases an antigenic polysaccharide (32) to which a specific monoclonal antibody (MAb) (WM-25) has been produced (33, 34). This MAb immunoprecipitates in agar gel with the polysaccharide produced by \textit{Mccp}, and is used to identify the causative agent in cases of CCPP, particularly when specimens are no longer suitable for culture because of deterioration during transit.

d) Isolation of mycoplasmas

i) Selection of samples

The necropsy samples of choice are lung lesions, particularly from the interface between consolidated and unconsolidated areas, pleural fluid, and mediastinal lymph nodes. If microbiological examination cannot be performed immediately, samples or whole lungs can be stored deep-frozen at $-20^\circ \text{C}$ for considerable periods (months) with little apparent loss of mycoplasma viability. During transport, samples should always be kept as cool as possible, as mycoplasma viability diminishes rapidly with increasing temperature. Lung samples can be dispatched to other laboratories in frozen form.

ii) Treatment of samples

Swabs are suspended in 2–3 ml of culture medium. Tissue samples are best minced using scissors, and then shaken vigorously, or pulverised in medium\textsuperscript{2} using 1 g of tissue to 9 ml of medium. Tissues should not be ground. The suspension is usually prepared with a mycoplasma medium, but if parallel bacteriological examination is to be carried out, a high quality bacteriological medium, such as nutrient broth, may be used to provide a suspension suitable for both forms of examination. Pleural fluid, or a tissue suspension or swab, is serially diluted through at least three tenfold steps (to a nominal $10^{-4}$) in the selected mycoplasma medium. Dilutions should also be plated on to solid medium.

iii) Mycoplasma media

The medium used by MacOwan & Minette to culture \textit{Mccp} organisms (19), is termed ‘viande foie goat’ (VFG), and includes goat-meat liver broth and goat serum. Alternative suitable media are WJ (12), modified Hayflick’s, and modified Newing’s tryptose (14)\textsuperscript{3}. Media enriched with 0.2% (or up to 0.8%) sodium pyruvate perform considerably better, both for primary isolation and antigen production of

\textsuperscript{2} For example, with the Stomacher 80, A.J. Seward, London, United Kingdom (UK).

\textsuperscript{3} A commercial medium called Mycoplasma Experience is also available from Mycoplasma Experience, Reigate, Surrey, UK.
Mccp (24, 25). This forms the basis for Thiaucourt’s medium (38, 40) and modified Thiaucourt’s medium (3). The formulations of the last two media are as follows:

- **Thiaucourt’s medium**
  A. Autoclaved portion (121°C for 15 minutes): Bacto PPLO (pleuropneumonia-like organisms) broth without crystal violet (Difco) (21 g); deionised water (700 ml).
  
  B. Membrane-filtered portion: Horse (alternatively pig or donkey) serum inactivated at 56°C for 30 minutes (200 ml); fresh yeast extract (100 ml); glucose (sterile solution 0.5 g/ml) (2 ml); and sodium pyruvate (sterile solution 0.5 g/ml) (8 ml).

  Part B is added to A aseptically. Ampicillin (0.1 g/litre) and thallium acetate (250 mg/litre) can be added to prevent contamination in primary isolations. The final pH of the medium should be 7.4–7.6.

- **Modified Thiaucourt’s medium**
  A. Autoclaved portion (121°C for 15 minutes): Bacto PPLO broth without crystal violet (Difco) (17.5 g); glass distilled water (650 ml).
  
  B. Membrane-filtered portion: Horse (alternatively pig or donkey) serum inactivated at 56°C for 30 minutes (250 ml); fresh yeast extract (100 ml); 50% glucose (4 ml); 25% sodium pyruvate (8 ml); 5% thallium acetate (4 ml); ampicillin (250 mg); and 0.5% phenol red (4 ml). The pH is adjusted to 7.8 with sodium hydroxide or hydrochloric acid. Part B is added to A aseptically.

Modified Newing’s tryptose broth (14) and agar plates (Gourlay’s medium) (9) are routinely used for isolation and maintenance of Mccp at the OIE Reference Laboratory in Kenya (see Table in Part 3 of this Terrestrial Manual).

iv) **Medium production, storage and quality control**

Certain medium components, particularly serum, yeast extract and deionised water, should be regularly monitored for growth-promoting capacity before incorporation into mycoplasma media. Low-passage field isolates should be used for this screening purpose.

Broth media may be stored for at least 6 months at –25°C before use, but penicillin or its analogues should not be added until final dispensing. Broth media are dispensed into bijoux (1.8 ml or 2.7 ml) or screw-capped tubes (4.5 ml), and stored for up to 3 weeks at 4°C. Solid media are best made with agarose (0.9% [w/v]), Noble agar (1.5% [w/v]), or purified agar (0.6% [w/v]). Plates, which are poured to a depth of 6–8 mm, should be as fresh as possible when used, and should be stored for no more than 2 weeks at 4°C before use. All culture media should be subjected to quality control and must support the growth of Mycoplasma spp. from small inocula. The reference stain should be cultured in parallel with the suspicious samples to ensure that the tests are working correctly.

v) **Cultivation**

Cultures are incubated at 37°C. Plates are best incubated in a humid atmosphere of 5% CO₂, 95% air or N₂, or in a candle jar with a moisture source.

Broth cultures are examined daily for evidence of growth – colour change and the appearance of floccular material. Gross turbidity indicates bacterial contamination; cultures showing this should be passed through a 0.45-µm membrane filter before subculture. Broth cultures are subcultured by inoculation of fresh broth medium with one-tenth of their volume, or by streaking agar medium with a loop.

Plate cultures are examined every 1–3 days using a stereo microscope (×5–50 magnification) and transmitted and incident light sources. If negative, the plates are discarded after 14 days. Subculture is carried out by the transfer of excised agar blocks bearing isolated colonies to either agar (on which the blocks are pushed, face down) or broth media. Alternatively, an agar plug bearing one colony is drawn into a Pasteur pipette and discharged into fresh broth medium.

Cloning and purification of isolates is performed by repeated transfer of single colonies representing each morphological type seen. Colony morphology varies with the medium used, the mycoplasma species, its passage level and the age of the culture.

In early passage, many mycoplasma species, including M. capricolum subsp. capricolum (Mcc), produce colonies of bizarre morphology, often small, centreless, and of irregular shape. This effect is often associated with the use of marginally suitable medium. With passage, such isolates demonstrate conventional ‘fried egg’ colony morphology, except M. ovipneumoniae, which retains centreless colonies. Colonies of M. mycoides subsp. mycoides Large Colony (MmmLC) and Mcc may be up to 3 mm in diameter.
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Filtration of broth cultures through 0.45 µm filters before subculture aids purification by excluding cell aggregates.

Cultures suspected of being L-forms of bacteria should be examined for reversion to bacterial form by three to five passages on solid mycoplasma medium from which antibiotics and thallium acetate have been omitted.

Broth media used for primary isolation and which have shown no indication of growth by 7 days, should be subcultured blind.

Cultures of each sample, including one blind subculture, should be examined for a minimum of 3 weeks before being discarded. Titrations in broths, if performed in full (to $10^{-10}$), are also read at 3–4 weeks and are expressed as colour-changing units per transfer volume. Growth on plates is expressed as colony-forming units (CFU) per ml.

e) Identification of mycoplasmas

i) Polymerase chain reaction

Once the organism has been cultured, verification of Mccp can be achieved in 1 day by PCR. The test is based on amplification of a segment of the 16S rRNA gene. The amplified fragment is common to the mycoides cluster. However, when the amplicon is digested with endonuclease PstI, a unique cleavage pattern of three fragments for Mccp is observed when the enzyme digests are analysed in agarose gel electrophoresis and stained with ethidium bromide (3).

Recently, PCR and sequencing has been used to establish the molecular epidemiology of CCPP. These tests can be performed on dried samples, such as pleural fluid on filter papers. The sequencing allows a precise identification of the species (the cleavage site for the 16S rRNA and a specific detection for the ‘locus H2′) (16, 28).

ii) Biochemical tests

Wild strains should be passaged, and preferably cloned, three times before identification is attempted.

Biochemical tests cannot identify an isolate unequivocally, which at present can only be done by serological or genetic means. Intraspecies variation in some biochemical reactions is often considerable, but some tests perform a useful function both as a preliminary screening system and in providing supportive data for serological findings.

The tests most commonly used are glucose breakdown, arginine hydrolysis, ‘film and spots’ formation, reduction of tetrathionate chloride (aerobically and anaerobically), phosphatase activity, serum digestion and digitonin sensitivity. The first three of these tests are performed routinely in isolation and cultivation procedures. Glucose breakdown is indicated by acid (yellow) changes, and arginine hydrolysis by alkaline (red) changes in broth media, using phenol red as indicator. Arginine use cannot be assessed on conventional medium for isolation and culture as the media for testing the arginine deaminase pathway should contain high concentrations of arginine and no glucose. Film and spots’ describes an apparent wrinkling of the agar surface due to the deposition on it of an iridescent film of lipid, together with the development of black spots within the medium in the vicinity of ageing colonies. This phenomenon, produced by three mycoplasmas of small ruminants, is demonstrated on agar media containing 20% or more serum, preferably of horse or pig origin. Supplementation of the medium with 10% egg yolk emulsion improves the sensitivity of the test.

The remaining biochemical tests require specific media or reagents. The test for tetrathionate reduction provides corroborative evidence of the mycoplasmal nature of M. agalactiae isolates, as this organism is neither glycolytic nor arginine-hydrolysing. Serum digestion (7) distinguishes members of ruminant mycoplasmas, and phosphatase production (4) separates Mcc from other members of this cluster. Digitonin sensitivity distinguishes members of the order Mycoplasmatales from those of the order Acholplasmatales (8).

iii) Serological identification

Mycoplasmal antigens used in hyperimmune serum production are often contaminated with medium constituents. The antibodies stimulated by these contaminants can cause false-positive reactions in serological identification tests. This problem is avoided by absorption of the antiserum with the medium used to produce the antigen (10 mg lyophilised medium per ml of antiserum), or by growing the mycoplasmas to be used as antigens in medium containing homologous animal components, e.g. growth in VFG medium to immunise goats.

Because of the close serological relationships between members of the ‘M. mycoides cluster’, isolates from cases of CCPP should, preferably, be identified by at least two of the three tests described below.
• Growth inhibition test
The growth inhibition test (GIT) is the simplest and most specific, but the least sensitive, of the tests available. It depends on the direct inhibition of growth on solid medium by specific hyperimmune serum, and detects primarily surface antigens (6).

Mccp appears to be highly homogeneous serologically and wide zones of inhibition free of ‘breakthrough’ colonies are observed with antiserum to the type strain, regardless of the source of the test strain (12). Mccp cross-reacts with Leach’s bovine group 7 (PG50), M. equigenitalium and M. primatum in the GIT when polyclonal antisera are used, but an MAb specific for Mccp in the GIT has been produced (33). The MAb reagent, WM25, is specific for (Mccp) isolates by the disc growth inhibition method, which will exclude M. agalactiae, Mcc and the other members of the ‘M. mycoides cluster’ associated with goats, but not bovine group 7 (not found in goats): the latter can be excluded, however, by colony indirect fluorescence tests (2). A small proportion of Mccp isolates also cross-react in the GIT with antiserum to Mcc, Group seven of Leach strains can sometimes be found in goats although it is rare. Results should be interpreted carefully as some bovine strains have been misidentified by the GIT using the ‘specific’ antiserum.

• Test procedure
i) Broth culture in mid-to-late logarithmic phase is used at three tenfold dilutions, the selection of which is related to the vigour of growth of the isolate on agar.
ii) Agar plates are dried for 30 minutes at 37°C.
iii) Sterile paper disks of 6–7 mm in diameter are impregnated with a drop (10–20 µl) of undiluted antiserum. Disks may be used wet, in which form they can be stored at –20°C, or they can be lyophilised (6), which allows storage at 4°C.
iv) Using a separate plate for each dilution of culture, 1 ml or 2.5 ml volumes are pipetted on to 5 cm or 10 cm diameter plates, respectively. The inoculum is dispersed evenly over the plate, then the excess is removed.
v) The plates are dried at 20–30°C for 15–20 minutes, preferably under a protective hood, until no visible liquid is present on the surface. Sufficient residual moisture should remain to enable freeze-dried disks to adhere to the agar surface.
vi) Several disks, each impregnated with a different antiserum (selected on the basis of sample source and the biochemical reactions and colony morphology of the isolate), are carefully placed on the agar plates; isolates from CCPP cases should be screened with antisera against Mccp, MmmLC, Mcc, M. mycoides subsp. capri (Mcc) and M. ovipneumoniae. A disk containing 1.5% digitonin should also be included on the plates.
vii) The plates are incubated at 37°C for 2–6 days. Initial overnight incubation at 27°C can increase the sensitivity of the test. Inhibition by digitonin is generally readily apparent; however, inhibition by antiserum may be more difficult to interpret, with suppression rather than total inhibition of growth, depending on the species of mycoplasma, colony density and potency of the antiserum. ‘Breakthrough’ colonies are commonly observed within zones of inhibition. Circular precipitin bands are occasionally seen around disks. Positive inhibition is regarded as a zone of 2 mm or more.

• Growth precipitation test
The growth precipitation test detects soluble cytoplasmic and extramembranous antigens released by growing cultures and allowed to diffuse through solid mycoplasma growth medium towards mycoplasma antiserum during growth (15). As with the gel precipitin test, there are strong cross-reactions within the mycoides cluster. If growth inhibition is performed using MAbs WM254, which is specific for Mccp, both specific inhibition and a growth precipitin line are achieved simultaneously.

• Indirect fluorescence antibody test
The direct and indirect fluorescent antibody tests are the most effective of the various serological methods for identifying most mycoplasmas (29). They are simple, rapid, and sensitive, yet economical in the use of antiserum. Several forms have been described, the most commonly used and perhaps best being the indirect fluorescent antibody (IFA) test applied to unfixed colonies on agar. Antiserum against a single strain is sufficient to identify field isolates of that species, and antisera are diluted before use. Cultures do not have to be cloned, but the test is usually applied only after several passages have indicated whether the culture contains more than one species and the growth characteristics of the organism(s) present.

• Test procedure
i) Two agar plates are predried at 37°C for 30 minutes. Each one is flooded with a different dilution of test broth culture, the dilutions being selected according to the vigour of growth of the strain on agar.

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4 Available from Kenya Agricultural Research Institute (KARI), P.O. Box 57811, Nairobi, Kenya.
medium. Alternatively, a drop of undiluted culture is spread over a single 5 cm plate using an L-shaped glass rod.

ii) The plates are incubated at 37°C until the first evidence of growth is observed. If the IFA test cannot be performed immediately, the plates can be stored at 4°C for up to 4 weeks.

iii) Several blocks of approximately 0.5–1 cm² are excised from areas where colonies are numerous, but not confluent. The blocks of each agar culture are cut to the same geometric shape to enable recognition of origin, a different shape being used for each isolate. Several blocks of each isolate are distributed (colony surface facing upwards) on to several different slides, each slide being used for a different mycoplasma antiserum. The colony surface of each block is identified for future reference by undercutting one corner.

iv) Rabbit anti-mycoplasma (ra-m) serum or normal rabbit serum (NRS; as a control on a duplicate block) at a suitable dilution in normal saline or phosphate buffered saline (PBS), pH 7.2, is gently pipetted on to each agar block until the surface area is totally covered. The optimal dilution of ra-m is determined by chequerboard titration against the fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin serum (a-r lg-FITC) used.

v) The flooded blocks are incubated on their slides at room temperature for 30 minutes in a humid chamber.

vi) All blocks on one slide are tipped into a 10 ml tube containing approximately 7 ml of PBS.

vii) The plugged tubes are rotated at 18–30 rpm for 10 minutes. The PBS is then decanted and replaced with fresh PBS, and the tubes are rotated again for 10 minutes.

viii) The PBS is decanted and the blocks are placed colony surface facing upwards on their respective slides. Excess moisture is blotted off.

ix) All blocks are flooded with a-r lg-FITC at optimal dilution.

x) The blocks are incubated again for 30 minutes at room temperature in a humid chamber, then tipped into tubes containing fresh PBS, and washed twice by rotating, as before.

xi) The blocks, replaced colony surface facing upwards on their respective slides, are examined by an epi-immunofluorescent microscope using the settings recommended by the manufacturer for FITC.

**Notes on the indirect fluorescent antibody test**

xii) Working dilutions of ra-m and a-r lg-FITC should be kept at 4°C, which limits their shelf life to approximately 1 week.

xiii) Isolates from CCPP should be examined using antisera against Mccp, MmmLC, Mmc (M. mycoides subsp. capri) and Mcc, positive control cultures should comprise their type strains, namely Mccp, Y goat, PG3 and California kid, respectively.

xiv) A negative (NRS-treated) control should always be incorporated for each culture.

xv) Interpretation of the IFA test can be difficult. Autofluorescence is produced by some species, particularly acholeplasmas. Even in pure cultures, a proportion of colonies may not stain positively with the relevant antiserum; this is particularly true of Mcc. Otherwise, poor results are usually ascribable to the use of an agar culture that has been allowed to grow for too long, or to the use of antiserum that has deteriorated with dilution and age.

**f) Other identification tests**

Metabolism inhibition (37) and tetrazolium reduction inhibition (35) are other tests sometimes used in the identification of caprine mycoplasmas. A gene probe, F38-12, capable of distinguishing Mccp has been developed (36).

A polysaccharide-specific antigen detection latex agglutination test has been developed to detect CCPP antigen (23). In this test, latex beads are coated with polyclonal immunoglobulin IgG (rabbit) directed against Mccp polysaccharide and used to detect the antigen in the serum of goats with CCPP. This test is proposed to be inexpensive, easy to carry out in the field and useful for detecting CCPP in its earliest stages.

**2. Serological tests**

Serology has not been widely applied to identifying the cause of outbreaks of pleuropneumonia in goats and sheep. Endemic infections with MmmLC and Mmc can produce a background of positive titres to these organisms in a proportion of apparently healthy animals (12), and under experimental conditions seroconversion to M. mycoides can occur in goats with no clinical signs of disease. Acute cases caused by Mccp rarely show
positive titres to the organism before death (21, 26, 38), perhaps because antibodies are 'eclipsed' by circulating mycoplasma antigens (26). Seroconversion to Mcpp in experimentally infected animals is observed, by the complement fixation (CF) test and indirect haemagglutination (IHA) test, to start 7–9 days after the appearance of clinical signs, to peak between days 22 and 30, and to decline rapidly thereafter (26). These various observations indicate that serology should be applied on a herd, not an individual basis, and that whenever possible, paired serum samples collected 3–8 weeks apart, should be examined.

a) Complement fixation test (the prescribed test for international trade) (19)

The CF test in various forms remains the most widely used serological test for diagnosis of contagious bovine pleuropneumonia (10, 27). In CCPP, the CF test was used for detection of Mcpp infection (19) and it has been found to be more specific, though less sensitive, than the IHA test (26). Its main disadvantage is the high level of technical expertise required to perform the test (10).

One method of performing the test is as follows. To prepare the antigen, 2 litres of culture of titre higher than $10^5$ CFU/ml is centrifuged at 40,000 $g$ for 1 hour at 5°C. The deposit is resuspended and washed three times in physiological saline prior to storage in 0.5–1.0 ml volumes at –20°C.

Sterile broth treated as above constitutes sediment antigen, and a freeze-dried broth reconstituted at 200 mg/ml constitutes a second control antigen. Prior to testing, the antigen is diluted 1/60 and ultrasonicated for 3 minutes at low power in a container of iced water. The sonicate is centrifuged at 1250 $g$ for 30 minutes to remove any debris, and stored at –20°C. If stored for more than 2–3 weeks the antigen should be recentrifuged.

Test procedure

Microtitre plate tests are performed using 0.025 ml volumes, two volumes containing three mean haemolytic doses of complement, and a 1.5% (v/v) final concentration of sheep red blood cells (SRBCs) in U-bottomed microtitre plates as follows:

i) The following are mixed and incubated at 37°C for 45 minutes:
   • 25 µl of doubling dilutions of test serum (heat inactivated at 56°C for 30 minutes) starting with 1/2 dilution;
   • 25 µl of antigen (the dilution of the antigen must be determined in a chequerboard titration using a known positive serum);
   • 25 µl of complement (3 haemolytic units).

ii) 25 µl of sensitised SRBCs, at a final concentration of 1.5% (v/v), is mixed and the plates are incubated at 37°C for 45 minutes.

iii) The plates are incubated at 4°C for 1 hour to allow the unlysed cells to settle.

iv) Reading the results: The titre will be the highest serum dilution that will fix 50% of the complement, i.e. 50% haemolysis.

Controls

In all CF tests a number of controls are required:

i) Indicator systems (RBCs + haemolysin) alone to ensure that RBCs do not lyse spontaneously.

ii) Indicator system with complement only to show that enough complement is present to lyse the cells.

iii) Indicator system with antigen only and no complement to show that antigen alone does not lyse the cells.

iv) Indicator system with serum alone and no complement to show that the serum alone does not lyse the cells.

v) Indicator system with complement and antigen to detect any anticomplementary activity of the antigen.

vi) Indicator system with the complement and serum to detect any anticomplementary activity of the serum.

b) Indirect haemagglutination test (26)

The IHA test (5) has been used for diagnosis of CCPP (26).
The IHA test is most commonly performed with RBCs that are either fresh and tanned, or treated with glutaraldehyde. The former are more sensitive but show greater variability between tests, and require sensitisation of cells with antigen each time the test is performed. Glutaraldehyde treatment of RBCs reduces sensitivity but produces a much more useful diagnostic test, as sensitised RBCs remain effective for 1 year or more if kept refrigerated, and require little further manipulation before use in the test.

The specificity of the IHA test for the \textit{M. mycoides} cluster has been evaluated using rabbit hyperimmune sera and glutaraldehyde-treated RBCs sensitised with lightly sonicated mycoplasma cell suspensions (12). \textit{Mnm} LC- and \textit{Mccp}-sensitised cells cross-reacted with antisera to the other three species, but \textit{Mmc} and \textit{Mcc}-sensitised cells each cross-reacted only with the antiserum to \textit{Mccp}.

The polysaccharide produced by \textit{Mccp} has been found to bind to untreated goat RBCs and, as such, was successfully used in an IHA test to identify animals with experimental and natural CCPP (32).

Cells sensitised with the four principal caprine mycoplasmas have been used in field studies in Oman and the Sudan. The conclusions from the two surveys differed. The Oman survey revealed widespread seropositivity to \textit{Mmc}, whereas \textit{Mcc} reactors were largely confined to herds in which the \textit{Mcc} had been shown to occur by cultural means (12). In contrast, more animals with signs of CCPP in the Sudan were seropositive to \textit{Mnm} LC than to any of the mycoplasmas tested, only 7\% reacting with \textit{Mcc} antigen. Seropositivity to two or more antigens was noted in a proportion of animals in both surveys.

c) \textbf{Latex agglutination test}

Latex beads sensitised with the polysaccharide produced by \textit{Mccp} and present in culture supernatant have been used in a slide agglutination test (11, 30). This test is presently used routinely in Kenya. It is a very useful test in an outbreak because it can be performed at the penside using a drop of whole blood.

Both CF test and IHA test findings emphasise the difficulties inherent in the serological diagnosis of CCPP when using whole cell or membrane preparations as antigen. The use of the more defined antigen, the polysaccharide elaborated by \textit{Mccp}, provides greater specificity, as there is no cross-reactivity with sera against the other three principal caprine mycoplasmas.

d) \textbf{Competitive enzyme-linked immusorbent assay (39)}

This test is based on the specificity of the MAb for the \textit{Mccp} epitope and the ability of \textit{Mccp}-infected goats to make antibodies to this epitope. The two antibodies are made to compete for the \textit{Mccp} epitope coated on to plates. The test has been described to be specific and to detect antibodies for long periods after infection. However, as with the other serological tests, it does not detect all reactors (it detects between 30\% and 60\% in an infected herd) (39). The competitive enzyme-linked immusorbent assay is easier to perform than the CF test, and is suitable for testing many samples at one time. It is therefore a suitable test for epidemiological investigations. Validation of the procedure is still in process.

\section*{C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS}

The first experimental vaccine against \textit{Mccp} comprised live \textit{Mccp} in high passage (22). When inoculated intratracheally, it proved innocuous and protected goats against experimental challenge. More recent work has concentrated, however, on inactivated forms of vaccine. The current form used in Kenya (where inactivated \textit{Mccp} vaccines have been in use for several years) contains lyophilised \textit{Mccp} suspended in saponin; this formulation has a shelf life of at least 14 months. The optimal dose of 0.15 mg of mycoplasma provides protection for over 1 year (31).

1. Seed management

a) Characteristics of the seed

The master seed was isolated from the lungs of a sick goat. It is a Yatta isolate and has been confirmed to be \textit{Mccp} by GIT and PCR, after 15 passages in cell culture. The master seed should also be pure and free from other contaminants.

b) Method of culture

The master seed is established and stored in a freeze-dried form in 1 ml ampoules. The working seed is prepared by amplifying the master seed in modified Newing’s broth to make a bulk of 4 litres. Its growth is arrested at the growth phase before filament formation.
This culture is tested for sterility before it is distributed in aliquots of 20 ml volumes and stored frozen at –20°C.

c) **Validation as a vaccine**

The master seed should be prepared from lung specimens or pleural fluids of a goat that dies of pneumonia, showing all the clinical signs of CCPP. The isolate must be confirmed by GIT or by PCR to be **Mccp**. It must be checked for sterility, safety, potency and extraneous agents.

2. **Method of manufacture**

For vaccine production, a working seed is first established by amplifying an aliquot of freeze-dried master seed in modified Newing’s broth (14), to make a bulk of 4 litres of culture. This culture is aliquoted in 20 ml volumes and frozen at –20°C. The vaccine is grown in 5 litre pots containing 4 litres of modified Newing’s broth. Each pot is sampled aseptically for sterility testing before 20 ml of working seed is inoculated into each 4 litres of medium. These pots are incubated at 37°C for 4–6 days depending on how fast the mycoplasma grows. After the filaments sediment, the antigen is harvested by centrifugation at 2600 g from pots that are not contaminated. (The filaments are thin and white and sometimes unite to form a resemblance of an inverted pine tree. This occurs on days 4–6 and it is at this point that they become heavy and sediment.) The pellet is resuspended in sterile normal saline and centrifuged to remove the remnants of the growth medium. The pellet is resuspended again in a small volume of sterile normal saline to make a viscous suspension.

Saponin is added to inactivate the mycoplasma at 3 mg saponin to 1 ml of antigen. It is left agitating overnight at 4°C using a magnetic stirrer.

**NOTE:** Saponin also acts as an adjuvant. Three samples are taken aseptically from this suspension and tested for sterility, for protein estimation and for the innocuity test.

The amount of protein is estimated by using the standard opacity tubes and expressed in mg/ml and this determines the total number of doses in the batch of vaccine.

3. **In-process control**

During production, the following tests are carried out to ensure that the product remains pure and safe. These tests are carried out by quality assurance staff. Each pot is sampled aseptically before inoculation to test for sterility.

After maturity of culture, only pots that have not shown signs of contamination are pooled for centrifugation; the contaminated pots are decontaminated and discarded after autoclaving.

After inactivation with saponin, a sample is taken aseptically for sterility, another one for innocuity and another one for the protein estimation test.

a) **Sterility test**

This test is aimed at verifying the absence of fungal and bacterial contaminants. Two tubes of thioglycollate are inoculated with 1 ml of the sample each. The tubes contain about 15 ml of broth. These are incubated at 37°C to eliminate aerobics, microaerophils and anaerobics. The other medium used is soybean casein digest broth. Four tubes are inoculated with 12 ml of sample each.

Two tubes are incubated at 37°C and another two at room temperature (25°C) to eliminate bacterial and fungal contaminants. All media are incubated for 14 days with controls. Absence of any growth shows that the sample is not contaminated.

b) **Innocuity test**

This test aims to demonstrate the presence of living mycoplasma in the vaccine: 0.3 ml of inactivated antigen is added to 2.7 ml of modified Newing’s broth in a tube and tenfold dilutions are made from 10⁻¹ to 10⁻⁹. A positive control is set up using a viable culture of *M. capripneumoniae* and a negative control using three uninoculated tubes of medium. All are incubated at 37°C for 12 days. If there is no growth in the tubes inoculated with test sample, the vaccine passes the test.

c) **Protein content estimation**

This test uses Brown’s opacity tubes. The antigen is dispensed to a similar tube as those containing standards, and its opacity is compared visually with the opacity of each standard opacity tube starting with
the lowest. After estimation, the antigen is diluted using sterile normal saline to make 2 mg of protein/ml as the final vaccine.

4. Batch control

a) Sterility

The sterility test on the final batch is carried out as described in Section C.3.a. except that four bottles of each batch are pooled together and samples of the pool are used.

b) Safety

Every batch of CCPP vaccine must be proven to be safe in laboratory animals. Two guinea-pigs are each inoculated by the intramuscular route in the hind leg and another two guinea-pigs are inoculated by the peritoneal route with 0.5 ml containing five doses of vaccine.

If the vaccine is safe, the guinea-pigs should not show any sign of disease for a 14-day observation period and, on post-mortem, there should be no abscesses on the site of inoculation and in the peritoneal cavity, respectively. If any vaccine-related deaths occur during the observation period, the vaccine fails. If on post-mortem examination, abscesses are observed at the site of inoculation and the peritoneum, the vaccine also fails.

c) Potency

Research is in progress at the Kenya Agricultural Research Institute, Muguga, Kenya, to develop a test for potency.

d) Duration of immunity

The vaccine protects goats for 14 months. It is, however, recommended to boost immunity after 1 year.

e) Stability

If stored at 4°C, the vaccine has a shelf life of 1 year. Before use the vaccine should be shaken thoroughly, for even distribution of antigen.

f) Preservatives

At present preservatives are not used in the vaccine.

g) Precautions

Side-effects of the CCPP vaccine include development of a swelling at the site of inoculation and fever for 1–2 days following vaccination, accompanied with inappetance. The swelling may last between 1 and 14 days and is due to the saponin adjuvant. Accidental self-injection causes severe irritation.

5. Tests on the final product

a) Safety

Every batch of vaccine should be tested for safety in laboratory animals as described in C.4.b.

b) Potency

Once the potency test has been developed, every batch of vaccine will be required to be tested for potency.

REFERENCES


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**NB:** There are OIE Reference Laboratories for Contagious caprine 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 2.4.7.

ENZOOTIC ABORTION OF EWES
(ovine chlamydiosis)

SUMMARY

The diagnosis of enzootic abortion, also known as ovine chlamydiosis or chlamydial abortion of ewes, depends on the isolation and identification of the causative agent or detection of the agent or its nucleic acid in the products of abortion or vaginal excretions of freshly aborted females. A humoral antibody response may be detected following abortion. Goats as well as sheep and, less commonly, cattle and deer, can be affected. Chlamydiosis of small ruminants is a zoonosis and the organism must be handled with biosafety precautions. Pregnant women are particularly susceptible. The causative organism, Chlamydia abortus, was formerly known as Chlamydia psittaci immunotype 1.

Identification of the agent: The basis for a positive diagnosis of infection with C. abortus depends on a history of abortion in sheep or goats (often in late pregnancy), evidence of necrotic placentitis, and the demonstration of large numbers of the organism in stained smears of affected placentae. The still moist fleece of fetuses or vaginal swabs of females that have freshly aborted are also useful. Care is needed to distinguish cotyledonary damage caused by Toxoplasma gondii and, in stained smears, to be aware of the morphological similarities between C. abortus and Coxiella burnetii, the agent of Q fever.

Chlamydial antigen can be detected by enzyme-linked immunosorbent assay, or the fluorescent antibody test, whereas chlamydial DNA can be detected by the polymerase chain reaction, the former two being available in kit form.

Chlamyphilia abortus can be isolated only in living cells; thus facilities for growth in chicken embryos or cell cultures, with appropriate biohazard containment, are required.

Serological tests: A rise in antibody titre to C. abortus, detected by the complement fixation (CF) test, is common after abortion or stillbirth, but this does not occur in every case. Chlamydia abortus shares common antigens with C. pecorum and some Gram-negative bacteria, so that the CF test is not wholly specific, nor does it distinguish between responses to vaccination and to infection. Low CF titres need to be interpreted with caution, particularly if these are encountered in individual animals or in flocks with no history of abortion.

Alternative serological tests have been developed, but none has been sufficiently appraised so far for field use. A delayed hypersensitivity reaction to chlamydial antigen can be elicited in infected sheep, but the procedure is not amenable to routine use.

Requirements for vaccines and diagnostic biologicals: Inactivated and live vaccines are available that have been reported to prevent abortion and to reduce excretion. They assist in control of the disease but will not eradicate it. Serological screening during the period after parturition helps to identify infected flocks, to which control measures can then be applied.

A. INTRODUCTION

Chlamydial abortion (enzootic abortion of ewes, ovine enzootic abortion) in late pregnancy causes serious reproductive wastage in many sheep-rearing areas of the world, particularly where flocks are closely congregated during the parturient period (1, 16). Infected animals show no clinical illness prior to abortion. Pathogenesis commences around day 90 of gestation coincident with a phase of rapid fetal growth when chlamydial invasion of placentomes produces a progressively diffuse inflammatory response, thrombotic vasculitis and tissue necrosis.
Milder changes occur in the fetal liver and lung and, in cases in which placental damage is severe, there may be evidence of hypoxic brain damage (5). Abortion probably results from a combination of impairment of maternal-fetal nutrient and gaseous exchange, disruption of hormonal regulation of pregnancy and induced cytokine aggression (7).

Chlamydial abortion also occurs in goats and, less frequently, cattle and deer may be affected. In sheep, abortion in late pregnancy with expulsion of necrotic fetal membranes are key diagnostic indicators, with care being needed to distinguish the diffuse pattern of necrosis from that caused by Toxoplasma gondii (cottonyons only). Distinction from other infectious causes of abortion such as brucellosis (see Chapter 2.4.2), coxiellosis (see Chapter 2.2.10.) or other bacterial pathogens (Campylobacter [see Chapter 2.10.8.], Listeria [see Chapter 2.10.14.], Salmonella [see Chapter 2.10.3.]) can be achieved by microscopy and/or culture.

Taxonomically, the family Chlamydiaceae is divided into two genera and nine species based on sequence analysis of the 16s and 23s rRNA genes (9). The genus Chlamydia includes C. trachomatis (humans), C. suis (swine) and C. muridarum (mouse and hamster). The genus Chlamydophila includes C. psittaci (avian), C. felis (cat), C. abortus (sheep, goat and cattle), C. caviae (guinea-pig), the former species C. pecorum (sheep and cattle) and C. pneumoniae (humans). The two genera and nine species have merit both on the basis of molecular structure and for the purposes of host range and clinical disease. The species show a marked degree of correlation with host range, disease syndrome and virulence, thus assisting in understanding the epidemiology of the various species and serovars affecting mammals and birds. The terms ‘chlamydiosis’ and ‘chlamydia(e)’ are used to refer to members of either of the two genera. However, a binomial of the generic and specific names is used when referring to a particular chlamydial species.

Infected females shed vast numbers of infective C. abortus at the time of abortion or parturition, particularly in the placenta and uterine discharges (20). Human infection may be acquired from such sources or from carelessly handled laboratory cultures of the organism, with effects that range from subclinical infection to acute influenza-like illness. Appropriate precautions should be taken when handling cultures and potentially infected tissues (see Chapter I.1.6 Human safety in the veterinary microbiology laboratory). Authenticated cases of human placentitis and abortion caused by C. abortus of ovine origin indicate that pregnant women are at special risk and should not be exposed to sources of infection (6, 16).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Smears

Where the clinical history of the flock and the character of lesions in aborted placentae suggest enzootic abortion, a diagnosis can be made by microscopic examination of smears made from affected chorionic villi or adjacent chorion. Several staining procedures are satisfactory, for example, modified Machiavello, Giemsa, Brucella differential, or modified Ziehl–Neelsen stains (24). In positive cases stained by the latter method and examined under a high-power microscope, large numbers of small (300 nm) coccoid elementary bodies are seen singly or in clumps stained red against the blue background of cellular debris. Under dark-ground illumination, the elementary bodies are pale green. If placental material is not available, smears may be made from vaginal swabs of females that have aborted within the previous 24 hours, or from the moist fleece of a freshly aborted or stillborn lamb that has not been cleaned by its mother. In general, such preparations contain fewer organisms than placental smears.

In morphology and staining characteristics, C. abortus resembles the rickettsia Coxiella burnetii, which, in some circumstances, may provoke abortion and which, in humans, causes Q fever. Care must be taken to differentiate between these two organisms in cases lacking a good history or evidence of chlamydial-induced placental pathology. Antigenic differences between C. abortus and Coxiella burnetii can be detected serologically. Fluorescent antibody tests (FATs) using a specific antiserum or monoclonal antibody may be used for identification of C. abortus in smears.

b) Antigen detection

Several chlamydial genus-level antigen-detection tests are available commercially. A comparative assessment of several such assays, on non-ovine material, indicated that those using enzyme-linked immunosorbent assay (ELISA) methodology were more sensitive than kits employing an FAT (29). Under the test conditions used, a kit (Clearview, Unipath) that detects chlamydial lipopolysaccharide (LPS) was judged to be the most sensitive of the rapid ELISA-based systems investigated. Though occasionally yielding false-positive results, particularly with avian faecal samples, the kit also gave satisfactory results with ovine placental samples (28).
c) DNA

Amplification of chlamydial DNA by polymerase chain reaction (PCR) provides an alternative approach for verifying the presence of chlamydiae in biological samples without resorting to culture. PCR is highly sensitive for this purpose (27), but has the attendant risk of cross-contamination between samples, so appropriate measures must be taken to avoid this happening (see Chapter I.1.8.). Another potential problem is in the production of false negatives resulting from PCR-inhibitory substances in the samples. Methods for discriminating between amplified DNA sequences originating from C. abortus and C. pecorum have been described (8, 14, 15). At present, these are mainly research techniques, but they are beginning to be introduced into diagnostic laboratories.

d) Tissue sections

Intracellular chlamydial inclusions can be demonstrated by Giemsa staining of thin (≤4 µm) sections taken from target tissues that have been suitably fixed in fluids such as Bouin or Carnoy. More striking results can be obtained by immunological staining procedures. The direct immunoperoxidase method (10) is rapid and simple, while the method with streptavidin–biotin is more complex (25). Electron microscopy can also be performed using negative contrast, to differentiate chlamydiae from Coxiella burnetii.

e) Isolation of the agent

*Chlamydia abortus* can be isolated in embryonated chicken eggs or in cell cultures, the latter being the method of choice for isolation of new strains. The causative agent of chlamydiosis is zoonotic (16) and thus isolation and identification procedures must be carried out under the appropriate containment level as described in Chapter I.1.6. Human safety in the veterinary microbiology laboratory.

Tissue samples, such as diseased cotyledons, placental membranes, fetal lung or liver, or vaginal swabs, that may be subject to any delay before isolation procedures begin, should be maintained in a suitable transport medium in the interim period. The most satisfactory medium is sucrose/phosphate/glutamate medium supplemented with 10% fetal bovine serum, antibiotic (streptomycin and gentamycin are suitable, but not penicillin), and a fungal inhibitor (23). A tissue:medium ratio of 1:10 is commonly employed. Alternatively, approximately 1 g of tissue is ground with sterile sand in 8 ml of transport medium.

*Chicken embryos*: Test samples are prepared as 10% suspensions in nutrient broth containing streptomycin (not penicillin) (200 µg/ml); 0.2 ml of suspension is inoculated into the yolk sac of 6–8-day old embryos, which are then further incubated at 37°C. Infected embryos die between 4 and 13 days after inoculation. Smears prepared from their vascularised yolk sac membranes reveal large numbers of elementary bodies.

*Cell cultures*: *Chlamydia abortus* of ovine origin can be isolated in a variety of cell types, but McCoy, BGM or baby hamster kidney (BHK) cells are most commonly used. For confirmatory diagnosis, cultured monolayers are suspended in growth medium at a concentration of 2 x 10⁵ cells/ml. Aliquots of 2 ml of the suspension are dispensed into flat-bottomed glass Universal bottles, each containing a single 16 mm cover-slip. Confluent cover-slip monolayers are achieved after incubation for 24 hours at 37°C. The growth medium is removed and replaced by 2 ml of test inoculum, which is then centrifuged at 2500 g for 30 minutes on to the cover-slip monolayer to promote infection. After further incubation for 2–3 days, the cover-slip monolayers are fixed in methanol and stained with Giemsa or according to the method of Gimenez (3, 11). After methanol fixation, infected cultures contain basophilic (Giemsa) or eosinophilic (Gimenez) intracytoplasmic inclusions. Similar procedures are used in culturing *C. abortus* for antigen preparation. FAT techniques can also be used and are equally effective.

Chlamydial activity can be further enhanced by chemical treatment of cultured cells, before or during infection, to favour chlamydial growth. Various substances that have been described for incorporation into the infective inoculum to which cover-slip monolayers are exposed include: cycloheximide (0.5 µg/ml) for 5 minutes before infection, and 5-iodo-2-deoxyuridine (80 µg/ml) for 3 days prior to infection. Unless preconditioned cells are available, the latter isolation procedure requires increased time for successful agent isolation.

2. Serological tests

Complement fixation (CF) is the most widely used procedure for detecting infection (sheep and goats are generally tested within 3 months of abortion or parturition). The test will also detect evidence of vaccination. Infection is evident principally during active placental infection in the last month of gestation and following the bacteraemia that often accompanies abortion. Consequently, paired sera collected at the time of abortion and again at least 3 weeks later may reveal a rising CF antibody titre that will provide a basis for a retrospective diagnosis. Antigenic cross-reactivity between *C. abortus* and *C. pecorum*, as well as with some Gram-negative bacteria (e.g. *Acinetobacter*), can give rise to low false-positive CF test results. Thus, titres less than 1/32 in
individual animals should be considered to be nonspecific for \( C. \) abortus, although they could also be due to a low grade infection with \( C. \) abortus.

Antigen is prepared from heavily infected yolk sac membranes obtained from chicken embryos that have been inoculated in the same manner as those used to isolate the organism from field material. The preparation of the antigen should be carried out in a biosafety cabinet with the appropriate biosecurity precautions to prevent human infection (see Chapter I.1.6.). Chopped and ground membranes are suspended in phosphate buffer, pH 7.6, at the rate of 2 ml per membrane. After removal of crude debris, the supernatant fluid is centrifuged at 10,000 \( g \) for 1 hour at 4°C, the deposit is resuspended in a small volume of saline, and a smear of this is examined to ensure a high yield of chlamydiae. The suspension is held in a boiling water bath for 20 minutes, or is autoclaved, and sodium azide (0.3%) is added as a preservative. Antigen may also be prepared from cell cultures infected with \( C. \) abortus. Infected monolayers are suspended in phosphate buffer, pH 7.6, and the cells are disrupted by homogenisation or ultrasonication. Gross debris is removed and subsequent procedures are as for the preparation of antigen from infected yolk sacs. In either case, CF tests with standardised complement and antiserum will establish the optimal working dilution for each batch of antigen.

The serological responses to \( C. \) abortus and \( C. \) pecorum can be resolved by indirect micro-immunofluorescence, but the procedure is too time-consuming for routine diagnostic purposes. ELISAs developed independently by several research groups have not been adapted for general diagnostic work, partly because of difficulties associated with the use of particulate antigens. However, a novel ELISA that incorporates a stable, solubilised antigen has been used to test experimental and field samples, and has given results that, though lacking species specificity, have a higher sensitivity than the CF test (2, 13). Of note has been the recent development of monoclonal antibody technology in a competitive ELISA (22) and recombinant antigen technology in indirect ELISAs (4, 17, 18) to discriminate between antibodies to \( C. \) abortus and \( C. \) pecorum.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Inactivated vaccines can be prepared from infected yolk sacs or cell cultures (12) and incorporate whole organisms or fractions of them (26), using the appropriate biosecurity precautions to prevent human infection (see Chapter I.1.6.). A recombinant vaccine against \( C. \) abortus remains a future goal. An alternative approach has been development of a chemically induced temperature-sensitive mutant of the organism (21). Currently, two types of vaccine are available commercially, to be administered intramuscularly or subcutaneously before breeding to aid in the prevention of abortion. Each has a role to play in controlling disease, but neither confers absolute protection against challenge. One vaccine incorporates inactivated \( C. \) abortus within an oil adjuvant suspension and protection for at least 2 years following primary vaccination is claimed. The other uses a live lyophilised temperature-sensitive mutant of \( C. \) abortus that is reconstituted in diluent immediately before administration. Vaccinates exposed to infection experience lower abortion rates and reduced excretion of chlamydiae for at least three lamblings after vaccination. Operator care in handling and administration of the live vaccine is advised. It has been reported that the live vaccine strongly reduces the excretion of the \( Chlamydia \) as well as preventing abortion and it has been claimed that it could be an aid to eradication (19).

1. Seed management

a) Characteristics of the seed

One or more ovine abortion isolates that consistently grow productively in the chosen substrate are suitable, and an early passage of the seed stock can be established. Alternatively, an isolate that has been adapted to the chicken embryo by multiple passage (>100) can be used. This permits more of the embryo to be used for vaccine production. Although adaptation to the embryo may diminish the isolate's virulence for sheep, there is no evidence that such change reduces its protective efficacy as an inactivated vaccine.

b) Method of culture

For low passage isolates, the procedures described for the preparation of CF antigen are suitably adapted and amplified for bulk production. Once the final harvest suspension is obtained, an aliquot is removed for titration of its infectivity. The bulk is treated with formalin to a final concentration of 0.4%, and stored until sterility tests confirm complete inactivation.

c) Validation as a vaccine

Before inoculation of large numbers of embryos or cell cultures, the viability and freedom from contamination of seed stock should be verified. It may be convenient to collect the total harvest in separate manageable lots. In this case, the infectivity of an aliquot of each lot should be separately titrated to ensure that each matches the requirements (see Section C.2. below). Store under refrigerated conditions.
Chapter 2.4.7. — Enzootic abortion of ewes (ovine chlamydiosis)

2. Method of manufacture

The inactivated harvest is centrifuged and resuspended in phosphate buffered saline containing 0.2% formalin to a volume representing a preinactivation infectivity titre of approximately $10^8$ infectious units/ml. Usually, the aqueous suspension is blended with an oil adjuvant, either directly or after precipitation by potassium alum ($\text{AlK}[\text{SO}_4]_2\cdot12\text{H}_2\text{O}$). A preservative, such as 0.01% thiomersal, may also be added.

3. In-process control

The main requirements are to ensure adequate growth of C. abortus, avoidance of extraneous infection of the culture substrate, completeness of inactivation and biohazard awareness by process workers.

4. Batch control

Each separate batch of manufactured vaccine should be tested for sterility, safety and potency.

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter I.1.5.

b) Safety

Subcutaneous inoculation into two or more seronegative sheep of twice the standard dose (usually 1.0 ml) of manufactured vaccine should elicit no systemic reaction, but oil-adjuvant vaccines can cause a nonharmful swelling at the inoculation site.

c) Potency

At present, potency is judged by the occurrence of a serological response in previously unvaccinated sheep given 1 ml of vaccine subcutaneously. Blood samples taken before and 28 days after vaccination are compared. Ultimately, potency has to be judged against experimental challenge or field performance, but no in vitro correlation of protective efficacy has yet been established.

d) Duration of immunity

No firm data are available, but revaccination is recommended after 1–3 years, according to the exposure risk.

e) Stability

Vaccine stored under refrigeration ($5\pm3^\circ\text{C}$) should remain stable for at least 1 year. Before use it should be held at room temperature for 24 hours, and the container should be shaken vigorously immediately before vaccine is withdrawn.

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

REFERENCES


Chapter 2.4.7. — Enzootic abortion of ewes (ovine chlamydiosis)


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CHAPTER 2.4.8.

SCRAPIE

SUMMARY

Scrapie is a naturally occurring infectious neurodegenerative disease of sheep and goats characterised by vacuolar or spongy changes in the central nervous system (CNS). It has been recognised for over two and a half centuries. It is caused by a transmissible agent, the prion, the molecular and biochemical nature of which remain unclear.

The disease is endemic in many parts of the world although, Australia and New Zealand have maintained freedom from scrapie. The infection in sheep may be passed from ewe to lamb in the period from parturition to weaning. Infection can also pass to unrelated sheep or goats, especially when parturition occurs in confined areas. Fetal membranes are thought to be a source of infection. The incubation time between primary infection and clinical disease is nearly always longer than 1 year and may sometimes exceed the commercial lifespan of the sheep. The majority of cases occur in sheep between 2 and 5 years of age. Clinical disease only develops if the infection enters the CNS.

The disease is recognised by the clinical signs, which start insidiously with behavioural abnormalities. These may initially go unrecognised, but usually progress to more obvious neurological signs, including pruritus and incoordination. Loss of bodily condition is also common. The clinical disorder is variable in duration and in the range of signs presented, but it is inevitably fatal.

Identification of the agent: Clinical diagnosis is supported by the immuno-detection of disease-specific accumulations of prion protein in the brain or peripheral lymphoreticular tissues or by histopathological diagnosis of spongiform encephalopathy. Brain pathology is characterised by bilateral and usually symmetrical neuronal vacuolation and spongiform change of grey matter in the brain stem. The nature of the agent causing scrapie and similar transmissible spongiform encephalopathies (TSEs) is unclear, but during the development of the disease an abnormal form of a host membrane glycoprotein, called prion protein or PrP, accumulates in certain tissues, notably the CNS and peripheral nervous system, and, dependent upon the genotype of the sheep, in lymphoreticular organs. This abnormal isoform (PrPres also referred to as PrPSc) is a component, perhaps the sole molecule, of the causative agent. Its detection in extracts or tissue sections of diseased brains is therefore a disease-specific diagnostic criterion. PrPres can be detected in unfixed brain material by detergent extraction, enzymatic digestion, electrophoresis and immunoblotting. A morphologically distinctive form of PrPres can be visualised, using an electron microscope from detergent- and protease-treated extracts of unfixed brain tissue, as scrapie-associated fibrils. Immunohistochemistry may be used to detect abnormal accumulations of PrP in routine histologically prepared brain and lymphoreticular tissue sections. Detection of such accumulation in lymphoreticular tissues during the incubation period of scrapie offers a means of preclinical diagnosis of scrapie infection by proxy and may be particularly useful for surveillance purposes when performed on biopsied tissue.

Scrapie can be transmitted to laboratory rodents by injecting them with infected tissue, but the variable efficiency of primary isolations coupled with incubation times of 1–2 years preclude this as a practical diagnostic procedure.

Serological tests: Scrapie infection is not known to elicit any specific immune response and there is no basis for establishing a diagnosis by detecting specific antibodies.

Genetic tests: Several polymorphisms have been identified within the protein-coding region of the sheep PrP gene. Epidemiological studies have established an association between the occurrence
of scrapie and specific polymorphisms in several breeds of sheep. PrP genotyping has the potential to be a valuable tool in the control of clinical scrapie. However, evidence is accumulating to suggest that no genotype is completely resistant to infection, although it has yet to be determined whether natural disease occurs in resistant genotypes. Sheep of more susceptible genotypes are found in geographical areas of the world where scrapie does not occur, thus challenging suggestions that scrapie may occur spontaneously.

Requirements for vaccines and diagnostic biologicals: There are no biological products available.

A. INTRODUCTION

Scrapie is a naturally occurring progressive, fatal, infectious neurodegenerative disease of sheep and goats. It is characterised by vacuolar or spongy changes in the central nervous system (CNS). In countries of Western Europe, it has been known to occur in sheep for at least the past two and a half centuries. It is therefore, in retrospect, the first transmissible spongiform encephalopathy (TSE) or prion disease of mammals to be recognised.

Scrapie remains endemic in many European countries and has also been reported in several countries throughout most other continents. However, Australia and New Zealand have maintained freedom from the disease through rigorous preventative policies. Because of the absence of procedures for surveillance for scrapie in many countries, their scrapie status is unknown. Within the European Union, scrapie has been a notifiable disease since January 1993.

Scrapie, like other TSEs is generally believed to be caused by a transmissible agent (the prion), the precise molecular and biochemical nature of which remains unclear. A minority view is that the scrapie agent possesses a conventional nucleic acid genome (13). Reviews of this well known yet enigmatic disease provide details of its clinical signs, epidemiology, pathogenesis, pathology, diagnosis and control (10, 19, 20, 36, 50, 71, 87, 89).

Most cases of clinical scrapie occur in sheep 2–5 years of age (36). Rarely, cases present in sheep under 1 year of age. In some instances the commercial lifespan of the sheep may be too short to allow the clinical disease to develop (8). Animals incubating the disease and even such animals that never develop clinical signs may still be a source of infection to others. Most breeds are affected, although in some there is a clear genetic basis for resistance or low prevalence of clinical disease (see below). Scrapie has also been described in the moufflon (Ovis musimon), a primitive type of sheep (90). The infection in domestic sheep may be passed from ewe to lamb in the period from parturition to weaning. Infection can also pass to unrelated sheep or goats, especially when parturition occurs in confined areas. Fetal membranes are thought to be a source of infection. Flock recording indicates that in sheep the disease tends to be linked with certain family lines. Clinical disease only develops if the infection enters the CNS.

The clinical signs (10, 20, 21, 49, 62, 63) usually start insidiously, often with behavioural changes that are evident only from repeated inspections. While a flock is being driven, an affected sheep may lead or trail the rest of the flock, it may show abnormal reactions to the sheep dog, appearing momentarily confused or anxious and may disengage from the flock. Sheep may have a staring gaze. These subtle presenting features progress to a more definite neurological illness frequently characterised by signs of pruritus and an ataxia or incoordination of gait. Either the pruritus or the ataxia usually emerges to dominate the clinical course (62). Often, the earliest detectable change is weight loss.

Pruritus is recognised principally by compulsive rubbing or scraping against fixed objects, nibbling at the skin and scratching with a hind foot. This results in extensive loss of wool, particularly over the lateral thorax, flanks and hindquarters. The persistence of pruritus often causes excoriations with the development of localised self-inflicted skin lesions. These may occur in areas of wool loss and on the poll, face, ears and limbs. A characteristic ‘nibbling reflex’ can often be elicited by palpation of the lumbar region, and may also be evoked by the sheep’s own scraping movements.

Ataxia or incoordination of gait is first apparent as an awkwardness of turning, with difficulty in positioning the hind limbs, swaying of the hindquarters and a high stepping or trotting gait of the forelimbs. Stumbling and falling occur, but the sheep is generally able to quickly regain a standing posture. These signs progress to weakness and recumbency.

Other neurological signs may include teeth grinding (bruxism), an abnormally low carriage of the head and ears, a fine tremor, seizures, and blindness. There may also be apparent hyperaesthesia to sound, movement, or touch. Polydipsia and polyuria may be evident. In most cases, there is also a loss of bodily condition, but
significant weight loss may be apparent only in the late clinical stages. This preterminal decline may, it is considered, be associated with the observation of diminished rumination time in sheep with scrapie (3).

The variety of names that have been used in different languages to describe scrapie, for example, la tremblante (trembling), Traberkrankheit (trotting disease), or Gnuibeberkrankheit (nibbling disease) reflects the diversity of the presenting and predominant clinical signs in different sheep populations. Progress of the clinical disease is very variable, lasting for a week or up to several months, with an inevitably fatal outcome. There is also variation in the clinical signs among individual animals and in different breeds of sheep. These variations may be due to the influence of host genotype and strain of agent, variables that in rodent models of scrapie are well known to determine the phenotype of disease observed. Environmental factors may also have an influence on the disease course. The clinical diagnosis of individual cases of scrapie can therefore be difficult. The clinical signs may, especially in the early phase of the disease, resemble those of some other conditions of adult sheep. These include: ectoparasitism, pseudorabies (Aujeszky’s disease), rabies, encephalitic listeriosis, ovine progressive pneumonia (maedi-visna), pregnancy toxxaemia (ketosis), hypomagnesaemia and chemical and plant intoxications (19).

The biohazard of scrapie diagnostic testing appears to be limited, but appropriate precautions should be taken. The existence of natural scrapie in domestic sheep for over the past two and a half centuries and the failure of many investigations to show any epidemiological link between scrapie and the human spongiform encephalopathies, provide a strong indication of negligible risks to those working with the agent (11). Most specifically, Creutzfeldt-Jakob disease has been found to occur at no greater frequency in those with occupations providing closest contact with the agent than in other population groups. However, the extreme chemical and physical resistance of the scrapie agent and the fact that it is experimentally transmissible by injection to a wide spectrum of mammalian species, suggest the prudence of preventing human exposure. Because of the now established link between bovine spongiform encephalopathy (BSE) and a variant form of the human TSE, Creutzfeldt-Jakob disease (vCJD), BSE and related agents are now categorised, with respect to biohazard, with the human TSEs (1). While scrapie is excluded from this categorisation, adoption of containment measures similar to those used for the other TSE agents is recommended when dealing with tissues from natural scrapie cases in the laboratory. This is particularly relevant in those countries that have experienced cases of BSE in their endemic cattle population. As sheep may have been exposed to the same contaminated feedstuffs considered to be the source of infection in cattle, those working directly with infected tissues should therefore wear appropriate protective clothing and observe standard decontamination procedures for the spongiform encephalopathy agents (1, 74, 82). Such an approach also ensures that attempts to characterise the scrapie agent are not compromised by cross-contamination.

**B. Diagnostic Techniques**

1. Identification of the agent

The aetiological agent of scrapie is as yet incompletely characterised. It is known to be a transmissible agent, but the molecular identity remains uncertain. A modified form (PrP\textsuperscript{res}) of a host-coded, highly conserved, membrane glycoprotein (PrP\textsubscript{c}) of unknown function, is the only macromolecular constituent of the infectious agent identified. The protein only or “prion” hypothesis proposes that the causative agent is composed entirely or principally of the disease-specific isoform of PrP (prion protein) and that the altered form is capable of inducing conversion of the normal form. Other theories as to the nature of the agent suggest that a nucleic acid is associated with the prion protein, or that the prion protein is merely a by-product of infection caused by another agent.

Transmission from infected tissues, usually to laboratory rodents by injection, is the only available means of detection of infectivity and requires incubation periods of 1–2 years. Although mice have proved the most useful species for this purpose, attempts to transmit natural scrapie to mice are not always successful. It is seldom practical to use the criterion of transmissibility for diagnosis. Immunohistochemistry has been used in experimental situations to detect the abnormal form of PrP in biopsy samples of tonsil (72, 73) or third eyelid (61, 84). The extent to which such tests are readily applied for field case diagnosis varies from country to country as their effectiveness is influenced by genotype, and hence breed. There is therefore no practical definitive diagnostic test available based on isolation or detection of the agent by culture, nor are there immunological or nucleic acid recognition methods. Subject to proof of the prion hypothesis, demonstration of the pathological form of PrP would constitute identification of the agent.

Unlike BSE infection in cattle, but similar to natural sheep scrapie, experimental BSE in sheep of susceptible genotype results in disseminated disease-specific PrP accumulation in a wide range of tissues (25, 37, 47). Preliminary studies suggest that the conformation of disease-specific PrP produced in BSE-infected sheep is different from that of disease-specific PrP found in natural sheep scrapie (4, 5, 31, 44, 46, 47, 77). These conformational differences may be revealed by immunoblotting or immunohistochemistry techniques using peptide-specific antibodies to the flexible tail of the disease-specific PrP. Other approaches, generally also
making use of the different susceptibilities of PrP\textsuperscript{res} to enzymatic action, are under consideration. However, none of these techniques has been validated. 

In the absence of in-vitro methods for isolation of the causative agent (53), the diagnosis of scrapie has been reliant on the demonstration of pathological changes. Historically the only such method was demonstration of the histopathological changes in the CNS. While clinically suspect cases of scrapie should, where suitable samples are available, continue to be investigated initially by histopathological examination, diagnostic criteria should also now include the demonstration of accumulation of the abnormal isoform of PrP. This latter approach not only offers capability for confirming a clinical diagnosis post-mortem by demonstration of PrP accumulation in the CNS, but can also provide the basis of an ante-mortem diagnosis in the preclinical phase of disease by detection of abnormal PrP in lymphoid tissues, including that of the tonsil, regional lymph nodes or mictitating membrane, obtainable by biopsy (61, 72, 73, 84). However, the practicality of collection of such tissue must be considered, particularly as accumulation of disease-specific PrP in peripheral lymphoid tissues does not occur in all sheep that progress to clinical disease (2, 86).

a) **Tissue preparation**

Methods for the routine laboratory diagnosis of scrapie (74) are summarised as follows. Sheep in which clinical disease is suspected should be killed by intravenous injection of barbiturate and the brain and a portion of cervical spinal cord should be removed by standard necropsy procedures as soon after death as possible. Methods of subdividing the brain and cord tissue for application of PrP detection requiring fresh tissue and for histological techniques are dependent on the optimum sensitivities of each of the tests for different brain areas and the compromise that precisely the same area cannot be used for both techniques. Early accumulation of PrP in the CNS is very restricted neuroanatomically. The diagnostic sensitivity of any test is therefore dependent on the accuracy with which the test sample is collected, in relation to the extent of anatomical involvement. The following protocol is suggested but may be subject to modification to satisfy the particular portfolio of tests applied.

For the detection of PrP (or scrapie-associated fibrils), fresh tissue samples are taken and stored frozen prior to extraction. Care should be taken to ensure that samples for immunohistochemistry and histopathological examination are not frozen. Samples should include approximately 3 g of cervical spinal cord and caudal medulla. If the medulla at the level of the obex is selected, this will compromise histopathological and immunohistochemical examination of key target areas. Additional samples are advocated to minimise false-negative diagnoses, taking into account the possibility that there may be strain-specific targetting of other parts of the brain (9). These can be taken from the cerebral cortex (75, 77, 80) or cerebellum (9). When immunohistochemical examination for PrP is to be employed, it is advisable to select the blocks to be used from the fresh brain and to process these after primary fixation for only 3–5 days. The single medulla block at the obex may well be sufficient for this purpose and indeed, as indicated above, for the morphological diagnosis.

b) **Histological examination**

The remaining brain tissue is fixed in approximately 10 times its volume of 10% formal saline for at least 1 week and then cut transversely as required to obtain blocks for histological processing to paraffin wax. The initial sampling may be confined to a single block of the medulla (see Chapter 2.3.13. BSE, Fig. 1) or extended to additional areas of the brain stem and, as necessary, to representative blocks of all major brain regions. Sections, cut at 5 µm thickness, are stained with haematoxylin and eosin and examined for the morphological changes outlined above.

There are no specific gross pathological changes seen in scrapie. The histological lesions are confined to the CNS and most reports of the changes have described those occurring in the brain (26, 87, 91, 92). The lesions are neurodegenerative, with no specific inflammatory changes and no primary degenerative changes of white matter. The most striking change is a vacuolation of neurons. Neuronal perikarya are distended by characteristic single or multiple vacuoles. Vacuolation in neuronal processes produces the distinctive appearance of spongiform change in the neuropil of grey matter. Vacular changes are accompanied by variable and usually less conspicuous microscopic features, which include other forms of neuronal degeneration, notably the occurrence of ‘dark’, shrunken neurons. There may also be neuronal loss, gliosis (particularly an astrocytic reaction), and cerebrovascular amyloidosis. Typically, the lesions have a bilaterally symmetrical distribution. There is considerable variation in the distribution pattern of vacuolation and other changes, but lesions are usually most apparent in the brain stem. Recent evidence suggests that vacuolation patterns are influenced by agent strain and host genotypes, although other host factors independent of these two variables are also considerably influential (6, 54). It is therefore unlikely that vacuolation patterns alone will permit scrapie strain identification as has traditionally been done in mice.

Although it is principally the vacuolar changes on which the laboratory diagnosis has been based, several factors may contribute to difficulties in making the histopathological diagnosis. Vacuolation of neuronal perikarya of the type seen in scrapie is not pathognomonic because it is often present in the brains of...
c) Detection of disease-specific forms of PrP

Methods for the demonstration of accumulation of disease-specific forms of PrP can now provide independent evidence for the diagnosis (19, 74). PrPres can be detected after partial purification from detergent and protease-treated extracts of unfixed, affected brain. The modified protein is demonstrated by electrophoresis and immunoblotting (23, 78). The accumulation of disease-specific PrP in scrapie-affected brain is also demonstrated by immunohistochemistry, which can be carried out on routinely formalin-fixed material by the application of a variety of epitope demasking techniques and the use of appropriate antibodies to PrP (34, 56–58). PrP detection by immunohistochemistry has also been demonstrated in lymphoid tissue (85) and is considered to have potential for the preclinical ante-mortem diagnosis of scrapie antibodies to PrP (34, 56–58). PrP detection by immunohistochemistry has also been demonstrated in lymphoid tissue (85) and is considered to have potential for the preclinical ante-mortem diagnosis of scrapie. Applications of PrP detection to the preclinical diagnosis of scrapie are promising, but preliminary, and require a better understanding of the variability of pathogenesis before their sensitivity relative to incubation period can be assessed. In particular, certain genotypes of sheep when infected with natural scrapie or with BSE do not appear to have a peripheral phase of replication (2, 86, 87).

A diagnosis based on PrPres detection by Western blotting requires that a wide region of immunostained bands corresponding to proteins of molecular mass 27–30 kDa be present in the proteinase-K-treated scrapie sample lanes only and that control sample lanes provide appropriate comparisons. The original technique used for diagnosis of BSE, which has been referred to as ‘the OIE Western immunoblotting technique’ relied on detergent extraction of a large amount of fresh brain material (4 g) followed by ultracentrifugation to concentrate the PrP and then treatment with proteinase K enzyme to digest any normal host protein. This would leave only PrPres to be bound by a specific antibody and detection system in positive brain samples (79) (see also Chapter 2.3.13. BSE).

Rapid immunodiagnostic tests have been developed and are currently being used for BSE surveillance (68). These tests have been evaluated for BSE diagnosis by the European Union (59, 60, 69). The rapid tests rely on the optimisation of the reagents used for extraction and digestion and a specific monoclonal antibody for detection, which negates the need for lengthy ultracentrifugation steps. The tests require at least 1 g of fresh medulla taken at the obex or just caudal to the obex. Some laboratories use the OIE Western immunoblotting technique (if enough tissue is available) to confirm any weak-positive samples that are initially detected using a rapid test. The concentration of PrPres by ultracentrifugation from a larger aliquot of brain tissue can give improved sensitivity (23). Immunohistochemical methods and the rapid tests are discussed in more detail in Chapter 2.3.13. BSE.

A morphological form of the pathological PrP can also be demonstrated in unfixed brain extracts in the form of scrapie-associated fibrils (SAF) visualised by negative stain electron microscopy (27, 75, 79). The latter may be particularly useful when available brain tissue is unsuitable for histological examination due to post-mortem changes (14, 80). The immunoblotting method can also be applied to autolysed material (65). Both immunoblotting and SAF examination can also be applied in circumstances when, sometimes in error, at post-mortem, CNS material intended for fixation and histological examination has been frozen. With
modification the method for SAF detection may also be applied successfully to formalin-fixed tissue (12). Caution should be exerted over the use of SAF alone; it should only be used in conjunction with at least one other confirmatory method: either histopathology or immunochemical detection of disease-specific PrP-sized.

Detection of PrP-sized in spleen and lymph nodes by immunoblotting has also been used in the diagnosis of scrapie at post-mortem (43, 58, 64), but it is not practical under most circumstances to obtain such tissues surgically to effect an ante-mortem diagnosis. The detection of PrP-sized by immunoblotting in placenta (correlating with the presence of infectivity in the tissue) from scrapie-affected sheep, may offer a non-invasive means of ante-mortem diagnosis and disease surveillance in flocks (66).

Histopathological examination, the rapid immunoblot test and immunohistochemistry are the three tests used for the statutory diagnosis of scrapie in the United Kingdom. Results from a blind test correlation study have shown that the results for the three tests give a high degree of correlation in clinically suspect scrapie cases (17). The sensitivity and specificity of tests for scrapie diagnosis have been studied in large populations of negative sheep and within flocks with scrapie (16, 79) and for an abattoir survey (76). Publications would also suggest that in preclinical cases of scrapie, PrP formation precedes vacuolation and clinical signs, making the immuno-based tests a more sensitive option (4, 32). Confirmation by SAF, which is now largely confined to confirmation of autolysed samples, requires the identification of the characteristic fibrils on a systematic search of the specimen grid in the electron microscope (27, 75). If histopathological examination and immunohistochemistry results cannot be achieved due to the poor state of the sample, i.e. badly autolysed cases, immunoblotting and SAF detection may be the only test options available. Confirmation of scrapie from immunohistochemical preparations must depend not only on the presence of immunologically specific PrP staining, but also on the recognition of neuroanatomically and morphologically disease-specific patterns of staining (67). It should be noted that although the rapid tests and immunohistochemistry are being widely used for the statutory diagnosis of scrapie, no formal validation or test correlation data for scrapie diagnosis has been published in the public domain. A small number of ovine scrapie samples were tested and the results reported as part of the validation of rapid tests for BSE (68).

A wide range of antisera and monoclonal antibodies for PrP detection by immunochemical methods are now in use, but not all of them react satisfactorily with ovine PrP (33). Some (MAb 6H4, L42 and F89/160.1.5) are commercially available. Improved availability and characterisation of antisera, and the further standardisation and validation of the methodologies are required. Positive and negative controls are essential. However, the inclusion of both immunoblotting and immunohistochemical approaches in the tests applied for routine scrapie diagnosis by many laboratories will enable the relative performance of each to be further assessed. Failure to observe characteristic histological changes or to detect disease-specific PrP/SAF does not provide evidence of the absence of the disease, but agreement between the results of multiple diagnostic approaches provides the best assurance of accuracy. Clearly, in surveillance situations where monitoring is for the purpose of obtaining evidence of freedom from scrapie in small ruminant species, it is necessary to apply multiple diagnostic criteria and to use at least two laboratory methods on CNS tissue (histopathological and immunohistochemical, or immunoblotting) to maintain a high degree of confidence in negative results.

Unlike in BSE, it is unwise to rely solely on screening examinations of the CNS to detect all exposed animals. Several studies have shown that disease-specific PrP accumulation can be detected in tissues of the lymphoreticular system and peripheral nervous systems prior to the accumulation of disease-specific PrP in the CNS (2, 35, 45, 47, 85). When these studies are compared, the tissue site at which first detection of disease-specific PrP was identified varied, but included tonsil, mesenteric lymph node, retropharyngeal lymph node, Peyer’s patch of intestine, spleen and brain/spinal cord. However, these studies employed different sheep breeds and genotypes, routes of infection and agent strains. The genotype of sheep also appears to have an effect on the age at which first detection of disease-specific PrP may be found. The effect of a number of key factors on peripheral pathogenesis is therefore presently insufficiently understood to permit accurate selection of peripheral tissues on which tests might be applied. The methodologies to perform large-scale testing for disease-specific PrP accumulation in the periphery are also not yet developed.

Whereas large-scale testing to determine freedom from scrapie must include targeted examination of peripheral tissues of younger animals, surveillance for prevalence of disease could potentially limit tissue examination to the CNS of adult sheep. However, testing to estimate disease prevalence needs to take into account a number of factors, including the stratification of the sheep-farming industry, dose or level of infection within particular flocks, frequency of disease in different genotypes and the effect of agent strain on incubation period (38). It is likely that sheep homozygous for VRQ allele provide the most susceptible group (22, 39–42). In a heavily infected environment, these animals may have a shorter mean incubation period than those of less susceptible genotypes (22), but even sheep homozygous for the VRQ allele may escape infection, especially where infection pressures are low. In contrast, sheep homozygous for the ARR allele are generally considered to be resistant to disease, although in some breeds this allele is rare or
absent (48, 83). Whether or not resistance to clinical disease equates with resistance to infection is now being challenged and requires further investigation.

Prospects for more sensitive and specific diagnostic tests for scrapie and other TSEs are mainly directed at present on the development of new approaches to the detection of disease-specific forms of PrP (70). The evaluation of rapid tests by the European Union for the diagnosis of BSE (59, 60, 69) will now be followed by an evaluation of their performance in the detection of scrapie.

2. Serological tests

A serological immune response to the scrapie agent has not been detected.

3. Genetic tests

In addition to the identification of scrapie-infected sheep based on disease-specific PrP detection methods, scrapie control and elimination strategies based on genetic selection for resistance are also being developed. The sheep PrP gene is highly polymorphic. Common polymorphisms have been identified within the protein-coding region of the sheep PrP gene at codons 136 – alanine/valine, 154 – arginine/histidine, and 171 – arginine/glutamine/histidine (28–30, 52). The incidence of scrapie in various breeds of sheep has been associated with some of these polymorphisms (30, 42, 51). The codon 171 polymorphisms are of particular significance in determining overall risk (7, 15, 30, 88). PrP genotyping can therefore be used as an aid to the control of scrapie: breeding stock, particularly rams, of appropriate PrP genotype can be selected to produce progeny with reduced risk of developing disease (18). Such genotyping services are available on a commercial basis in North America and in several countries in Europe. The test is performed using DNA extracted from white blood cells obtained from ethylene diamine tetra-acetic acid (EDTA)-treated blood samples. Some individual farmers and breed societies are already basing selection of breeding stock on the most scrapie-resistant animals, homozygous for arginine at codon 171. These animals are not common in most flocks, and in some breeds the genotype is actually absent. A strategic approach to eliminating scrapie infection from national flocks or geographical regions by adopting a similar genetic breeding programme is possibly premature. The shortage of sheep that are homozygous for arginine at codon 171 is one factor. The lack of data on the effects of a high prevalence of such a genotype on productivity, resistance to diseases other than scrapie and viability in general is another factor. Decisions on the appropriateness of such programmes must be based on local pressures.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available.

REFERENCES


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NB: There are OIE Reference Laboratories for Scrapie (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 2.4.9.

OVINE PULMONARY ADENOMATOSIS
(adenocarcinoma)

SUMMARY

Ovine pulmonary adenomatosis (OPA), also known as ovine pulmonary adenocarcinoma and jaagsiekte, is a contagious tumour of sheep and, exceptionally, of goats. It is a progressive respiratory disease, principally affecting adult animals. The disease occurs in many regions of the world. Two viruses, a herpesvirus and a retrovirus, have been associated with the disease, but only the latter has an aetiological role. The retrovirus of OPA is distinct from the non-oncogenic ovine lentivirus, and classified as a betaretrovirus.

Identification of the agent: The betaretrovirus of OPA cannot yet be propagated in vitro, therefore routine diagnostic methods, such as virus isolation, are not available for diagnosis. Diagnosis relies, at present, on clinical history and examination, as well as on the findings at necropsy and by histopathology. Viral DNA or RNA can be detected in tumour, draining lymph nodes, and peripheral blood mononuclear cells by polymerase chain reaction.

Serological tests: Antibodies to the retrovirus have not been detected in infected sheep and, therefore, serological tests are not available for diagnosis.

Requirements for vaccines and diagnostic biologicals: There are no vaccines or diagnostic biologicals available.

A. INTRODUCTION

Ovine pulmonary adenomatosis (OPA), also known as ovine pulmonary adenocarcinoma, jaagsiekte (Afrikaans = driving sickness) and ovine pulmonary carcinoma (OPC), is a contagious lung tumour of sheep and, to a lesser extent, of goats. It is the most common pulmonary tumour of sheep and occurs in many countries around the world. It is absent from Australia and New Zealand and has been eradicated from Iceland.

A number of different viruses have been linked aetiologically to OPA, including a herpesvirus and lentiviruses that have been propagated from tumour tissue. However, the former does not have an aetiological role in OPA and the latter exhibit characteristics of non-oncogenic lentiviruses. It has been demonstrated, recently, that OPA is caused by a betaretrovirus that cannot yet be cultured in vitro, but the virus has been cloned and sequenced. The term jaagsiekte sheep retrovirus (JSRV) is used in referring to this virus.

B. DIAGNOSTIC TECHNIQUES

At present, diagnosis of OPA relies on clinical and pathological investigations. In flocks in which the disease is suspected, its presence must be, at least once, confirmed by histopathological examination of affected lung tissue. For such an examination, it is imperative to take specimens from several affected sites and, if possible, from more than one animal. This is because secondary bacterial pneumonia, which might be the immediate cause of death, often masks the lesions (both macroscopic and microscopic) of the primary disease. In the absence of specific serological tests, which can be used for the diagnosis of OPA in live animals, disease control relies on regular flock inspections and prompt culling of suspected cases and, in the case of ewes, their offspring.
**1. Identification of the agent**

Although ovine herpesvirus 1 (OvHV-1) had been isolated exclusively from OPA tumours, epidemiological studies and experimental infections provide no evidence for a role in the aetiology of OPA. Ovine herpesvirus 2 (OvHV-2) is the sheep-associated malignant catarrhal fever herpesvirus and has never been linked to OPA.

The association of retroviruses with OPA has been recognised for several years. Ovine lentiviruses have been isolated on a number of occasions, but these viruses have no aetiological role in OPA (15, 16).

For many years, the inability to culture JSRV and the lack of antibodies to the virus in affected sheep impeded the confirmation of this virus as the aetiological agent. However, molecular biological techniques provided a key advance, namely, the cloning and sequencing of the 7.5 kb JSRV genome following purification of virions from lung washes of naturally affected sheep (26). JSRV has been designated as a betaretrovirus because of its genetic organisation and its structural proteins. Although cloned JSRV genes, used as hybridisation probes, have revealed a range of homologous endogenous sequences in the genome of both healthy and OPA-affected sheep (1, 7, 26), JSRV is clearly exogenous and associated exclusively with OPA (12). JSRV is detected constantly in the lung fluid, tumour, peripheral blood mononuclear cells, and lymphoid tissues of sheep affected by OPA or unaffected in-contact flockmates, and never in sheep from unaffected flocks with no history of the tumour. Recently, a full-length proviral clone of JSRV has been obtained from OPA tumour DNA. JSRV virus particles, prepared from this clone by transient transfection of a cell line, were used for intratracheal inoculation of neonatal lambs. OPA tumour was induced in two of four lambs, thus demonstrating that JSRV is the causal agent of OPA (14).

What contribution, if any, the endogenous viral sequences make to the aetiology of OPA remains unclear, but their expression in the fetus may, by induction of tolerance, account for the apparent lack of immune response of mature animals to exogenous JSRV.

**a) Nucleic acid recognition methods**

Sequencing of JSRV and endogenous sequences in the sheep genome has led to the development of polymerase chain reaction (PCR) tests that specifically detect JSRV (1, 12). Using this sensitive procedure, JSRV has been detected in peripheral blood mononuclear cells of unaffected in-contact sheep from flocks with OPA, as well as experimentally infected lambs (5, 9). This finding holds promise that it may be possible to develop a molecular test for identifying infected animals during the preclinical stages, thus offering hope that this will provide a method to diagnose OPA.

**b) Animal inoculation**

OPA can be transmitted only with material that contains JSRV, such as tumour homogenates and, more significantly, concentrated cell-free lung fluid from natural cases of OPA. The infectious fraction of the fluid contains JSRV virions and reverse transcriptase (RT) activity. Following the experimental inoculation of adult sheep, clinical disease develops only after several months or years. Similar inoculation of newborn lambs results in a reduced incubation period of 3–6 weeks, and many of the transformed epithelial cells contain intracytoplasmic retrovirus particles (21, 25). At this time there is no practical animal inoculation method for the diagnosis of OPA.

**c) Virus isolation**

Some cell cultures prepared from the tumours occurring in young lambs can support virus replication for a short period (10, 22). Following intratracheal inoculation of concentrated supernatant fluids of such cultures into three lambs within 24 hours of birth, clear histological evidence of OPA was detected in one lamb 6 months later (19). These are preliminary studies and at this time there is no established method to isolate the virus.

**d) Clinical signs and pathology**

As there is no reliable laboratory method for the diagnosis of OPA at this time, clinical signs and post mortem lesions are the primary method for the diagnosis of the disease. As OPA has a long incubation period, clinical disease is encountered most commonly in sheep over 2 years of age, with a peak occurrence at the age of 3–4 years. In exceptional cases, the disease occurs in animals as young as 2–3 months of age. The cardinal signs are those of a progressive respiratory embarrassment, particularly after exercise; the severity of the signs reflects the extent of tumour development in the lungs. Accumulation of fluid within the respiratory tract is a prominent feature of OPA, giving rise to moist râles that are readily detected by auscultation. Raising the hindquarters and lowering the head of affected sheep may cause frothy mucoid fluid to run from the nostrils. Coughing and inappetence are not common but, once clinical signs are evident, weight loss is progressive and the disease is terminal within weeks or months. Death is often precipitated by a superimposed bacterial pneumonia, particularly that due to *Mannheimia* (formerly *Pasteurella*) *haemolytica*. In clinically affected animals, a peripheral lymphopenia characterised by a
Chapter 2.4.9. – Ovine pulmonary adenomatosis (adenocarcinoma)

reduction in CD4+ T lymphocytes and a corresponding neutrophilia may assist clinical diagnosis, but the changes are not pathognomonic and are not detected during early experimental infection (17, 23).

In some countries, another form of OPA (atypical OPA) occurs, which generally presents as an incidental finding at necropsy or the abattoir (3, 4).

e) Necropsy

OPA lesions are in most cases confined to the lungs, although intra- and extrathoracic metastasis to lymph nodes and other tissues can occur. In typical cases, affected lungs are considerably enlarged and heavier than normal due to extensive nodular and coalescing firm grey lesions affecting much of the pulmonary tissue. Usually lesions are present in both lungs, although the extent on either side does vary. Tumours are solid, grey or light purple with a shiny translucent sheen and often separated from the adjacent normal lung by a narrow emphysematous zone. The presence of frothy white fluid in the respiratory passages is a prominent feature and is obvious even in lesions as small as a few millimetres. In advanced cases, this fluid flows out of the trachea when it is cut or pendant.

Pleurisy may be evident over the surface of the tumour and in some cases abscesses are present in the adenomatous tissue.

In atypical OPA, tumours comprise solitary or aggregated hard white nodules that have a dry cut surface and show clear demarcation from surrounding tissues. The presence of excess fluid is not a prominent feature.

Adult sheep, which on post-mortem examination appear to have died from acute pasteurellosis, should have their lungs examined carefully, as lesions of OPA may be masked by coexisting bronchopneumonia, verminous pneumonia, chronic progressive pneumonia (maedi-visna) or combinations of these. Samples should be taken at necropsy for PCR for retrovirus genome examination. Storage of these samples at –80°C is recommended, prior to RNA extraction and testing.

f) Histopathology

Histologically, the lesions are characterised by proliferation of mainly type II pneumocytes, a secretory epithelial cell in the pulmonary alveoli. Nonciliated (Clara) and epithelial cells of the terminal bronchioli may be involved. The cuboidal or columnar tumour cells replace the normal thin alveolar cells and sometimes form papilliform growths that project into the alveoli. Intrabronchiolar proliferation may be present. In advanced cases, extensive fibrosis may develop and, occasionally, nodules of loose connective tissue in a mucopolysaccharide substance may be present.

A prominent feature is the accumulation of large numbers of alveolar macrophages in the alveoli adjacent to the neoplastic lesions.

Where maedi-visna is concurrent, perivascular, peribronchiolar and interstitial lymphoid infiltrates may be prominent.

The histological appearance of atypical OPA is essentially the same as classical OPA, but with an exaggerated inflammatory response (mostly lymphocytes and plasma cells) and fibrosis (3, 4).

For more detailed accounts of the clinical, post-mortem and histopathological aspects of OPA, the reader is referred elsewhere (4, 18, 20, 24).

There appears to be a synergistic interaction between OPA and maedi-visna. Lateral transmission of maedi-visna virus appears to be enhanced in sheep affected by OPA (2, 6).

2. Serological tests

At present, there are no laboratory tests to support a clinical diagnosis of OPA in the live animal. JSRV has been associated exclusively with both typical and atypical forms of OPA, but antibodies to this virus have not been detected in the sera of affected sheep, even with highly sensitive assays such as immunoblotting (11).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no vaccines or diagnostic biologicals available at the present time.
Chapter 2.4.9. – Ovine pulmonary adenomatosis (adenocarcinoma)

REFERENCES


CHAPTER 2.4.10.
NAIROBI SHEEP DISEASE

See Chapter 2.10.2. Bunyaviral diseases of animals (excluding Rift Valley fever)

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CHAPTER 2.4.11.

SALMONELLOSIS
(S. abortusovis)

See Chapter 2.10.3. Salmonellosis
SECTION 2.5.

EQUINE DISEASES IN LIST B

CHAPTER 2.5.1.

CONTAGIOUS EQUINE METRITIS

SUMMARY

Contagious equine metritis is an inflammation of the endometrium of mares caused by Taylorella equigenitalis, which usually results in temporary infertility. It is a nonsystemic infection, the effects of which are restricted to the reproductive tract of the mare.

The chief clinical signs are a slight to copious mucopurulent vaginal discharge and a variable cervicitis and vaginitis. Recovery is uneventful, but prolonged asymptomatic carriage is established in a proportion of infected mares. The infection is most frequently transmitted by sexual contact with carrier stallions, which are always asymptomatic and in which the principal sites of T. equigenitalis colonisation are the urogenital membranes (urethral fossa, urethral sinus, urethra and penile sheath). Inadequate hygiene during the cleansing or examination of the genitalia of horses can also be responsible for the transmission of infection. The sites of persistence of T. equigenitalis in the mare are urogenital membranes, principally in the clitoral sinuses and fossa and very frequently in the uterus. Foals born of carrier mares may also become carriers. The organism can infect equid species other than horses, e.g. donkeys.

Washing with disinfectants combined with local and systemic antibiotic treatment can eliminate T. equigenitalis. Vaccination has been found to be ineffective.

The principal means of control is through preventing transmission by establishing that stallions and mares are free from T. equigenitalis before breeding commences. Detection of the carrier state depends on the culture of T. equigenitalis from urogenital swabs of mares and stallions and its accurate identification. Serum antibody to T. equigenitalis can be detected in mares for 3–7 weeks after infection and can also be demonstrated in the occasional carrier mare, but never in the stallion. Serology is of value in detecting recent, but not chronic, infection in the mare, but the emphasis for control of the disease should be on the detection of carriers by culture.

Identification of the agent: Swabs must be transported to the laboratory with precautions to avoid loss of viability. Growth of T. equigenitalis is likely to take at least 72 hours and may take up to 14 days, but usually does not take longer than 6 days at 37°C on medium enriched with heated blood and an atmosphere of 5–10% CO₂. An incubation time of at least 10 days is advisable before certifying cultures negative for T. equigenitalis. After 72 hours under appropriate culture conditions colonies may be small – up to 2–3 mm in diameter – watery to opaque and yellowish grey, and are smooth with an entire edge. Taylorella equigenitalis is a Gram-negative, small, coccoid rod that is sometimes pleomorphic and exhibits bipolar staining. It produces catalase and phosphatase, and is strongly oxidase positive. It is otherwise unreactive biochemically, and identification is finally dependent on antigenic characterisation of an isolate using specific antibodies. The fastidious nature of T. equigenitalis makes it difficult to isolate and test-breeding of stallions for detection of the carrier state has been used as a valuable adjunct to cultural examination.
Recently another species of Taylorella, T. asinigenitalis, has been isolated from male donkeys in the United States of America. This newly described bacterium reportedly does not produce disease but resides in the genital tract of male donkeys and can be passed to other horses during mating.

Antibody to whole killed T. equigenitalis and a latex agglutination kit employing these antibodies may be used. Specificity for the organism and evidence of its failure to react with other oxidase positive/catalase positive, Gram-negative bacteria that might be cultured from the urogenital tract of horses is essential. Monoclonal antibodies have been developed that can be used successfully to identify T. equigenitalis and distinguish it from strains of T. asinigenitalis.

Serological tests: No serological test described to date will, by itself, reliably detect infection for diagnosis and control purposes. However, serological tests can be used as an adjunct to culture for T. equigenitalis in screening mares recently bred with a carrier stallion, but must not be used as a substitute for culture.

Requirements for vaccines and diagnostic biologicals: Effective vaccines that protect against contagious equine metritis or prevent colonisation by T. equigenitalis are not yet available.

A. INTRODUCTION

Contagious equine metritis was first described in the United Kingdom (UK) in 1977 (11), after which it was diagnosed world-wide. It was first seen as disease outbreaks characterised by a mucopurulent vaginal discharge originating from inflammation of the endometrium, and caused temporary infertility. The fastidious nature and slow growth of the causative bacterium, *Taylorella equigenitalis*, caused difficulties in initial attempts at culture (19), but the disease was reproduced by experimental clitoral challenge with isolated laboratory-grown bacteria (18, 20, 24). Using appropriate culture conditions, *T. equigenitalis* can be isolated from infective vaginal discharge. Mares may encounter more than one episode of the disease in a short period of time (29). Serum antibody persists for 3–7 weeks after infection, but often it is not detectable for up to 15–21 days after recovery from acute infection in the mares (13). Most mares recover uneventfully, but some may become carriers of *T. equigenitalis* for many months (18). Colonisation by *T. equigenitalis* is most consistently demonstrated by culture of swabs taken from the recesses of the clitoral fossa and sinuses, but it may be recovered from the cervix and endometrium in more pure culture (18). Carriage does not always affect conception (30), and in such cases pregnancy may proceed so that foals are born, become infected by passage down the birth canal, and thereby also become long-term and asymptomatic carriers (27). Many primary cases of infection with *T. equigenitalis* in the mare are subclinical, and a frequent indicator of infection is a mare returning in early oestrus after being bred to a putative carrier stallion.

Carrier mares and stallions act as reservoirs of infection, but stallions, because they mate with numerous mares, act as the principal means of transmission. The urogenital membranes of stallions become contaminated at coitus, leading to a carrier state that may persist for many months or years (22). Unhygienic examination of mares and insanitary washing of the stallion’s penis may also spread infection. Other sites of the horse body are not known to harbour *T. equigenitalis*. Most carrier females are clitoral carriers of *T. equigenitalis*. Long-term persistence of the organism in the uterus is not common. However, there are carrier mares that harbour this pathogen in the uterine area. To detect these carriers of *T. equigenitalis*, cervical or endometrial swab samples should be taken routinely in addition to sampling the clitoral area of all mares. *Taylorella equigenitalis* can cause abortion in the mare but this is a rare occurrence.

B. DIAGNOSTIC TECHNIQUES

Prior infection and vaccination are not fully protective (14), and failure of antibody to persist has meant that control of infection has relied on prevention of transmission through the detection of *T. equigenitalis* in swabs of urogenital membranes. In spite of difficulties with the culture of *T. equigenitalis*, screening mares and stallions prior to and while on the stud farm has successfully eliminated the disease from thoroughbred horses in countries using a voluntary code of practice. Many of these are based on the widely adopted UK’s Horserace Betting Levy Board’s Code of Practice, which is updated annually; the key recommendations of the Code are summarised below.

At the start of the breeding season, swabs are taken from all stallions, including those in their first season, from the urethra, urethral fossa, penile sheath and pre-ejaculatory fluid, on two occasions no fewer than 7 days apart. Mares are classified according to the degree of risk that they represent, and the intensity of sampling is adjusted accordingly. High-risk mares are defined as: (a) those from which *T. equigenitalis* was isolated in the past 7 years; (b) those found to be positive over 7 years ago but not mated since (and hence that have not recently had their fertility tested); (c) any mare mated to a stallion that transmitted infection in the previous year;
(d) barren and maiden mares arriving from outside the countries adopting the Code if mated; and (e) mares mated in the previous year to a stallion from outside of these countries. All other mares are considered to be of low risk. A clitoral swab is taken from high-risk mares at home and a negative result should be obtained before the mare is transported to the stallion stud farm. Two further clitoral swabs are taken at the stallion stud farm; one on arrival, and the second during oestrus prior to mating, at which time the endometrium is also swabbed. All swabs should be negative before mating is permitted. In low-risk mares, one clitoral swab (taken at home or at the stallion breeding farm) and one endometrial swab are taken and a result is not required before mating is allowed to proceed.

This Code of Practice applies only to thoroughbred horses and it does not exist for the warm and cold blood horse breeding associations. There is a potentially serious problem with contagious equine metritis in this group of horses.

Results of laboratory tests for *T. equigenitalis* should be entered on a certificate, which is sent to the veterinarians and stallion stud farm managers who are supervising the breeding. The certificate should record the animal’s name, the sites and date of swabbing, the name of the veterinarian taking the swab, details of the test laboratory, the date the swabs were received and cultured by the laboratory, and whether the swabs were negative or positive, or whether the culture was overgrown by other bacteria to an extent that the laboratory could not be confident that small numbers of *T. equigenitalis* would be detected and, therefore, requiring another set of swabs to be collected.

Difficulties with the culture of *T. equigenitalis* caused by its fastidious nature necessitate the use of a quality control system that should be approved before a laboratory is permitted to undertake testing for contagious equine metritis and to issue certificates of the results obtained. The task of quality control should be undertaken by an experienced, reliable, and impartial microbiology laboratory, authorised for the purpose, which is not involved in routine testing of contagious equine metritis swabs. At 6-month intervals, swabs inoculated into mixed cultures that are designed to test a laboratory’s ability to recover and identify *T. equigenitalis* in the face of contaminants, as well as procedures for reporting results, should be sent to laboratories wishing to participate. A list of those laboratories satisfactorily passing the quality control should be published in a veterinary journal that is widely read by the national veterinarians. The veterinarians and stallion stud farm managers who are supervising the breeding season should accept only certificates provided by laboratories currently approved for that purpose.

Any mares with abnormal vaginal exudate, or returning to oestrus prematurely, should be investigated and managed as though infected with *T. equigenitalis* until results of laboratory testing prove otherwise. Other causes of outbreaks of endometritis include *Pseudomonas aeruginosa*, *Streptococcus zooepidemicus* and certain capsule types of *Klebsiella pneumoniae*. Swabs should be examined for these bacteria, and an attempt should be made to culture and identify *K. pneumoniae* and *P. aeruginosa* so as to establish a differential diagnosis.

If carriers of *T. equigenitalis* are detected, the organism can be eliminated by treatment with systemic antibiotics combined with disinfectant washing of exposed genital membranes (1). Particular attention should be paid to cleansing of the recesses of the clitoral fossa and sinuses of mares, where colonisation by *T. equigenitalis* is frequently found in carrier animals. A course of treatment may take several weeks and may need to be repeated before intensive swabbing consistently fails to recover *T. equigenitalis* (12). A significant number of carrier mares can be refractive to several courses of treatment. These may require surgery and ablation of the clitoral sinuses for permanent elimination of the carrier state in such animals.

Control measures for countries regarded as free from *T. equigenitalis* infection should be based on the screening of animals prior to importation and/or during a post-importation quarantine period using swabbing and testing regimes broadly based on those described above for breeding populations.

1. **Identification of the agent (the prescribed test for international trade)**

Various bacteria may be present on the urogenital membranes of horses as harmless commensals that interfere with the culture of *T. equigenitalis*. Some may be present in small numbers, but grow in the swab before it is cultured thereby obscuring the presence of *T. equigenitalis*. Swabs must be placed in a transport medium with activated charcoal, such as Amies medium, to absorb inhibitory by-products of bacterial metabolism (25). Numbers of *T. equigenitalis* decline in swabs with time, and this effect is more pronounced at higher temperatures (21). Swabs must be kept cool during transportation and should arrive at the laboratory no later than 24–48 hours after they were taken. Negative culture results from swabs plated out more than 48 hours after they were taken are unreliable. Antibiotic treatment for whatever cause should cease at least 7 days before swabbing. The presence of antibiotics may damage *T. equigenitalis*, which nonetheless persists in the urogenital membranes but will not grow on laboratory media.

Each swab must be inoculated on to 5% (v/v) heated blood agar plates with trimethoprim, clindamycin and amphotericin B (MECA+CTA) as described by Timoney et al. (28). This is the preferred medium for isolating...
T. equigenitalis; this medium has been used successfully to isolate equally well both biotypes of this pathogen and to eliminate the growth of many commensal bacteria. As inhibitors may prevent the isolation of some strains of T. equigenitalis, swabs should also be inoculated on to plates of 5% heated blood agar with a rich peptone agar base containing additional cysteine (0.83 mM), sodium sulphite (1.59 mM) and a fungicide (5 µg/ml amphotericin B). Taylorella equigenitalis will grow on blood agar, but it can tolerate less than ideal conditions better when grown on heated blood agar (‘chocolate’ blood produced by heating at 70–80°C for 12 minutes). Some manufacturers produce a peptone agar base that is quality controlled for its ability to support the growth of T. equigenitalis. The quality of the commercial agar should be confirmed in the using laboratory. An important feature of all good T. equigenitalis media is the absence of fermentable carbohydrates. These do not enhance the growth of T. equigenitalis, but their fermentation by other bacteria inhibits T. equigenitalis growth (4, 14). A third media containing streptomycin sulphate (200 µg/ml) is sometimes used as some isolates of T. equigenitalis are resistant to this concentration of antibiotic, which serves to reduce the extent of growth of other bacteria that might otherwise obscure the presence of small numbers of T. equigenitalis (25). However, a streptomycin-resistant biotype is now the most common isolate and will not be detected on this media; consequently, it should only be used in conjunction with medium without streptomycin. Growth by other bacteria, for example Proteus mirabilis, however, may be so extensive that the laboratory should record that they cannot issue a negative result. In this event, further swabs should be requested in the hope that the problem will not recur.

All culture media prepared should be subjected to quality control and must support growth of the suspect organism from a small inoculum. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are working correctly.

The fastidious nature of T. equigenitalis makes it difficult to isolate. Test breeding of stallions for detection of the carrier state has been used to increase the sensitivity and serves as a valuable adjunct to cultural examination. The numbers of Taylorella mechanically carried by stallions that do not have overt signs, is very low and can be missed by culturing swabs, but can be detected after multiplication in the mare that has been test breed. The use of test breeding as a diagnostic tool can be especially important in countries that are considered free from contagious equine metritis.

Occasionally, the urogenital membranes will be persistently colonised by another bacterium that interferes with diagnosis, and it will prove necessary to attempt to eliminate this by washing and antibiotic treatment. Swabbing for T. equigenitalis should not recommence until at least 7 days after treatment has stopped. A medium containing trimethoprim (1 µg/ml), clindamycin (5 µg/ml), and amphotericin B (5 µg/ml), to which all T. equigenitalis are resistant, has been developed (25) and assessed (4), and is claimed to overcome this difficulty in most cases. This medium is usefully included routinely in the initial culturing. Additional plates should be inoculated with a culture of T. equigenitalis to check that each batch of medium will support growth.

Plates must be incubated at 35–37°C in 5–10% (v/v) CO₂ in air or by use of a candle jar. At least 72 hours is normally required before colonies of T. equigenitalis become visible, after which time daily inspection is needed. Visual detection of colonies may take up to 14 days (31). A standard incubation time of at least 10 days is advisable before certifying cultures negative for T. equigenitalis. Plates should be examined for contaminants after the first 24 hours’ incubation. Colonies of T. equigenitalis may be up to 2–3 mm in diameter, smooth with an entire edge, glossy and yellowish grey. Laboratories should be aware that certain countries require the prolonged incubation period as a standard procedure and should therefore ascertain the particular import requirements and/or indicate the incubation period on which their judgement is made.

Taylorella equigenitalis is a Gram-negative, nonmotile, bacillus or coccobacillus that is often pleomorphic (up to 6 µm long) and may exhibit bipolar staining. It is catalase positive, phosphatase positive, and strongly oxidase positive (see ref. 5 for methods for examining catalase, phosphatase and oxidase activities). It is otherwise inert in tests for biochemical activity. If a slow-growing organism is isolated that fits the description for cellular morphology and that is strongly oxidase positive, it should be tested for reactivity with T.-equigenitalis-specific antiserum.

A variety of serotyping tests have been developed ranging in complexity from slide agglutination to direct or indirect immunofluorescence. Each method has its advantages and disadvantages. The disadvantage of the slide agglutination test is that occasionally autoagglutination of isolates occurs: culturing in bottled CO₂ in air, as opposed to in a candle jar, may reduce autoagglutination (26). It has been suggested that immunofluorescence can be used to identify autoagglutinating isolates, some workers have reported cross-reaction with Pasteurella haemolytica but this is very rare. If a cross-reaction is suspected, it may be necessary to repeat the test using adsorbed antisera (26). The immunofluorescence test can be improved by the use of monoclonal antibodies, which are now available.

1 For example, Mast Diagnostics, Mast House, Derby Road, Bootle, Merseyside L20 1EA, United Kingdom (UK), and Lab M, Tomley House, Wash Lane, Bury BL9 6AU, UK.
Antiserum is produced by vaccinating rabbits with killed *T. equigenitalis*. A number of different immunisation regimes can be employed, ranging from those used for producing *Escherichia coli*-typing antisera (23), to immunisation together with an adjuvant, such as Freund’s incomplete. Monoclonal antibodies are available commercially that provide a highly specific means of identifying *T. equigenitalis*. A standard strain, such as NCTC 11184\(^2\), should be used for immunisation. However, the most important consideration is the specificity of the antiserum produced. It should agglutinate *T. equigenitalis*, but fail to agglutinate other bacteria that might be cultured from horse urogenital membranes, even if rarely. In particular, it should not agglutinate any oxidase-positive and Gram-negative rods, such as *Pasteurella haemolytica*, *Actinobacillus equuli*, *Bordetella bronchiseptica* (to which *T. equigenitalis* is closely related, see ref. 8), and *Pseudomonas aeruginosa*. Recently another species of *Taylorella*, *T. asinigenitalis*, has been isolated from male donkeys in the United States of America (15). This newly described bacterium reportedly does not produce disease but resides in the genital tract of male donkeys and can be passed to other horses during mating. Moreover it has similar, though not identical, colonial appearance and cultural characteristics and gives identical biochemical test results to those used to confirm the identity of *T. equigenitalis*. There is even serological cross-reactivity between the two organisms. Laboratories should be aware of the existence of this newly described *Taylorella* and its significance. Differentiation of *T. asinigenitalis* from *T. equigenitalis* is possible using specific monoclonal antibodies.

A latex agglutination kit for the antigenic identification of *T. equigenitalis* can be purchased\(^3\) that is based on polyclonal antibodies produced using methods similar to those described above. This is widely used by routine testing laboratories for the confirmation of the identity of colonies growing on selective medium that give a biochemical reaction consistent with *T. equigenitalis*. As *T. equigenitalis* is antigenically relatively distinct, and small amounts of cross-reactive antibody are easily absorbed during production of the reagent, the test has proved to be highly specific and sensitive. It should be emphasized that it will not necessarily distinguish strains of *T. equigenitalis* from *T. asinigenitalis*.

A polymerase chain reaction (PCR) method has been used for detecting *T. equigenitalis* and was compared with culture methods in the Netherlands and Japan (2, 6, 9). In these studies, a much higher rate of detection by PCR was found than by culture, even among horses imported from a source without previous evidence of *T. equigenitalis* infection or clinical disease. The authors proposed that carriage is more widespread than previously believed, and that recently discovered genetic variation among strains (7, 16) may relate to differences in pathogenicity. The PCR has also been used in the UK (10). It was highly specific and was able to detect very small numbers of *T. equigenitalis* in the presence of very large numbers of bacteria arising from the background flora harvested from culture plates inoculated with samples of the equine urogenital tract. Recently in Japan, the field application of the PCR in the eradication of contagious equine metritis was evaluated. It was demonstrated that the PCR was more sensitive than culture for the detection of *T. equigenitalis* from genital swabs of horses in the field (2, 3, 17). This promising technique needs to be more fully and widely evaluated.

2. Serological tests

No serological test described to date will, by itself, reliably detect infection for diagnosis and control. However, the complement fixation test has been used successfully as an adjunct to culture for *T. equigenitalis* in screening mares recently bred with a carrier stallion.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Effective vaccines that protect against contagious equine metritis or prevent colonisation by *T. equigenitalis* are not yet available.

REFERENCES


\(^{2}\) Obtainable from the National Collection of Type Cultures, Colindale, London, UK.

\(^{3}\) Mono Tayl, Obtainable from Bionor, Stromdaljordet 4, Postboks 1868, Gulset, N-3701 Skein, Norway.


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**NB:** There are OIE Reference Laboratories for Contagious equine metritis (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 2.5.2.

DOURINE

SUMMARY

Dourine is a chronic or acute contagious disease of breeding solipeds that is transmitted directly from animal to animal during coitus. The causal organism is Trypanosoma equiperdum (Doflein, 1901).

Dourine is the only trypanosomosis that is not transmitted by an invertebrate vector. Trypanosoma equiperdum differs from other trypanosomes in that it is primarily a tissue parasite that rarely invades the blood. There is no known natural reservoir of the parasite other than infected equids. It is present in the genital secretions of both infected males and females. The incubation period, severity, and duration of the disease vary considerably; it is often fatal, but spontaneous recoveries do occur. Subclinical infections occur, and donkeys and mules are more resistant than horses and may remain inapparent carriers. Infection is not always transmitted by an infected animal at every copulation. Rats can be infected experimentally, and can be used to maintain strains of the parasite indefinitely. Trypanosoma equiperdum strains are best stored in liquid nitrogen.

The clinical signs are marked by periodic exacerbation and relapse, ending in death or, possibly, recovery. Fever, local oedema of the genitalia and mammary glands, cutaneous eruptions, incoordination, facial paralysis, ocular lesions, anaemia, and emaciation may all be observed. Oedematous cutaneous plaques, 5–8 cm in diameter and 1 cm thick, are pathognomonic.

Identification of the agent: Definitive diagnosis depends on the recognition of clinical signs and identification of the parasite. As this is rarely possible, diagnosis is usually based on clinical signs, together with serological evidence from complement fixation (CF) tests.

Serological tests: Humoral antibodies are present in infected animals whether or not they display clinical signs. The CF test is used to confirm infection in clinical cases or in latent carriers. Noninfected animals, especially donkeys, often yield unclear results. The indirect fluorescent antibody test can be used to confirm infection or resolve inconclusive CF test results. Enzyme-linked immunosorbent assays are also used.

Requirements for vaccines and diagnostic biologicals: There are no vaccines available. The only effective control is through the slaughter of infected animals. Good hygiene is essential during assisted matings because infection may be transmitted through contaminated fomites.

A. INTRODUCTION

Dourine is a chronic or acute contagious disease of breeding solipeds that is transmitted directly from animal to animal during coitus. The causal organism is Trypanosoma equiperdum (Doflein, 1901). Dourine is also known under other names: mal de coït, el dourin, morbo coitale maligno, Beschäleuche, slapsiekte, sluchnaya bolyezn, and covering disease (1, 7).

Although the disease has been known since ancient times, its nature was established only in 1896 when Rouget discovered trypanosomes in infected Algerian horses. Dourine is the only trypanosomosis that is not transmitted by an invertebrate vector. Trypanosoma equiperdum differs from other trypanosomes in that it is primarily a tissue parasite that is rarely detected in the blood. There is no known natural reservoir of the parasite other than infected equids.

Infection is transmitted during copulation, more commonly from stallion to mare, but also from mare to stallion, due to the presence of the parasite in the seminal fluid and mucous exudate of the penis and sheath of the infected male, and in the vaginal mucus of the infected female. Initially, parasites are found free on the surface of
the mucosa or between the epithelial cells of a newly infected animal. Invasion of the tissues takes place, and oedematous patches appear in the genital tract. Parasites then may pass into the blood, where they are carried to other parts of the body. In typical cases, this metastatic invasion gives rise to characteristic cutaneous plaques.

The incubation period, severity and duration of the disease vary considerably. In South Africa, the disease is typically chronic, usually mild, and may persist for several years (5). In other areas, such as northern Africa and South America, the disease tends to be more acute, often lasting only 1–2 months or, exceptionally, 1 week. Although dourine is a fatal disease with an average mortality of 50% (especially in stallions), spontaneous recovery can occur. Subclinical infections are recognised. Donkeys and mules are more resistant than horses.

As trypanosomes are not continually present in the genital tract throughout the course of the disease, transmission of the infection does not necessarily take place at every copulation involving an infected animal. Transmission of infection from mare to foal can occur via the mucosa, such as the conjunctiva. Mares' milk has been shown to be infectious. Animals other than equids can be infected experimentally. Rat-adapted strains can be maintained indefinitely; infected rat blood can be satisfactorily cryopreserved. Antigens for serological tests are commonly produced from infected laboratory rats.

The disease is marked by stages of exacerbation, tolerance or relapse, which vary in duration and which may occur once or several times before death or recovery. The signs most frequently noted are: pyrexia, tumefaction and local oedema of the genitalia and mammary glands, oedematous cutaneous eruptions, knuckling of the joints, incoordination, facial paralysis, ocular lesions, anaemia, and emaciation. A pathognomonic sign is the oedematous plaque consisting of an elevated lesion in the skin, up to 5–8 cm in diameter and 1 cm thick. The plaques usually appear over the ribs, although they may occur anywhere on the body, and usually persist for between 3 and 7 days. They are not a constant feature.

Generally, the oedema disappears and returns at irregular intervals. During each recess, an increasing extent of permanently thickened and indurated tissue can be seen. The vaginal mucosa may show raised and thickened semitransparent patches. Folds of swollen membrane may protrude through the vulva. It is not uncommon to find oedema of the mammary glands and adjacent tissues. Depigmentation of the genital area, perineum, and udder may occur. In the stallion, the first clinical sign is a variable swelling involving the glans penis and prepuce. The oedema extends posteriorly to the scrotum, inguinal lymph nodes, and perineum, with an anterior extension along the inferior abdomen. In stallions of heavy breeds, the oedema may extend over the whole floor of the abdomen.

Pyrexia is intermittent; nervous signs include incoordination, mainly of the hind limbs, lips, nostrils, ears, and throat. Facial paralysis is usually unilateral. In fatal cases, the disease is usually slow and progressive, with increasing anaemia and emaciation, although the appetite remains good almost throughout.

At post-mortem examination, gelatinous exudates are present under the skin. In the stallion, the scrotum, sheath, and testicular tunica are thickened and infiltrated. In some cases the testes are embedded in a tough mass of sclerotic tissue and may be unrecognisable. In the mare, the vulva, vaginal mucosa, uterus, bladder, and mammary glands may be thickened with gelatinous infiltration. The lymph nodes, particularly in the abdominal cavity, are hypertrophied, softened and, in some cases, haemorrhagic. The spinal cord of animals with paraplegia is often soft, pulpy and discoloured, particularly in the lumbar and sacral regions.

## B. DIAGNOSTIC TECHNIQUES

### 1. Identification of the agent

A definitive diagnosis depends on the recognition of the clinical signs and the demonstration of the parasite. This is rarely possible because: (a) although the clinical signs and gross lesions in the developed disease may be pathognomonic, they cannot always be identified with certainty, especially in the early stages or in latent cases; they can be confused with other conditions, such as coital exanthema (moreover, in some countries [e.g. in South America], *T. evansi* infections give rise to similar clinical signs); (b) the trypanosomes are only sparsely present and are extremely difficult to find, even in oedematous areas; and (c) the trypanosomes are only fleetingly present in the blood, and in small numbers that defy detection. For unknown reasons, no parasite strain of *T. equiperdum* has been isolated in any country of the world since 1982 and most of the strains currently available in national veterinary diagnostic laboratories are related to *T. evansi* (3).

In practice, diagnosis is based on clinical evidence supported by serology. Recently, other approaches have been studied and reported on (3).
In infected animals, trypanosomes are present, in low numbers only, in lymph and oedematous fluids of the external genitalia, in the vaginal mucus, and fluid contents of plaques. They are usually undetectable in the blood, but may be found in the urethral or vaginal mucus collected from preputial or vaginal washings or scrapings 4–5 days after infection. Later, parasites may be found in the fluid contents of oedemas and plaques, especially shortly after their eruption. The skin of the area over the plaque should be washed, shaved and dried, and the fluid contents aspirated by syringe. Blood vessels should be avoided. The fresh aspirate is examined microscopically for motile trypanosomes. These are present for a few days only, so that lesions should be examined at intervals. The parasite is rarely found in thick blood films, but is sometimes detectable after centrifuging blood and examining the recentrifuged plasma.

As dourine is the only trypanosome to affect horses in temperate climates, the observation of trypanosomes in thick blood films is sufficient for a positive diagnosis. In countries where nagana or surra occur, it is difficult to distinguish *T. equiperdum* microscopically (morphology, motility) from other members of the subgenus Trypanozoon (*T. evansi*, *T. brucei*). In particular, *T. equiperdum* and *T. evansi* cannot be differentiated on the basis of morphological criteria. Both are monomorphic, slender tryomastigotes with a free flagellum, although pleomorphic, stumpy, proteonuclear forms are recognised.

Typical strains of the parasite range in length from 15.6 to 31.3 μm.

2. Serological tests

Humoral antibodies are present in infected animals, whether they display clinical signs or not. The complement fixation (CF) test (11) is used to confirm clinical evidence and to detect latent infections. Uninfected equids, particularly donkeys and mules, often give inconsistent or nonspecific reactions because of the anticomplementary effects of their sera. In the case of anticomplementary sera, the indirect fluorescent antibody (IFA) test is of advantage. There is no internationally adopted protocol. Cross-reactions are possible due to the presence in some countries of other trypanosomes, for example, *T. cruzi* and *T. evansi*. Enzyme-linked immunosorbent assays (ELISAs) are also used. *Trypanosoma equiperdum* is closely related to other Old World trypanosomes, including *T. brucei* and *T. evansi*. Members of this genus all share conserved cytoskeletal elements that provoke a strong and cross-reactive serological response. All diagnostic antigens and antisera (monoclonal and polyclonal) currently available for use in serodiagnostic testing contain these conserved elements or antibodies to them, and therefore none of the serological procedures described below is specific for dourine. The diagnosis of dourine must include history, clinical, and pathological findings as well as serology. Significant improvements in dourine serodiagnosis will require development of more trypanosome-specific subunit antigens and antibodies to them.

a) Complement fixation test (the prescribed test for international trade)

Standard or microplate techniques may be used (6). Guinea-pig serum (available commercially) is used as a source of complement. Other reagents are sheep red blood cells (RBCs) washed in veronal buffer, and rabbit haemolytic serum (i.e. rabbit anti-sheep RBC) (commercial).

- **Antigen production**
  i) A rat is inoculated with *T. equiperdum* cryopreserved stock. The rat must be free from *T. lewisi*, which could be achieved by injection with neoarsphenamine, but is better accomplished by using specific-pathogen free rats. Adult rats receive 0.5–1.0 of rapidly thawed frozen stablate, intraperitoneally. At maximum parasitaemia, blood is collected into an anticoagulant, such as heparin, which will serve as a stock culture for the inoculation of additional rats.
  ii) Twenty large rats are inoculated intraperitoneally with 0.3 ml of this stock culture. All rats are to have a heavy infection concurrently. Rats usually die within 3–5 days; prior to this, blood is taken from the tail for thick smears and examined microscopically. When parasitaemia is maximal, the rats are bled into citrated saline. If parasitaemia is not synchronous, rats can be bled and their blood held in acid–citrate–dextrose saline at 4°C until they have all been bled.
  iii) The blood is filtered through muslin gauze and centrifuged at 800 g for 4 minutes. The RBCs are mostly deposited while the trypanosomes remain in suspension.
  iv) The supernatant fluid is transferred to a fresh tube, the upper layer of RBCs is mixed with trypanosomes to a second tube, and the next layer to a third. Citrate saline is added to tubes 2 and 3 to prevent clotting of cells. All tubes are mixed and centrifuged at 1500 g for 5 minutes.
  v) The supernatant fluid is discarded and the upper white layer of trypanosomes is transferred from all tubes into a clean tube. The next pink layer is transferred into a second tube, and the lower layer to a third tube.
  vi) Physiological saline is added and mixed and the tubes are centrifuged again at 1500 g for 5 minutes to separate the trypanosomes. The washing step is repeated until all the trypanosomes are collected.
as a pure white mass. Ten rats should produce 3–5 g of antigen. This purification procedure can also be carried out using a column of DEAE (diethylaminoethyl) cellulose in a solution of phosphate buffered saline (PBS) containing glucose, pH 8.0 (10).

vii) The concentrated trypanosomes are diluted with two volumes of veronal buffer and 5% polyvinylpyrrolidone as a cryopreservative. Before use in CF tests, the antigen must be dispersed to a fine suspension with a hand-held or motorised ground glass homogeniser chilled in ice (13). This antigen may be divided into aliquots, frozen and lyophilised.

The antigen is standardised by titration against a 1/5 dilution of a standard low-titre antiserum.

Sera: Positive and negative sera should be inactivated at 58°C for 30 minutes before being used in the tests. Mule and donkey sera are normally inactivated at 62°C for 30 minutes. Dilutions of sera that are positive in the screening test are titrated against two units of antigen. Test sera are screened at a dilution of 1/5. Sera showing more than 50% complement fixation at this dilution are usually deemed to be positive.

Anticomplementary sera: If the anticomplementary control shows only a trace, this may be ignored. For all other anticomplementary sera, the activity must be titrated. A duplicate series of dilutions is made and the sample is retested using *T. equiperdum* antigen in the first row and veronal buffer only in the second. The second row gives the titre of the anticomplementary reaction. Provided the first row shows an end-point that is at least three dilutions greater than the second, the anticomplementary effect may be ignored and the sample rated as positive. If the results are any closer, a fresh sample of serum must be requested. Dilution of the serum 1/2 and heat inactivation at 60–63°C for 30 minutes may result in reduction or removal of the anticomplementary effect.

Buffers and reagents: 0.15 M veronal buffered saline, pH 7.4, is used for diluting reagents and for washing sheep RBCs. Antigen is pretested by checkerboard titration, and two units are used in the test. Guinea-pig complement (C) is tested for its haemolytic activity, and diluted to provide two units for the test. Sheep RBCs in Alsever’s solution are washed three times. A 3% solution is used for the haemolytic system. Titrated rabbit-anti-sheep RBCs – the rabbit haemolytic serum – is taken at double the concentration of its haemolytic titre (two units). All test sera, including positive and negative control sera, are inactivated at a 1/5 dilution before testing.

- Primary dilutions
  i) 100 µl of test serum is diluted with 400 µl of veronal buffer (1/5).
  ii) 100 µl of both positive and negative control sera is diluted with 400 µl of veronal buffer (1/5).
  iii) The solutions are incubated in a water bath at 58°C for 30 minutes to inactivate complement and destroy anticomplementary factors.

- Screening test procedure
  i) 25 µl of inactivated test serum is placed in each of three wells.
  ii) 25 µl of inactivated control serum is placed in each of three wells.
  iii) 25 µl of *T. equiperdum* antigen diluted to contain two units is placed in the first well only for each serum.
  iv) 25 µl of complement diluted to contain two units is added to the first two wells only for each serum.
  v) 25 µl veronal buffer, pH 7.4, is added to the second well for each serum (anticomplementary well).
  vi) 50 µl veronal buffer, pH 7.4, is added to the third well for each serum (lysis activity well).
  vii) The complement control is prepared.
  viii) The plate is shaken on a microshaker sufficiently to mix the reagents.
  ix) The plate is incubated for 1 hour in a water bath at 37°C.
  x) The haemolytic system is prepared. After the first 50 minutes of incubation, the sheep RBCs are sensitised by mixing equal volumes of rabbit haemolytic serum, diluted to contain two units per 50 µl, and a 3% suspension of washed RBCs; the solution is mixed well and incubated for 10 minutes at 37°C.
  xi) After incubation, 50 µl of haemolytic system is added to each well.
  xii) The plate is shaken on a microshaker sufficiently to mix the reagents.
  xiii) The plate is incubated for 30 minutes at 37°C.
xiv) Reading the results: the plate is viewed from above with a light source beneath it. The fixation in every well is assessed by estimating the proportion of cells not lysed. The degree of fixation is expressed as 0, $1^+$, $2^+$, $3^+$, $4^+$ (0%, 25%, 50%, 75% or 100% cells not lysed). Reactions are interpreted as follows: $4^+$, $3^+$, $2^+$ = positive, $1^+$ = suspicious, trace = negative, complete haemolysis = negative.

xv) End-point titration: All sera with positive reactions at 1/5 are serially double diluted and tested according to the above procedure for end-point titration.

b) Indirect fluorescent antibody test

An IFA test for dourine can also be used (11) as a confirmatory test or to resolve inconclusive results obtained by the CF test. The test is performed as follows:

**Antigen:** (For method, see preparation of CF test antigen in Section B.2.a.) Blood is collected into heparinised vacutainers or into a solution of acid–citrate–dextrose from an animal in which the number of trypanosomes is still increasing (about ten parasites per 10$^3$ microscope field should be present).

i) The blood is centrifuged for 10 minutes at 800 g.

ii) One to two volumes of PBS is added to the packed RBCs, the mixture is agitated, and smears are made that evenly cover the whole slide.

iii) The smears are air-dried and then wrapped in bundles of four, with paper separating each slide. The bundles of slides are wrapped in aluminium foil, sealed in an airtight container over silica gel, and stored at $-20^\circ$C or $-76^\circ$C.

iv) Slides stored at $-20^\circ$C should retain their activity for about 1 year, at $-76^\circ$C they should remain useable for longer.

**Acid–citrate–dextrose solution:** Use 15 ml per 100 ml of blood.

**Conjugate:** Fluorescein-labelled sheep anti-horse immunoglobulins.

- **Test procedure**
  i) The antigen slides are allowed to reach room temperature in a desiccator.
  ii) The slides are marked out.
  iii) Separate spots of test sera diluted in PBS are applied, and the slides are incubated in a humid chamber in a water bath at 37°C for 30 minutes.
  iv) The slides are washed in PBS, pH 7.2, three times for 5 minutes each, and air-dried.
  v) Fluorescein-labelled conjugate is added at the correct dilution. Individual batches of antigen and conjugate should be titrated against each other using control sera to optimise the conjugate dilution. The slides are incubated in a humid chamber in a water bath at 37°C for 30 minutes.
  vi) The slides are washed in PBS, three times for 5 minutes each, and air-dried.
  vii) The slides are mounted in immerson oil (commercially available).
  viii) The slides are then examined under UV illumination. Incident light illumination is used with barrier filter K 530 and exciter filter BG 12. Slides may be stored at 4°C for 4–5 days. Sera diluted at 1/80 and above showing strong fluorescence of the parasites are usually considered to be positive. Estimating the intensity of fluorescence demands experience on the part of the observer.

Standard positive and negative control sera should be included in each batch of tests, and due consideration should be given to the pattern of fluorescence in these controls when assessing the results of test sera.

c) Enzyme-linked immunosorbent assay

The ELISA has been developed and compared with other serological tests for dourine (12, 14).

**Carbonate buffer, pH 9.6, for antigen coating on to microtitre plates:** Na$_2$CO$_3$ (1.59 g); NaHCO$_3$ (2.93g); and distilled water (1 litre).

**Blocking buffer:** Carbonate buffer + 3% fetal calf serum (FCS).
PBS, pH 7.4, with Tween 20 (PBST) for washing: KH$_2$PO$_4$ (0.2 g); Na$_2$HPO$_4$ $\times$ 12 H$_2$O (2.94 g); NaCl (8.0 g); KCl (0.2 g in 1 litre distilled water), and Tween 20 (0.5 ml).

**Sample and conjugate buffer:** PBST + 6% FCS.

**Citric phosphate buffer:** Citric acid monohydrate (4.2 g in 200 ml distilled water); Na$_2$HPO$_4$ $\times$ 12 H$_2$O (in 200 ml distilled water). Both components are mixed at equal volumes.

**Substrate indicator system:** ABTS (2,2’-Azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) (40 mg) is dissolved in citric phosphate buffer (100 ml), and stored at 4°C in the dark. Just before use, 100 µl of 1/40 H$_2$O$_2$ is added to 10 ml of ABTS.

**Conjugate:** Rabbit anti-horse IgG (H+L) PO or IgY anti-horse Ig-PO.

**Antigen:** Lyophilised *T. equiperdum* antigen (0.5 ml) is reconstituted with coating buffer (5 ml), sonicated twice for 10 seconds each at 12 µm peak to peak, and centrifuged at 10,000 g for 4 minutes. The supernatant is further diluted to a pretested working dilution (e.g. 1/500).

- **Test procedure**
  i) Wells in columns 2, 4, 6, etc., are charged with 50 µl of antigen, columns 1, 3, 5, etc., are charged with the same amount of carbonate buffer. The plate is incubated for 40 minutes at 37°C in a humid chamber, washed in tap water, and 50 µl of blocking buffer is added to each well. The plate is incubated for 20 minutes, washed in tap water followed by three wash cycles with PBST, with soaking times of 3 minutes/cycle.
  ii) 50 µl of test samples and equine control sera prediluted 1/100 in sample/conjugate buffer is added in parallel to wells with and without antigen. The plate is incubated for 30 minutes, washed in tap water, followed by three wash cycles with PBST.
  iii) Properly diluted conjugate in sample/conjugate buffer is added in volumes of 50 µl to all wells. The plate is incubated for 30 minutes with subsequent washing as above.
  iv) 100 µl of substrate indicator system is added to all wells.
  v) The reaction is stopped after 15 minutes at room temperature by the addition of 25 µl of 37 mM NaCN. Alternatively, commercially available detergents can be used after pretesting. The results are read photometrically at a wavelength of 405 nm.
  vi) **Calculation of results:** absorbance (with antigen) minus absorbance (without antigen) = net extinction. A reaction exceeding a net extinction of 0.3 is regarded as a positive result.

A competitive ELISA has also been described for detecting antibody against *Trypanosoma equiperdum* (9).

**d) Other serological tests**

Other serological tests have been used, including radioimmunoassay, counter immunoelectrophoresis and agar gel immunodiffusion (AGID) tests (2, 4). The AGID has been used to confirm positive tests and to test anticomplementary sera. A seven-well pattern in 0.8% agarose in Tris buffer is used, with the CF test antigen in the centre well and positive control sera and unknown sera in alternate peripheral wells. A method has been published for diagnosing equine piroplasmosis, glanders and dourine at the same time, using immunoblotting (8).

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

There are no biological products available. Control of the disease depends on compulsory notification and slaughter of infected animals. Good hygiene at assisted matings is also essential.

**REFERENCES**


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** NB: There is an OIE Reference Laboratory for Dourine (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 2.5.3.

EQUINE ENCEPHALOMYELITIS
(Eastern and Western)

SUMMARY

Eastern and Western equine encephalomyelitis viruses belong to the genus Alphavirus of the family Togaviridae. These viruses cycle between birds and mosquitoes. The disease occurs sporadically in horses and humans from mid-summer to late autumn. Horses and humans are tangential dead-end hosts. The disease in horses is characterised by fever, anorexia, and severe depression. Eastern equine encephalomyelitis (EEE) virus infection in horses is often fatal, while Western equine encephalomyelitis (WEE) virus can cause a subclinical or mild disease with less than 30% mortality. EEE and WEE have been reported to cause disease in poultry, game birds and ratites.

EEE and WEE viruses cause disease in humans; severe infections and death in laboratory workers have been reported. Work with these viruses should be performed only by immunised personnel using certified biosafety cabinets following containment level 3 procedures (see Chapter I.1.6. Human safety in the veterinary microbiological laboratory).

Identification of the agent: A presumptive diagnosis of EEE or WEE can be made when susceptible horses display the characteristic somnolence and other signs of neurological disease in areas where haematophagous insects are active. There are no characteristic gross lesions. Histopathological lesions can provide a presumptive diagnosis. EEE virus can usually be isolated from the brain and sometimes other tissues of dead horses, however WEE virus is rarely isolated. EEE and WEE viruses can be isolated from field specimens by inoculating newborn mice, embryonating chicken eggs, cell cultures, or newly hatched chickens. The virus is identified by complement fixation (CF), immunofluorescence, or plaque reduction neutralisation (PRN) tests. EEE and WEE viral RNA may also be detected by reverse-transcription polymerase chain reaction methods.

Serological tests: Antibody can be identified by PRN, haemagglutination inhibition, CF tests, or IgM capture enzyme-linked immunosorbent assay.

Requirements for vaccines and diagnostic biologicals: EEE and WEE vaccines are safe and immunogenic. They are produced in cell culture and inactivated with formalin.

A. INTRODUCTION

Eastern equine encephalomyelitis (EEE) and Western equine encephalomyelitis (WEE) viruses are members of the genus Alphavirus of the family Togaviridae. The viruses cycle between birds and mosquitoes; from mid-summer to late autumn, clinical disease may be observed in humans and horses, both of which are dead-end hosts for these agents. EEE is usually a fatal disease of horses and has been diagnosed in Quebec and Ontario in Canada, Texas and the states east of the Mississippi River in the United States of America (USA), the Caribbean Islands, Mexico, and Central and South America. WEE can be subclinical, and the mortality in horses with clinical disease is less than 30%. Disease caused by the WEE virus has been reported in the western USA and Canada, Mexico, and Central and South America (8, 10, 16). Highlands J virus, antigenically related to WEE virus, has been isolated in eastern USA. Although it is generally believed not to cause disease in mammals, it has been isolated from the brain of a horse dying of encephalitis in Florida (4).

Even though the mortality is lower for WEE, the clinical signs of EEE and WEE can be identical. Following an incubation period of 5–14 days, clinical signs include fever, anorexia, and depression. In severe cases, the disease in horses progresses to hyperexcitability, blindness, ataxia, severe mental depression, recumbency,
convulsions, and death. A presumptive diagnosis of EEE or WEE virus infection in unvaccinated horses can be made if the characteristic somnolence is observed during the summer in temperate climates or the wet season in tropical and subtropical climates, when the mosquito vector is plentiful. However, a number of other diseases, such as West Nile virus, produce similar clinical signs and the diagnosis must be confirmed by the described diagnostic test methods. WEE virus infection in horses is often observed over a wide geographical area, e.g. sporadic cases over 1000 square miles. EEE virus infections are usually observed in limited geographical areas. WEE and EEE virus infections have been reported to cause high mortality in captive-raised game birds, primarily pheasants, chukars and quail. Most encephalomyelitis infections in domestic fowl are caused by EEE virus and occur on the east coast states of the USA. The virus is introduced by mosquitoes, but transmission within the flocks is primarily by feather picking and cannibalism. Both EEE and WEE viruses have caused a fatal disease in raptors. Haemorrhagic enteritis has been observed in emus infected with EEE and WEE viruses, and morbidity and mortality rates may be greater than 85%. Recently, Highlands J and EEE viruses have been found to produce depression, somnolence, decreased egg production, and increased mortality in turkeys (3). EEE virus has been reported to cause disease in swine (2).

EEE virus causes severe disease in humans with a mortality rate of 30–70% and a high frequency of permanent sequelae in patients who survive. WEE is usually mild in adult humans, but can be a severe disease in children. The fatality rate is between 3 and 14%. Severe infection and death caused by EEE and WEE viruses have been reported in laboratory workers; therefore, any work with these viruses must be performed at containment level 3 (see Chapter I.1.6. Human safety in the veterinary microbiological laboratory). It is recommended that personnel be immunised against EEE and WEE viruses (14). Precautions should also be taken to prevent human infection when performing post-mortem examinations on horses suspected of being infected with the equine encephalomyelitis viruses.

Gross pathological lesions are rarely observed in horses and, if present, consist only of the congestion of the brain and meninges. Ecchymotic haemorrhages of traumatic origin may be observed. Microscopic lesions are usually found throughout the central nervous system and can be diagnostic. There is widespread evidence of a severe inflammatory response involving the grey matter. Neuronal degeneration with infiltration by polymorphonuclear leukocytes, diffuse and focal gliosis, and perivascular cuffing with lymphocytes and neutrophils are seen. Also observed are neuronophagia and liquefaction of the neuropil. The extent of the lesions depends on the severity of the infection and the duration of the neurological involvement (16). Immunohistochemical procedures for diagnosis of EEE have been described (9).

Brain lesions caused by WEE virus infection are focal and have lymphocytic infiltrations. Brain lesions caused by EEE virus infection are more severe and are found throughout the grey matter. They are characterised by a larger number of neutrophils among the inflammatory cells.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The most definitive method for diagnosis of EEE or WEE is the isolation of the viruses. EEE virus can usually be isolated from the brains of horses, unless more than 5 days have elapsed between the appearance of clinical signs and the death of the horse. EEE virus can frequently be isolated from brain tissue even in the presence of a high serum antibody titre. WEE virus is rarely isolated from tissues of infected horses. Brain is the tissue of choice for virus isolation, but the virus has been isolated from other tissues, such as the liver and spleen. It is recommended that a complete set of these tissues be collected in duplicate, one set for virus isolation and the other set in formalin for histopathological examination. Specimens for virus isolation should be sent refrigerated if they can be received in the laboratory within 48 hours of collection; otherwise, they should be frozen and sent with dry ice. A complete set of tissues will allow the performance of diagnostic techniques for other diseases. For isolation, a 10% suspension of tissue is prepared in phosphate buffered saline (PBS), pH 7.8, containing bovine serum albumin (BSA) (fraction V; 0.75%), penicillin (100 units/ml), and streptomycin (100 µg/ml). The suspension is clarified by centrifugation at 1500 g for 30 minutes.

The newborn mouse is considered to be a sensitive host system. Inoculate intracranially one or two litters of 1–4-day-old mice with 0.02 ml of inoculum using a 26-gauge 3/8 inch (9.3 mm) needle attached to a 1 ml tuberculin syringe. The inoculation site is just lateral to the midline into the midportion of one lateral hemisphere. Mice are observed for 10 days; dead mice are collected daily and frozen at –70°C. Mouse brains are harvested for virus identification by aspiration using a 20-gauge 1 inch (2.5 cm) needle attached to a 1 ml tuberculin syringe. A second passage is made only if virus cannot be identified from mice that die following inoculation.

The chicken embryo is considered to be less sensitive than newborn mice when used for primary isolation of EEE and WEE viruses. Tissue suspensions can be inoculated by the yolk-sac route into 6–8-day-old embryonating chicken eggs. There are no diagnostic signs or lesions in the embryos infected with these viruses. Inoculated embryos should be incubated for 7 days, but deaths usually occur between 2 and 4 days post-inoculation. Usually only one passage is made unless there are dead embryos from which virus cannot be isolated. Newly hatched chickens are susceptible and have been used for virus isolation. If this method is used,
precautions must be taken to prevent aerosol exposure of laboratory personnel, as infected birds can shed highly infectious virus.

EEE and WEE viruses can also be isolated in a number of cell culture systems. The most commonly used cell cultures are primary chicken or duck embryo fibroblasts, continuous cell lines of African green monkey kidney (Vero), rabbit kidney (RK-13), or baby hamster kidney (BHK-21). Isolation is usually attempted in 25 cm² cell culture flasks. Confluent cells are inoculated with 1.0 ml of tissue suspension.

Following a 1–2-hour absorption period, maintenance medium is added. Cultures are incubated for 7 days, and one blind passage is made. EEE and WEE viruses will produce a cytopathic change in cell culture. Cultures that appear to be infected are frozen. The fluid from the thawed cultures is used for virus identification.

When the complement fixation (CF) test is used, EEE or WEE viruses can be identified in infected mouse or chicken brains, cell culture fluid, or amnionic-allantoic fluid. A 10% brain suspension is prepared in veronal (barbitone) buffer; egg and cell culture fluids are used undiluted or diluted 1/10 in veronal buffer. The fluid or suspension is centrifuged at 9000 g for 30 minutes, and the supernatant fluid is tested against hyperimmune serum or mouse ascitic fluid prepared against EEE and WEE viruses using a standard CF procedure (13). The CF test requires the overnight incubation at 4°C of serum-antigen with 7 units of complement. Virus can be identified in cell culture by direct immunofluorescent staining. The less commonly used method of virus identification is the neutralisation test, as outlined below.

EEE virus nucleic acid in mosquitoes and tissues has been identified by the polymerase chain reaction (PCR) using primers selected from the capsid gene (15). RNA is extracted using guanidium iso-thiocyanate-acid phenol. Forty repetitions of the three-step amplification cycle of nucleic acid denaturation, primer annealing and primer extension are used. Temperature and duration of each step are optimised for the specific primer pair, reagents and thermal cycler used in the PCR cycles. Reaction products or their fragments are analysed on 2.0–2.6% agarose gels that have been stained with 1 µg/ml of ethidium bromide. An alternate identification procedure is by hybridisation with an oligonucleotide probe. A reverse-transcription PCR method for detection of WEE RNA and alternative methods for EEE RNA detection have also been described (5, 7).

Antigen-capture enzyme-linked immunosorbent assay (ELISA) has been developed for EEE surveillance in mosquitoes. This can be used in countries that do not have facilities for virus isolation or PCR (1).

2. Serological tests

Serological confirmation of EEE or WEE virus infection requires a four-fold or greater increase or decrease in antibody titre in paired serum samples collected 10–14 days apart. Most horses infected with EEE and WEE virus have a high antibody titre when clinical disease is observed. Horses infected with EEE or WEE virus usually have antibody titres in the acute stage of the disease. Consequently, a presumptive diagnosis can be made if an unvaccinated horse with appropriate clinical signs has antibody against only EEE or WEE virus. The detection of IgM antibody by the ELISA can also provide a presumptive diagnosis of acute infection (11). The plaque reduction neutralisation (PRN) test or, preferably, a combination of PRN and haemagglutination inhibition (HI) tests is the procedure most commonly used for the detection of antibody against EEE and WEE viruses. There are cross-reactions between antibody against EEE and WEE virus in the CF and HI tests. CF antibody against both EEE and WEE viruses appears later and does not persist; consequently, it is less useful for the serological diagnosis of disease.

a) Complement fixation

The CF test is frequently used for the demonstration of antibodies, although the antibodies detected by the CF test may not persist for as long as those detected by the HI or PRN tests. A sucrose/acetone mouse brain extract is commonly used as antigen. The positive antigen is inactivated by treatment with 0.1% beta-propiolactone.

In the absence of an international standard serum, the antigen should be titrated against a locally prepared positive control serum. The normal antigen, or control antigen, is mouse brain from un inoculated mice similarly extracted and diluted.

Sera are diluted 1/4 in veronal buffered saline containing 1% gelatin (VBSSG), and inactivated at 56°C for 30 minutes. Titrations of positive sera may be performed using additional twofold dilutions. The CF antigens and control antigen (normal mouse brain) are diluted in VBSSG to their optimal amount of fixation as determined by titration against the positive sera; guinea-pig complement is diluted in VBSSG to contain 5 complement haemolytic units-50% (CH₅₀). Sera, antigen, and complement are reacted in 96-well round-bottom microtitre plates at 4°C for 18 hours. The sheep red blood cells (SRBCs) are standardised to 2.8% concentration. Haemolysin is titrated to determine the optimal dilution for the lot of complement used. Haemolysin is used to sensitize 2.8% SRBCs and the sensitis ed cells are added to all wells on the microtitre plate. The test is incubated for 30 minutes at 37°C. The plates are then centrifuged (200 g), and
the wells are scored for the presence of haemolysis. The following controls are used: (a) serum and control serum each with 5 CH50 and 2.5 CH50 of complement; (b) CF antigen and control antigen each with 5 CH50, and 2.5 CH50 of complement; (c) complement dilutions of 5 CH50, 2.5 CH50, and 1.25 CH50; and (d) cell control wells with only SRBCs and VBSG diluent. These controls test for anticomplementary antigen, anticomplementary serum, activity of complement used in the test, and integrity of the SRBC indicator system in the absence of complement, respectively.

To avoid anticomplementary effects, sera should be separated from the blood as soon as possible. Positive and negative control sera should be used in the test.

b) Haemagglutination inhibition

The antigen for the HI test is the same as described above for the CF test. The antigen is diluted so that the amount used in each haemagglutinating unit (HAU) is from four to eight times that which agglutinates 50% of the RBCs in the test system. The haemagglutination titre and optimum pH for each antigen are determined with goose RBCs diluted in pH solutions ranging from pH 5.8 to pH 6.6, at 0.2 intervals.

Sera are diluted 1/10 in borate saline, pH 9.0, and then inactivated at 56°C for 30 minutes. Kaolin treatment is used to remove nonspecific serum inhibitors. Sera should be absorbed before use by incubation with a 0.05 ml volume of washed packed goose RBCs for 20 minutes at 4°C.

Following heat inactivation, kaolin treatment and absorption, twofold dilutions of the treated serum are prepared in borate saline, pH 9.0 with 0.4% bovalbumin. Serum dilutions (0.025 ml/well) are prepared in a 96-well round-bottom microtitre plate in twofold dilutions in borate saline, pH 9.0, with 0.4% bovalbumin. Antigen (0.025 ml/well) is added to the serum. Plates are incubated at 4°C overnight. RBCs are derived from normal white male geese1 and washed three times in dextrose/gelatin/veronal (DGV), and a 7.0% suspension prepared in DGV. The 7.0% suspension is then diluted 1/24 in the appropriate pH solution, and 0.05 ml per well is added immediately to the plates. Plates are incubated for 30 minutes at 37°C. 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. Titres of 1/10 and 1/20 are suspect, and titres of 1/40 and above are positive.

c) Enzyme-linked immunosorbert assay

The ELISA is performed by coating flat-bottomed plates with anti-equine IgM capture antibody (11). The antibody is diluted according to the manufacturer’s recommendations in 0.5 M carbonate buffer, pH 9.6, and 50 µl is added to each well. The plates are incubated at 37°C for 1 hour, and then at 4°C overnight. Prior to use, the coated plates are washed twice with 200 µl/well of 0.01 M PBS containing 0.05% Tween 20. After the second wash, 200 µl/well of PBS/Tween/5% nonfat dried milk is added and the plates are incubated at room temperature for 1 hour. Following incubation, the plates are washed again three times with PBS/Tween. Test and control sera are diluted 1/100 and 1/1000 in 0.01 M PBS, pH 7.2, containing 0.05% Tween 20, and 50 µl is added to each well. The plates are incubated at 37°C for 2 hours and then washed three times. Next, 50 µl of viral antigen is added to all wells. (The dilution of the antigen will depend on the source and should be empirically determined.) The plates are incubated overnight at 4°C, and washed six times. Then, 50 µl of horseradish-peroxidase-conjugated monoclonal antibody (MAb) to encephalitis virus2 is added. The plates are incubated for 60 minutes at 37°C and then washed three times. Finally, 50 µl of freshly prepared ABTS (2,2'-Azino-bis-[3-ethylbenzo-thiazoline-6-sulphonic acid]) substrate + hydrogen peroxidase is added, and the plates are incubated at room temperature for 15–40 minutes The absorbance of the test serum is measured at 405 nm. A test sample is considered to be positive if the absorbance of the test sample in wells containing virus antigen is at least twice the absorbance of negative control serum in wells containing virus antigen and at least twice the absorbance of the sample tested in parallel in wells containing control antigen.

d) Plaque reduction neutralisation

The PRN test is very specific and can be used to differentiate between EEE and WEE virus infections. The PRN test is performed in duck embryo fibroblast, Vero, or BHK-21 cell cultures. The sera can be screened at a 1/10 and 1/100 final dilution. Endpoints can be established using the PRN or HI test. Serum used in the PRN assay is tested against 100 plaque-forming units of virus. The virus/serum mixture is incubated at 37°C for 75 minutes before inoculation on to confluent cell culture monolayers in 25 cm² flasks. The inoculum is adsorbed for 1 hour, followed by the addition of 6 ml of overlay medium. The overlay medium consists of two solutions that are prepared separately. Solution I contains 2 × Earle’s Basic Salts Solution

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1 RBCs from adult domestic white male geese are preferred, but RBCs from other male geese can be used. If cells from female geese are used, there may be more test variability. It has been reported that rooster RBCs cause a decrease in the sensitivity of the test.

2 Available from: Centers for Disease Control and Prevention, Biological Reference Reagents, 1600 Clifton Road NE, Mail Stop C21, Atlanta, Georgia 30333, United States of America.
without phenol red, 6.6% yeast extract lactalbumin hydrolysate, 4% fetal bovine serum, 800 units/ml penicillin, 400 µg/ml streptomycin, 200 µg/ml nystatin, 6% of a 7.5% solution of sodium bicarbonate, and 3.3% of a 1/1500 dilution of neutral red (1/8000). Solution II consists of 2% Noble agar that is sterilised and maintained at 47°C. Equal volumes of solutions I and II are adjusted to 47°C and mixed together just before use. The test is incubated for 48–72 hours, and endpoints are based on a 90% reduction in the number of plaques compared with the virus control flasks, which should have about 100 plaques.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Inactivated vaccines against EEE and WEE viruses are available commercially. Attenuated EEE and WEE virus vaccines have not proven satisfactory. The vaccines licensed for use in the USA are prepared using the following combinations: EEE and WEE, EEE, WEE, and Venezuelan equine encephalomyelitis (VEE); and EEE and VEE. In addition, tetanus toxoid and inactivated influenza virus have been combined with EEE and WEE or EEE, WEE, and VEE.

Early vaccines were produced from virus propagated in embryonating chicken eggs and inactivated with formalin. Current vaccines are prepared from virus propagated in cell culture, and inactivated with formalin (6) or monoethylamine.

1. Seed management
   a) Characteristics of the seed
      Standard strains of EEE and WEE viruses that were isolated over 20 years ago have been used for vaccine production and have been proven to produce good immunity. Strains of EEE virus that differ antigenically and in molecular structure have been identified from different geographical regions. However, the North American and Caribbean isolates appear to be similar (17). Strains of WEE virus isolated from different countries have been found to be similar both by Mab testing and RNA oligonucleotide fingerprinting analysis (10). A recent well-characterised isolate from the country where the vaccine is to be used would be advantageous. Viruses that are selected must be immunogenic and replicate to high titres in cell culture.

   b) Method of culture
      Primary chicken embryo fibroblasts and Vero cells have been used for propagation of viruses used for vaccine production. The fibroblasts should be prepared from specific pathogen free embryos. Other susceptible cell lines could also be used.

   c) Validation as a vaccine
      If a cell line is used, the master cell stock is tested to confirm the identity of the cell line, species of origin, and freedom from extraneous agents. If primary cell cultures are used, a monolayer from each batch of subculture should be tested for extraneous agents including bacteria, fungi, mycoplasma, and viruses. The master seed virus should also be tested to ensure freedom from bacteria, fungi, mycoplasma, and extraneous viruses.

      The vaccines are administered by the intramuscular (in most cases) or intradermal route in the cervical region in two doses given 2–4 weeks apart. Annual revaccination is recommended. All foals vaccinated before 1 year of age should be revaccinated before the next vector season.

2. Method of manufacture
   Details of the manufacture of vaccines currently on the market are not available. Consequently, the information provided here is intended only as background reference material on the vaccines and not as a method of manufacture. The virus and cell culture system should be selected so that a high virus titre, \( \geq 10^6 \) TCID\(_{50}\) (50% tissue culture infective dose) per ml, is obtained in under 48 hours. Virus for vaccine production can be prepared from the supernatant fluid from infected cell cultures. The fluid is harvested when 70–100% of the monolayers have the characteristic cytopathic changes. The virus titre is determined by titration in cell culture or mice. The fluid is clarified by low speed centrifugation and filtered through gauze. The virus is inactivated by adding formalin to a final concentration of 1/2000 (0.05%) and holding at 37°C for 24 hours. Residual formaldehyde is neutralised by sodium bisulphite (6). The residual free formalin content in the inactivated vaccine should not exceed 0.2% formaldehyde.

3. In-process control
   Cultures should be examined daily for virus-induced cytopathic effect. The harvested virus should be tested for microbial contamination. The efficacy of the inactivation process should be checked by testing for viable virus.
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 inactivated vaccine is safety tested by inoculating subcutaneously at least ten 6–12-hour-old chickens with 0.5 ml of the vaccine. The chickens are observed each day for 10 days for unfavourable reactions that are attributable to the vaccine (12). Safety testing can also be carried out by inoculating intracerebrally at least eight 1–4-day-old mice with 0.02 ml of the vaccine, and observing for 7 days. It is critical that safety tests be conducted on each lot of vaccine to insure that there is no residual virulent virus present.

c) Potency

Potency testing is performed by inoculating each of ten guinea-pigs with either EEE or WEE virus, using one-half the horse dose on two occasions, 14–21 days apart, by the route recommended for the horse. Serum samples from each vaccinate and each control are tested 14–21 days after the second dose using the PRN test. The EEE titres should be \( \geq 1/40 \), and the WEE titres should be \( \geq 1/40 \) (12), using Vero cells. If duck embryo fibroblasts are used in the PRN test, the titres will be lower. An alternative potency test is to use intracerebral challenge, 14–21 days after the second vaccination. Each guinea-pig is inoculated with 0.1 ml of virus containing 100 LD\(_{50}\) (50% lethal dose). Simultaneous titration is carried out. In order for the vaccine to be approved, 80% of the guinea-pigs must survive both viruses.

d) Duration of immunity

Comprehensive studies on duration of immunity are not available. An annual revaccination is recommended. Foals that are vaccinated before 1 year of age should be revaccinated before the next vector season.

e) Stability

The lyophilised vaccine is stable and immunogenic for 3 years if kept refrigerated at 2–7°C. After 3 years, vaccine should be discarded. The vaccines should be used immediately after reconstitution.

f) Preservatives

The preservatives used are thimerosal at a 1/10,000 dilution and antibiotics (neomycin, polymyxin amphotercin B and gentamicin).

g) Precautions (hazards)

Severe infection and death caused by EEE and WEE viruses have been reported in laboratory workers; therefore, any work with these viruses must be carried out at least in a biosafety level 2 laboratory using biological safety cabinets, and personnel should be immunised against EEE and WEE viruses (14).

Pregnant mares and foals under 2 weeks old should not be vaccinated.

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

REFERENCES


Chapter 2.5.3. — Equine encephalomyelitis (Eastern and Western)


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NB: There is an OIE Reference Laboratory for Equine encephalomyelitis (Eastern and Western) (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 2.5.4.

EQUINE INFECTION ANAEMIA

SUMMARY

Equine infectious anaemia (EIA) is caused by a lentivirus. It can be diagnosed on the basis of clinical signs, pathological lesions and serology. Infected horses remain viraemic carriers for life and will yield a positive serological result. Antibody response usually persists and antibody-positive animals, older than 6 months, are identified as virus carriers (below 6 months of age, serological reactions can be due to maternal antibodies). They can potentially transmit the infection to other horses.

**Identification of the agent:** Virus from a horse can be isolated by inoculating suspect blood into a susceptible horse or on to leukocyte cultures prepared from susceptible horses. Recognition of infection in horses that have been inoculated experimentally may be made on the basis of clinical signs, haematological changes and a positive antibody response determined by an immunodiffusion test or enzyme-linked immunosorbent assay (ELISA). A successful virus isolation in horse leukocyte cultures is confirmed by the detection of specific EIA antigen, by immunofluorescence assay, polymerase chain reaction, reverse-transcriptase assay, or by the inoculation of culture fluids into susceptible horses. Virus isolation is rarely attempted because of the time, difficulty and expense involved.

**Serological tests:** Agar gel immunodiffusion (AGID) tests and ELISAs are simple, reliable tests for the demonstration of EIA virus infection. When ELISAs are positive they should be confirmed using the AGID test. The precipitating antigen can be prepared from infected tissue cultures or by using recombinant DNA technology.

**Requirements for vaccines and diagnostic biologicals:** There are no biological products currently available.

A. INTRODUCTION

Equine infectious anaemia (EIA) occurs world-wide. The infection is limited to equids. The disease is characterised by recurrent febrile episodes, thrombocytopenia, anaemia, rapid loss of weight and oedema of the lower parts of the body; it tends to become an inapparent infection if death does not result from one of the acute clinical attacks. The incubation period is normally 1–3 weeks, but may be as long as 3 months. In acute cases, lymph nodes, spleen and liver are hyperaemic and enlarged. Histologically these organs are infiltrated with nests of immature lymphocytes and plasma cells. Kupffer cells in the liver often contain haemosiderin or erythrocytes. The enlarged spleen may be felt on rectal examination.

Once a horse is infected with EIA virus, its blood remains infectious for the remainder of its life. This means that the horse is a viraemic carrier and can potentially transmit the infection to other horses (4). Transmission occurs by transfer of blood from an infected horse. In nature, spread of the virus is most likely via interrupted feeding of bloodsucking horseflies on a clinically ill horse and then on susceptible horses, or from the use of contaminated needles. However, in utero infection of the fetus does occur (8). The virus titre is higher in horses with clinical signs and the risk of transmission is higher from these animals than the carrier animals with a lower virus titre.

B. DIAGNOSTIC TECHNIQUES

Agar gel immunodiffusion (AGID) tests (7) and enzyme-linked immunosorbent assays (ELISAs) (15) are accurate, reliable tests for the detection of EIA in horses, except for animals in the early stages of infection and foals of infected dams. In other rare circumstances, misleading results may occur when the level of virus circulating in the blood during an acute episode of the disease is sufficient to bind available antibody, and if initial
antibody levels never rise high enough to be detectable (16). Although the ELISA will detect antibodies somewhat earlier and at lower concentrations than the AGID test, positive ELISAs are confirmed using the AGID test. This is because false-positive results have been noted with the ELISA. The AGID test also has the advantage of distinguishing between EIA and non-EIA antigen–antibody reactions by lines of identity.

The EIA virus is a lentivirus, a subfamily of the Retroviridae, which also includes maedi-visna, caprine arthritis/encephalitis, bovine immunodeficiency and the human immunodeficiency viruses. Nucleic acid sequence comparisons have demonstrated a marked relatedness among these viruses.

1. Identification of the agent

Virus isolation is usually not necessary to make a diagnosis.

Isolation of the virus from suspect horses may be made by inoculating their blood on to leukocyte cultures prepared from horses free of infection. Virus production in cultures can be confirmed by detection of specific EIA antigen by ELISA (14), by immunofluorescence assay (18), or by subinoculation into susceptible horses. Virus isolation is rarely attempted because of the difficulty of growing horse leukocyte cultures.

A nested polymerase chain reaction assay to detect EIA proviral DNA from the peripheral blood of horses has been described (12).

When the exact status of infection of a horse cannot be ascertained, the inoculation of a susceptible horse with suspect blood should be employed. In this case a horse that has previously been tested for antibody and shown to be negative is given an immediate blood transfusion from the suspect horse, and its antibody status and clinical condition are monitored for at least 45 days. Usually, 1–25 ml of whole blood given intravenously is sufficient to demonstrate infection, but in rare cases it may be necessary to use a larger volume of blood (250 ml) or washed leukocytes from such a volume (5).

2. Serological tests

Due to the persistence of EIA virus in infected equids, detection of serum antibody to EIA virus confirms the diagnosis of EIA virus infection.

a) Agar gel immunodiffusion test (the prescribed test for international trade)

Precipitating antibody is rapidly produced as a result of EIA infection, and can be detected by the AGID test. Specific reactions are indicated by precipitin lines between the EIA antigen and the test serum and confirmed by their identity with the reaction between the antigen and the positive standard serum. Horses in the first 2-3 weeks after infection will usually give negative serological reactions. In rare cases the post-infection time prior to the appearance of detectable antibody may extend up to 60 days.

Reagents for AGID are available commercially from several companies. Alternately, AGID antigen and reference serum may be prepared as described below.

• Preparation of antigen

Specific EIA antigen may be prepared from the spleen of acutely infected horses (6), from infected equine tissue culture (10), from a persistently infected canine thymus cell line (3), or from proteins expressed in bacteria or baculovirus using the recombinant DNA technique (2, 9). Preparation from infected cultures or from recombinant DNA techniques gives a more uniform result than the use of spleen cells and allows for better standardisation of reagents.

To obtain a satisfactory antigen from spleen, a horse must be infected with a highly virulent strain of EIA virus. The resulting incubation period should be 5–7 days, and the spleen should be collected 9 days after inoculation, when the virus titre is at its peak and before any detectable amount of precipitating antibody is produced. Undiluted spleen pulp is used in the immunodiffusion test as antigen (6). Extraction of antigen from the spleen with a saline solution and concentration with ammonium sulphate does not give as satisfactory an antigen as selection of a spleen with a very high titre of EIA antigen.

Alternatively, equine fetal kidney or dermal cells or canine thymus cells are infected with a strain of EIA virus adapted to grow in tissue culture (American Type Culture Collection). Virus is collected from cultures by precipitation with 8% polyethylene glycol or by pelleting by ultracentrifugation. The diagnostic antigen, p26, is released from the virus by treatment with detergent or ether (10). EIA virus core proteins, expressed in bacteria or baculovirus, are commercially available and find practical use as high quality antigens for serological diagnosis. There is evidence of strain variation in the p26 amino acid sequence; however there is no evidence to indicate that this variation influences any of the diagnostic tests (19).
The p26 is an internal structural protein of the virus that is coded for by the gag gene. This gene is stable and no variation has been found between strains (11).

- **Preparation of standard antiserum**

A known positive antiserum may be collected from a horse previously infected with EIA virus. This serum should yield a single dense precipitation line that is specific for EIA, as demonstrated by a reaction of identity with a known standard serum. It is essential to balance the antigen and antibody concentrations in order to ensure the optimal sensitivity of the test. Reagent concentrations should be adjusted to form a narrow precipitation line approximately equidistant between the two wells containing antigen and serum.

- **Test procedure (1, 6, 13)**

  i) Immunodiffusion reactions are carried out in a layer of agar in Petri dishes. For Petri dishes that are 100 mm in diameter, 15–17 ml of 1% Noble agar is used. Six wells are punched out of the agar surrounding a centre well of the same diameter. The wells are 5.3 mm in diameter and 2.4 mm apart. Each well must contain the same volume of reagent.

  ii) The antigen is placed in the central well and the standard antiserum is placed in alternate exterior wells. Serum samples for testing are placed in the remaining three wells.

  iii) The dishes are maintained at room temperature in a humid environment.

  iv) After 24–48 hours the precipitation reactions are examined over a narrow beam of intense, oblique light and against a black background. The reference lines should be clearly visible at 24 hours, and at that time any test sera that are strongly positive may also have formed lines of identity with those between the standard reagents. A weakly positive reaction may take 48 hours to form and is indicated by a slight bending of the standard serum precipitation line between the antigen well and the test serum well. Sera with high precipitating antibody titres may form broader precipitin bands that tend to be diffuse. Such reactions can be confirmed as specific for EIA by dilution at 1/2 or 1/4 prior to retesting; these then give a more distinct line of identity. Sera devoid of EIA antibody will not form precipitation lines and will have no effect on the reaction lines of the standard reagents.

  v) **Interpretation of the results:** Horses that are in the early stages of an infection may not give a positive serological reaction in an AGID test. Such animals should be bled again after 3–4 weeks. In order to make a diagnosis in a young foal, it may be necessary to determine the antibody status of the dam. If the mare possesses any EIA antibody, then a period of about 6 months or longer after birth must be allowed for the maternal antibody to wane; the foal is then retested to determine whether an initial positive reaction was due to maternal antibody or infection.

b) **Enzyme-linked immunosorbent assay**

There are three ELISAs that are approved by the United States Department of Agriculture for the diagnosis of equine infectious anaemia and are available internationally; a competitive ELISA and two non-competitive ELISAs. The competitive ELISA and one non-competitive ELISA detect antibody produced against the p26 core protein antigen. The other non-competitive ELISA incorporates both p26 core protein and gp45 (viral transmembrane protein) antigens. Typical ELISA protocols are used in all tests. If commercial ELISA materials are not available, a non-competitive ELISA using p26 antigen purified from cell culture material may be employed (14).

A positive test result by ELISA should be retested using the AGID test to confirm the diagnosis because some false-positive results have been noted with the ELISA. The results can also be confirmed by the immunoblot technique. A standard antiserum for immunodiffusion, which contains the minimum amount of antibody that should be detected by laboratories, is available from the OIE Reference Laboratories (see Table given in Part 3 of this Terrestrial Manual). Uniform methods for EIA control have been published (17).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No biological products are available currently.

REFERENCES

1. **ASSOCIATION FRANÇAISE DE NORMALISATION (AFNOR) (2000). Animal Health Analysis Methods, Detection of Antibodies against Equine Infectious Anaemia by the Agar Gel Immunodiffusion Test. NF U 47-002. AFNOR, 11 avenue Francis de Pressensé, 93571 Saint-Denis La Plaine Cedex, France.**


* * *

NB: There are OIE Reference Laboratories for Equine infectious anaemia (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 2.5.5.
EQUINE INFLUENZA

SUMMARY

Equine influenza is an acute respiratory disease caused by two distinct subtypes (H7N7, formerly equi-1, and H3N8, formerly equi-2) of influenza A virus within the genus Influenzavirus A of the family Orthomyxoviridae. In fully susceptible animals, clinical signs include pyrexia, and a harsh dry cough followed by a mucopurulent nasal discharge. In partially immune vaccinated animals, one or more of these signs may be absent. Characteristically, influenza spreads rapidly in a susceptible population. Diagnosis of influenza virus infections is based on virus isolation from horses with acute respiratory illness, or on the demonstration of a serological response to infection. Ideally, both methods are used. Infection may also be demonstrated by detection of viral antigen in respiratory secretions using an enzyme-linked immunosorbent assay. Spread of infection and severity of disease may be reduced by the use of potent inactivated equine influenza vaccines containing epidemiologically relevant virus strains.

Identification of the agent: Embryonated hens’ eggs and/or cell cultures can be used for virus isolation from nasopharyngeal swabs or nasal and tracheal washes. Viral growth is monitored by haemagglutination (HA) or, in cell cultures, by haemadsorption (HAD) using chicken or guinea-pig red blood cells. Isolates can be characterised by haemagglutination inhibition (HI) using strain-specific antisera. Isolates should always be sent immediately to an International Reference Laboratory (OIE or World Health Organization). Samples that yield negative results should be repassedaged; up to five passages may be necessary to isolate viruses from vaccinated horses.

Serological tests: Diagnosis of influenza virus infections is usually only accomplished by tests on paired sera; the first sample should be taken as soon as possible after the onset of clinical signs and the second approximately 2 weeks later. Antibody titres are determined by HI or single radial haemolysis (SRH).

Requirements for vaccines and diagnostic biologicals: Inactivated equine influenza vaccines contain whole viruses or their subunits. The vaccine viruses are propagated in embryonated hens’ eggs or tissue culture, concentrated, and purified before inactivation with agents such as formalin or beta-propiolactone. Vaccines provide protection by inducing humoral antibody to HA. Responses are generally short-lived and multiple doses are required to maintain protective levels of antibody. An adjuvant is usually required to stimulate durable protective levels of antibody. A live attenuated vaccine has recently been licensed in some countries.

Influenza vaccines are widely available and are routinely used in competition horses in Europe, the Americas, and Asia. In some countries, vaccination is mandatory for sport horses that are competing under rules of equestrian organisations. Vaccines are not widely used on the Indian subcontinent, China or Australia, the latter region, along with New Zealand, having remained free from infection. Following a primary course of three doses at intervals of 0, 1 and 6 months, an annual booster is the minimum requirement. In some regions, repeated vaccinations are given every 3–6 months. Foals should not be vaccinated in the presence of maternal antibody.

Vaccines have been highly successful in preventing infection with H7N7 viruses, but frequent reports of vaccine breakdown occur during outbreaks of H3N8 infections. This has been attributed to inadequate vaccine potency, inappropriate vaccination schedules, and outdated vaccine viruses that have become irrelevant as a result of antigenic drift. An improved in-vitro potency test (single radial diffusion) has been developed that can be used for in-process testing of the product before addition of an adjuvant. Levels of antibody required for protection of horses have been identified. A
surveillance programme is underway to monitor antigenic drift among equine influenza viruses and to provide information on strain selection for vaccines.

A. INTRODUCTION

Equine influenza is caused by two subtypes: H7N7 (formerly subtype 1) and H3N8 (formerly subtype 2) of influenza A viruses (genus Influenzavirus A of the family Orthomyxoviridae). Although these are not genuine human pathogens, humans can become infected with equine influenza virus subtypes. Such infections are unusual and subclinical, but may represent a potential biohazard to laboratory personnel.

In fully susceptible animals, clinical signs include pyrexia, and a harsh dry cough followed by a mucopurulent nasal discharge. In partially immune vaccinated animals, one or more of these signs may be absent. Characteristically, influenza spreads rapidly in a susceptible population. Diagnosis of influenza virus infections is based on virus isolation from horses with acute respiratory illness, or on the demonstration of a serological response to infection. Ideally, both methods are used. Infection may also be demonstrated by detection of viral antigen in respiratory secretions using an enzyme-linked immunosorbent assay (ELISA).

B. DIAGNOSTIC TECHNIQUES

All influenza viruses are highly contagious for susceptible hosts, including embryonated hens’ eggs and cell cultures. Care must therefore be taken during the handling of infected eggs or cultures to avoid accidental cross-contamination (21). Standard strains should not be propagated in the diagnostic laboratory, at least never at the same time or in the same place where diagnostic samples are being processed. All working areas must be efficiently disinfected before and after virus manipulations, which should preferably be conducted within biohazard containment.

It is important to obtain samples as soon as possible after the onset of clinical signs. These samples include nasopharyngeal swabs and nasal or tracheal washings, the latter taken by endoscopy. Swabs consist of absorbent cotton wool sponge/gauze on wire, and should be passed via the ventral meatus into the nasopharynx and held there for approximately 1 minute to absorb respiratory secretions. Swabs should be transferred to a vial containing transport medium immediately after use. This medium consists of phosphate buffered saline (PBS) containing 40% glycerol, or PBS containing 2% tryptose phosphate broth, 2% antibiotic solution (penicillin [10,000 units], streptomycin [10,000 units] in sterile distilled water [100 ml]), and 2% fungizone (250 mg/ml stock). If the samples are to be inoculated within 1–2 days they may be held at 4°C, but, if kept for longer, they should be stored at –70°C or below. Preferably, samples should also be transported on ice.

Only one sample is processed at a time. The liquid is expelled from the swab, which is then disposed of suitably. Further antibiotics may be added if samples appear to be heavily contaminated. The remainder of the fluid is stored at –70°C. Samples treated with antibiotics are allowed to stand on ice for 30 minutes and are then centrifuged at 1500 g for 15 minutes to remove bacteria and debris; the supernatant fluids are used for inoculation. Filtration of samples is not advised as influenza virus may adsorb on to the filter and be lost from the sample.

1. Identification of the agent

Isolation of infectious virus may be carried out in embryonated hens’ eggs or cell cultures. Traditionally, eggs have been preferred for isolation of equine influenza. Comparison of H3N8 viruses isolated in eggs and Madin–Darby canine kidney (MDCK) cells indicated that MDCK cells are capable of selecting variant viruses that are not representative of the predominant virus in clinical specimens (6). However, in recent years some viruses have been successfully isolated in MDCK cells but not in eggs and selection of variants has occurred as a result of culture in eggs (18), therefore isolation should be attempted using both substrates. Recently, polymerase chain reaction techniques have been described for the identification of equine influenza virus from clinical specimens and for molecular epidemiology (4, 8, 17).

a) Embryonated hens’ eggs

Fertile eggs are set in a humid incubator at (37–38°C) and turned twice daily; after 10–11 days, they are examined by candling. Only live embryonated eggs are selected for use. The area above the air sac is cleansed with alcohol and a small hole is drilled through the shell. Three eggs/sample are inoculated (0.1 ml) in the amniotic cavity. The syringe is withdrawn approximately 1 cm and a further 0.1 ml is inoculated into the allantoic cavity. Alternatively, the sample may be inoculated into the allantoic cavity alone. The hole is sealed with wax or Sellotape, and the eggs are incubated at 34–35°C for 2–3 days.
Chapter 2.5.5. – Equine influenza

The eggs are then transferred to 4°C for 4 hours or overnight to kill the embryos and to reduce bleeding at harvest. The shells are disinfected, and the allantoic and amniotic fluid is harvested by pipette, each harvest being kept separate. These are tested for haemagglutination (HA) activity by mixing in equal volumes (0.025 ml) with chicken red blood cells (RBCs) (0.5% [v/v] packed cells in PBS) in V- or U-bottomed microtitre plates or 0.4% guinea-pig RBCs (0.4% [v/v] packed cells in PBS) in V- or U-bottomed plates. If chicken RBCs are used, the plates may be read by tilting to 70° so that non-agglutinated cells 'stream' to the bottom of the well. Non-agglutinated guinea-pig cells appear as a button at the bottom of the well and may take longer to settle. If there is no HA activity, aliquots of each harvest are pooled and passaged into further eggs. All HA positive samples are divided into aliquots and stored at −70°C; one aliquot is titrated for HA immediately. If the HA titre is 1/16 or more, the isolate is characterised immediately. If titres are low, positive samples should be passaged. Care should be taken to avoid generation of defective interfering particles by prediluting the inoculum 1/10, 1/100, 1/1,000. Positive samples arising from the highest dilution should be selected as stocks for storage. It may be necessary to undertake as many as five passages to isolate the virus, particularly from vaccinated horses. If virus has not been recovered by the fifth passage, further passages are unlikely to be successful.

b) Cell cultures

Cultures of the MDCK cell line (MDCK, ATCC CCL34) may be used to isolate equine influenza viruses. The cells are grown to confluence in tubes and then infected in triplicate with 0.25–0.5 ml of each sample, processed as described above. The cultures are maintained with serum-free medium containing 0.5–2 µg/ml trypsin (treated with TPCCK [L-1-tosylamine-2-phenylethyl chloromethyl ketone] to remove chymotrypsin, available pretreated, e.g. from Sigma), and examined daily for evidence of cytopathic effects (CPE). If positive, or after 7 days in any case, the supernatant fluids are tested for HA. Fluids with titres of 1/16 are characterised immediately. Negative fluids and those with titres <1/16 are repassaged up to five passages.

Alternatively, the cells are screened for evidence of haemadsorption (HAD). This procedure detects expression of viral antigens at the cell surface. The medium is removed from the cultures and the tubes are washed with PBS. One or two drops of a 50% suspension of chicken or guinea-pig RBCs are added, the tubes are rotated carefully, and kept at 22°C (±2°C) for 30 minutes. Unbound RBCs are washed off with PBS, and the cultures are examined microscopically for evidence of HAD.

c) Haemagglutinin

The HA subtype of new isolates of equine influenza viruses is best determined by haemagglutination inhibition (HI; Section B.2.a.) using H7N7- and H3N8-specific antisera. Isolates may first be treated with Tween 80/ether, which destroys viral infectivity and reduces the risk of cross-contamination. In the case of H3N8 viruses particularly, this treatment enhances the HA activity (7). However, treatment with Tween 80/ether may increase the variability of the results obtained. Standard antisera must be titrated in parallel with tests to identify viruses and should include H7N7 strains (e.g. A/eq/Prague/56, A/eq/Newmarket/77) and H3N8 strains (e.g. A/eq/Miami/63, A/eq/Fontainebleau/79, A/eq/Newmarket/2/93, and A/eq/Kentucky/94). Virus strains may be obtained from OIE Reference Laboratories (see Table given in Part 3 of this Terrestrial Manual). Additionally, recent isolates from the same geographical area should be included if available. The standard antisera should be treated with Tween 80/ether to avoid cross-contamination. Test antisera and standard antisera are always back-titrated to confirm their antigen content.

New isolates of equine influenza viruses may be further characterised by HI using strain-specific antisera. The species in which antibodies are raised will influence the cross-reactivity of the antiserum, with ferrets providing the most strain-specific antibody (11).

All isolates should be sent immediately to an International Reference Laboratory designated by OIE or the World Health Organization (WHO) for inclusion in the strain surveillance programme to monitor antigenic drift and emergence of new viruses.

d) Neuraminidase

Typing of neuraminidase requires specific antisera and no routine technique is available. Such typing is best done, therefore, in reference laboratories.

e) Detection of viral antigen by enzyme-linked immunosorbent assay

In situations where laboratory facilities for virus isolation are unavailable, influenza virus antigen in nasal secretions may be detected directly by an antigen-capture ELISA for the H3N8 virus employing a monoclonal antibody (MAb) against the nucleoprotein (2, 9). Reagents may be obtained from the OIE Reference Laboratory, Newmarket (see Table given in Part 3 of this Terrestrial Manual). Commercial self-contained kits for detecting human influenza are available and have been shown to cross-react with equine influenza (1).
This approach provides a rapid result on which management decisions may be based. It should not be used in preference to virus isolation, as it is essential that new viruses be isolated and sent to reference laboratories for characterisation as part of the surveillance programme to monitor antigenic drift and emergence of new viruses and to provide isolates for inclusion in updated vaccines. Positive ELISA results are useful in the selection of samples if resources are limited or if samples must be sent to a reference laboratory for virus isolation attempts.

- **Test procedure**
  i) Dispense 100 µl/well of nasal swab extract into triplicate wells of microstrips coated with polyclonal rabbit anti-H3N8, and incubate for 90 minutes at 22°C (±2°C). Wash the microstrips three times with PBS containing 0.05% (v/v) Tween 20 (PBST).
  ii) Add 100 µl/well of MAb–horseradish-peroxidase conjugate diluted 1/150 in dilution buffer then incubate for 1 hour at 37°C. Wash the microstrips six times with PBST.
  iii) Add 100 µl/well of tetramethyl benzidine and incubate for 10 minutes at 22°C (±2°C). Stop the reaction by adding 100 µl/well of 0.18 M sulphuric acid, and measure the absorbance at 450 nm.

2. **Serological tests**

Infections are detected by performing serological tests on paired sera to show a rise in antibody titre. These tests should be carried out whether virus isolation has been attempted or not. Two simple methods exist, HI and single radial haemolysis (SRH), each equally efficient and widely used. The complement fixation (CF) test can also be applied, but is not in general use. Both of the paired serum samples should be tested together at the same time to minimise variability. The standard antigens are described above (Section B.1.c.). If available, isolates from recent cases should be included. Freeze-dried post-infection equine antisera to A/eq/Newmarket/77 (H7N7), A/eq/Newmarket/1/93 ('American-like' H3N8) and A/eq/Newmarket/2/93 ('European-like' H3N8) and an influenza-negative equine serum, are available from the OIE Reference Laboratory, Newmarket (see Table given in Part 3 of this Terrestrial Manual). These sera have been assigned SRH values through an international collaborative study and can be used as primary reference sera for this assay.

a) **Haemagglutination inhibition test**

The antigen is first treated with Tween 80/ether in order to increase the sensitivity of the test, particularly for H3N8 viruses. The test is best done in microtitre plates using the appropriate dilution equipment. A macrotest may be used, for which antigen is diluted to a final HA titre of 1/8 per well and the volumes for PBS, sera and antigen are 0.5 ml. Sera are pretreated to remove nonspecific haemagglutinins, and inactivated at 56°C for 30 minutes. Pretreatments include the use of one of the following: (a) kaolin and RBCs absorption, (b) potassium periodate, or (c) *Vibrio cholerae* receptor-destroying enzyme. All three procedures yield similar results. The treated sera are diluted in PBS, a standard dose of antigen is added (HA titre of 1/4 per well for microtitration assay), and these are kept at 22°C (±2°C) for 30 minutes. After gentle mixing, RBCs are added and the test is read 30 minutes later. The HI titre is read as the highest dilution of serum giving complete inhibition of agglutination. Either chicken RBCs (1% [v/v] packed cells) in V-bottomed microtitre plates or guinea-pig RBCs (0.5% [v/v] packed cells) in V- or U-bottomed plates may be used. If chicken RBCs are used, the plates may read by tilting to 70° so that non-agglutinated cells ‘stream’ to the bottom of the well. Non-agglutinated guinea-pig cells appear as a ‘button’ in the bottom of the well and may take longer to settle. Titre increases of fourfold or more between paired sera indicate recent infection (21).

- **Tween 80/ether treatment of the virus**
  i) To 40 ml of infective allantoic fluid, add 0.5 ml of a 10% (v/v) suspension of Tween 80 in PBS to give a 0.125% (v/v) concentration of Tween 80.
  ii) After mixing gently at room temperature for 5 minutes, add 20 ml of diethyl ether to give a final concentration of 33.3% by volume, and mix the suspension well at 4°C for 15 minutes.
  iii) After allowing the layers to separate by standing, remove the aqueous layer containing the disrupted virus particles to a glass bottle with a loose lid and allow the excess ether to evaporate off overnight (9).
  iv) Store treated virus in aliquots at –70°C.

- **Titration of haemagglutination**
  i) Add 25 µl of PBS to all wells in a row of a microtitre plate.
  ii) Add 25 µl of virus to first well (dilution = 1/2) and titrate through, leaving the last well as a control.
  iii) Add an extra 25 µl of PBS to all wells.
iv) Add 50 µl of RBCs to all wells. Leave at 22°C (±2°C) for 30 minutes. The HA titre is taken as the last virus dilution giving partial HA.

• Pretreatment of sera
  i) Mix one volume (150 µl) of serum with two volumes (300 µl) of freshly prepared 0.016 M potassium periodate (0.38 g in 100 ml PBS), and leave at 22°C (±2°C) for 15 minutes.
  ii) Add a further one volume of 3% glycerol in PBS to neutralise any excess periodate solution, mix and leave at 22°C (±2°C) for 15 minutes.
  iii) Inactivate in a 56°C water bath for 30 minutes.

• Test procedure
  i) Dispense 25 µl of PBS to all wells of a microtitre plate.
  ii) Add serum (25 µl) to the first well of a row of 12, and titrate through, leaving the last well as a control (1/8 to 1/512, allowing for dilution of 1/4 from treatment of serum).
  iii) Dilute the antigen to give a dose of 4 HA units (4 × minimum agglutinating dose, i.e. titre/4).
  iv) Add 25 µl to each well, and incubate at 22°C (±2°C) for 30 minutes.
  v) Add 50 µl of RBCs to each well. Leave at 22°C (±2°C) for 30 minutes.
  vi) The plates may be read by tilting to 70° so that non-agglutinated cells ‘stream’ to the bottom of the well. No agglutination is recorded as a positive result.

b) Single radial haemolysis

In this test, viral antigens are coupled to fixed RBCs that are suspended in agarose containing guinea-pig complement (C'). Wells are punched in the agarose and filled with test sera. Influenza antibodies and C' lyse the antigen-coated RBCs, resulting in a clear, haemolytic zone around the well; the size of this zone is directly proportional to the level of strain-specific antibody in the serum sample (10, 19, 20).

Special immunodiffusion plates (Hyland, Miles Scientific) may be used for the assay, but simple Petri dishes are also suitable. Sheep RBCs collected into Alsever's solution are washed three times. The C' can be obtained commercially, or normal guinea-pig serum can be used. The antigens are allantoic fluids or purified preparations; the strains used are the same as for the HI tests. The viruses are coupled to RBCs by potassium periodate or by chromic chloride. The coupled antigen/RBCs preparations are mixed with C', together with a 1% solution of agarose (low melting grade) in PBS. Care must be taken to ensure that the temperature is not allowed to rise above 42°C at any time. The mixture is poured into plates and left overnight at 4°C. Wells of 3 mm in diameter and 12 mm apart are punched in the solidified agarose, at least 6 mm from the edge of the plates. Such plates may be stored at 4°C for 12 weeks. Plates are prepared for each antigen and pretested with known positive and negative antisera.

Sera are inactivated at 56°C for 30 minutes, but no further treatment is necessary. Paired sera should be assayed in duplicate on the same plate. As a minimum, a subtype-specific antiserum should be included as a control serum in one well on each plate. All sera are tested in a control plate containing all components except virus to check for nonspecific lysis. Alternatively, an unrelated virus, such as A/PR/8/34 (H1N1), may be used in the control plate. Sera that show haemolytic activity for sheep RBCs must be pre-absorbed with sheep RBCs. Zones of lysis should be clear and not hazy or translucent. All clear zones should be measured and the area of haemolysis calculated.

• Preparation of reagents
  i) Saline/HEPES: 0.85% NaCl (4.25 g/500 ml); 0.05 M HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid; 5.95 g/500 ml); and 0.02% sodium azide. Make to pH 6.5 with NaOH.
  ii) Saline/HEPES/BSA: as saline/HEPES with 0.2% (w/v) bovine serum albumin (BSA).
  iii) CrCl₃ stock solution (2.25 M) 6 g/10 ml: Make fresh 1/400 dilution in 0.85% NaCl for each assay.
  iv) PBS (London)/PBS ‘A’: NaCl (10.00 g); KCl (0.25 g); Na₂PO₄ (1.45 g); KH₂PO₄ (0.25 g); and Na azide (0.20 g). Make up to 1 litre with distilled water.
  v) Agarose in PBS: Place flask containing PBS ‘A’ on a stirrer. Slowly add 10 g agarose to the stirring solution. Liquefy in a pressure cooker. Dispense into glass bottles for storage at 22°C (±2°C).
  vi) Virus antigen: Allantoic fluid containing infectious virus is harvested and stored at –70°C. A short titration curve determines the optimum ratio of virus antigen to RBCs to be used when preparing sensitised sheep RBCs. The H7N7 influenza strains always produce clear zones; the H3N8 strains...
sometimes produce hazy zones, in which case it is necessary to concentrate the virus by centrifugation.

vii) Sheep blood: Collect blood into an equal volume of Alsever’s solution and store at 4°C. It may be necessary to test bleed several sheep, as characteristics of RBCs from individual sheep vary. Keep the blood for 2 days before use, it may then be usable for up to 3 weeks, providing sterility is maintained.

viii) Complement: Use commercially available guinea-pig complement or collect serum from young guinea-pigs of 300–350 g body weight and store in small volumes at –70°C. For use, thaw in cold water and hold at 4°C prior to mixing.

ix) Treatment of sera: Use undiluted sera heat inactivated at 56°C for 30 minutes. Avoid repeated freeze-thaw cycles.

• Test procedure
i) Wash sheep RBCs at least three times in saline/HEPES.
ii) Prepare an appropriate volume of 8% RBCs (v/v packed cells) in saline/HEPES, having first calculated the number of plates required and allowing 1 ml per 6 x 11 cm immunoplate and 1–2 ml extra.
iii) Add a predetermined volume\(^1\) of virus antigen to the 8% RBCs solution. Hold the mixture at 4°C for 10 minutes. Haemagglutination may be observed.
iv) SLOWLY add CrCl\(_3\) (1/400 in 0.85% NaCl) at half the total volume of virus/RBCs suspension. Hold at 22°C (±2°C) for 5 minutes with occasional mixing.
v) Sediment the sensitised RBCs by centrifugation at 1500 \(g\) for 5 minutes.
vi) Gently resuspend in saline/HEPES/BSA and centrifuge at 1500 \(g\) for 5 minutes.

During the sensitisation process, melt the agarose in a pressure cooker. Shortly before use, pipette 7.8 ml volumes to Universal bottles and retain at 42°C. Check that the agar has cooled to 42°C before use.

• Preparation of plates
i) Add 0.9 ml of virus-sensitised sheep RBCs to 7.8 ml of agarose (42°C). Mix quickly, but gently.
ii) Add 0.3 ml of undiluted guinea-pig serum. Mix again and pour into immunoplates on a levelling table. Allow to set and air dry without a lid for 5 minutes.
iii) Place lids on plates and store at 4°C in a humid box until used.
iv) Prepare control plates with unsensitised cells or cells sensitised with an unrelated virus. Batches of prepared plates can be stored for several weeks.
v) Punch 3 mm holes in the set gels to a prepared template, allow for 16 test sera and a positive control serum. On antigen control plates, prepare five rows of eight wells.
vi) Pipette 10 µl of heat-inactivated (56°C for 30 minutes) test sera and a positive control serum to appropriate wells. Incubate at 34°C for 20 hours in a humid box.

For results to be valid, positive and negative control sera should give results consistent with those expected on the basis of prior experience. Areas of haemolysis for the control sera should be clear and vary no more than 20% from the designated value for the control serum. Results may be expressed as mm\(^2\) or as a ratio of the control serum value. Sera giving positive results in the control plate should be adsorbed with sheep RBCs. For diagnostic purposes, acute and convalescent sera should be tested in duplicate on the same plate. Increase in zone areas produced by convalescent serum compared with acute serum is evidence of infection. The increase in area deemed to be significant depends on the reproducibility of the test within the laboratory, but should be equivalent to a twofold or more increase in antibody concentration. This area can be calculated from a standard curve generated from a dilution series of a standard antiserum.

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1 Prepare three plates by adding 0.6, 1.2 or 1.8 ml of virus antigen to 2 ml RBCs. Add 1.3, 1.6 and 1.9 ml CrCl\(_3\) respectively and resuspend to 2 ml in PBS ‘A’. Optimum volume of virus antigen is that which results in the largest and clearest zones with appropriate reference serum.
C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Equine influenza virus vaccines consist of inactivated whole viruses or their subunits, with or without adjuvant. A live attenuated influenza vaccine for intranasal administration has recently become available commercially in some countries. Requirements for such a vaccine may be anticipated to differ in some particulars from the following, which are appropriate to inactivated vaccines.

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

An ongoing surveillance programme by OIE and WHO Reference Laboratories aimed at providing information on suitable vaccine strains is being co-ordinated by the OIE Reference Laboratory (Newmarket) (15). Recommendations on vaccine strains made by the Expert Surveillance Panel will be published annually in the OIE Bulletin.

H7N7: Many vaccines still contain an H7N7 strain. However, the Expert Surveillance Panel has recommended that the H7N7 component should be omitted as no reports of infections with this subtype have been substantiated during the past 20 years.

H3N8: Antigenic variants of H3N8 viruses co-circulate (3). It is important to include recent isolates that are epidemiologically relevant to the region in which the vaccine is to be used. Typical European isolates include A/eq/Suffolk/89, A/eq/Borlange/91, and A/eq/Newmarket/2/93. A/eq/Kentucky/94 or A/eq/Newmarket/1/93 represent typical American isolates. However, it is recommended that vaccines contain representatives of both lineages (American-like and European-like), particularly where equine populations from different continents mix. Some vaccines also contain the original H3N8 prototype strain A/eq/Miami/63. However, this strain will not induce adequate protection against the predominant viruses currently circulating and should be omitted from vaccines.

b) Method of culture

Virus strains may be obtained from OIE Reference Laboratories (see Table given in Part 3 of this Terrestrial Manual). Viruses selected as vaccine strains should be described in terms of origin and passage history. The strains are propagated in the allantoic cavity of 10-day-old embryonated hens’ eggs or cell cultures, such as MDCK. All manipulations must be conducted separately for each strain. Viral growth is monitored by HA tests. Passed virus is identified by serological tests, such as HI or SRH. If vaccine virus is grown in cell culture, antigenic studies with ferret sera and MAbs should be undertaken to ensure that variant viruses have not been selected during passage to prepare master and working seed viruses. Master and working seed viruses are divided into aliquots and stored in freeze-dried form at –20°C or at –70°C following testing for extraneous agents. Records of storage conditions should be maintained.

The master seed lot of each vaccine strain selected should be processed at one time to assure a uniform composition, tested for extraneous agents, and fully characterised. Antisera or MAbs for use in HI tests to characterise vaccine strains may be obtained from OIE and WHO Reference Laboratories.

Working seed lots are derived from a master seed lot and should be of uniform composition, free from extraneous agents, and fully characterised. Aliquots of the working seed are used for production of vaccine.

Master and working seed lots should be prepared in specific pathogen free eggs or, as a minimum, in eggs derived from a healthy flock.

If MDCK cells are used to propagate vaccine virus, master cell lines should be established and stored in liquid nitrogen, and should be tested for freedom from extraneous agents according to National Control Authority Guidelines.

Examination of seed viruses for extraneous agents including mycoplasmas and other equine viruses should be performed by appropriate techniques, including inoculation of susceptible tissue cultures and examination for cytopathic effect or application of fluorescent antibodies for antigen detection.

The presence of other common equine respiratory pathogens, e.g. equine herpesviruses 1, 2, 4, equine picornaviruses, equine viral arteritis, and equine adenoviruses, should be specifically excluded.

The absence of bacteria should be confirmed by standard sterility tests and toxicity tests in small animals.
c) Validation as a vaccine

For each vaccine strain, a prototype batch should be prepared to establish its suitability as a vaccine strain, i.e. purity and safety should be confirmed by standard techniques. The ability of seed-lot viruses to grow to high titre and generate sufficient antigenic mass to stimulate adequate antibody responses in the target species, should be confirmed.

Additionally, vaccine virus derived in MDCK cells should be fully characterised to ensure that antigenic variants have not arisen during the culture process, such that the vaccine virus is no longer representative of the original isolate.

2. Method of manufacture

Production is based on a seed-lot system that has been validated with respect to the characteristics of the vaccine strains. Each strain of virus is inoculated separately into the allantoic cavity of 9–11-day-old embryonated hens’ eggs from a healthy flock. The eggs are incubated at a suitable temperature for 2–3 days, and the allantoic fluid is collected. The viral suspensions of each strain are collected separately and inactivated. If necessary, they may be purified. Suitable adjuvants and antimicrobial preservatives may be added.

Monovalent virus pools should be inactivated as soon as possible after their preparation, by a method approved by the National Control Authority. If formalin (40% formaldehyde) or beta-propiolactone (2-oxetanone) is used, the concentration by volume should not exceed 0.1%. Ideally, pools should be held at 4°C and should be inactivated within 5 days of harvest. Inactivation of the vaccine must be demonstrated. A suitable method consists of inoculating 0.2 ml of undiluted monovalent pool and 1/10 and 1/100 dilutions of the monovalent pool into the allantoic cavities of groups of fertile eggs (ten eggs in each group), and incubating the eggs at 33–37°C for 3 days. At least eight of the ten eggs should survive at each dosage level. A volume of 0.5 ml of allantoic fluid is harvested from each surviving egg. The fluid harvested from each group is pooled, and 0.2 ml of each of the three pools is inoculated, undiluted, into a further group of ten fertile eggs. Haemagglutinin activity should not be detected in these new groups of eggs. In some countries, the requirement that 80% of the eggs should survive during incubation may be impossible to satisfy, in which case the National Control Authority should then specify a modified requirement to be satisfied. Before inactivation, samples should be collected for bacterial and fungal sterility tests.

Monovalent material may be concentrated and purified by high-speed centrifugation or other suitable methods approved by the National Control Authority, either before or after the inactivation procedure. It is important to concentrate and purify the virus under optimum conditions, e.g. temperatures that preserve its antigenic properties.

The monovalent virus pool shall be shown not to contain viable influenza virus when tested by inoculation of embryonated hens’ eggs, by a method approved by the National Control Authority.

3. In-process control

Relevant in-process controls should be applied before and after inactivation and before and after concentration and purification.

In-process controls include: (a) identity of virus strains (tested by HI); (b) sterility; (c) virus titre; (d) haemagglutinin content (tested by chicken RBCs agglutinating units, CCA [chick cell agglutination]); and (e) immunologically active HA (tested by single radial diffusion [SRD] or another suitable immunochemical method).

- Single radial diffusion test

SRD is a reliable method for measuring immunologically active HA in terms of µg HA, and is used routinely for potency testing human influenza vaccines (24).

The potency of inactivated equine influenza vaccine depends on the concentration of immunologically active haemagglutinin (16, 22, 23).

Assessment of the antigenic content of the vaccine by CCA alone may be misleading, as the sensitivity of this assay is a reflection of the ability of virus strains to agglutinate RBCs. Disruption of virus may lead to an apparent increase in HA as measured by CCA. The CCA assay does not provide a measure of the antigenic properties of the HA (HA may retain its properties to bind to RBCs while losing its ability to stimulate antibody).

The composition of some equine influenza vaccines is unusual in that products may contain more than one variant of the H3N8 subtype. In this situation, it is not possible to judge the potency of individual H3N8
components from serological tests performed on sera collected from horses or small animals vaccinated with the final product, because of cross-reactivity between the two isolates of the same subtype. Thus, it is important that a reliable method, such as SRD, be used to measure the potency of individual components before and after inactivation and prior to mixing and formulation with adjuvant.

In the SRD test, virus preparations are compared with a calibrated reference preparation of known HA content. Antigens are allowed to diffuse through a gel containing an antiserum specific for a particular HA. The distance diffused by the antigen before precipitation by the antibody incorporated in the gel is directly related to the concentration of haemagglutinin in the antigen preparations (13).

Standard reagents for SRD testing are available from the WHO International Laboratory for Biological Standards2. Reagents for A/eq/Prague/56 (H7N7), and the H3N8 strains A/eq/Miami/63, A/eq/Kentucky/81, A/eq/Newmarket/1/93 (‘American-like’) and A/eq/Newmarket/2/93 (‘European-like’) are currently available.

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
i) Using no fewer than three horses, each horse is inoculated intramuscularly (at two different sites) with the dose of vaccine specified by the manufacturer; these inoculations are repeated 2–4 weeks later. The animals are kept under observation for 10 days after the second set of injections. No abnormal local or systemic reaction should ensue.

ii) If vaccine is to be used in mares, safety should be demonstrated by giving two doses of vaccine to no fewer than two pregnant mares at the prescribed interval within the trimester for which the vaccine is recommended.

c) Potency
Following mixing of viral antigens and adjuvants, aliquots should be potency tested in vivo using horses and guinea-pigs or a suitable alternative immunochemical assay. Adjuvants cause interference in quantitative in-vitro tests, such as CCA and SRD, although SRD may be used on the final product as a qualitative assay to demonstrate the presence of antigen for each vaccine strain. For repeated batch tests, only guinea-pigs or a suitable alternative immunochemical assay are used, subject to agreement of the National Control Authority.

i) Serological responses in horses
For a valid in-vivo potency test, naive seronegative horses must be selected for vaccination. Young horses or ponies (not less than 6 months old) should be screened for the presence of antibody using recently isolated viruses relevant to the area in which the horses were reared. If HI tests are used for screening, H3N8 viruses should be treated with Tween 80/ether to maximise the sensitivity of the test. Alternatively, SRH may be used to establish the seronegative status of animals to H7N7 and H3N8 viruses.

To test a vaccine for efficacy in horses, inject a volume corresponding to one vaccine dose by the recommended route into each of five susceptible seronegative horses. After the period recommended between the first and second doses, as stated on the label, a volume of vaccine corresponding to the second dose of vaccine is injected into each horse.

Three blood samples are collected from each animal, the first at the time of the first vaccination, the second 1 week after the first vaccination, and the third 2 weeks after the second vaccination.

The serological assay used to measure the antibody response to the viruses contained in the vaccine must be standardised for a valid in-vivo potency test, therefore the SRH assay (see Section B.2.b.) is preferred. Standard sera for the quality control of equine influenza vaccines are available from the European Pharmacopoeia3. These sera should be tested in parallel with the test sera to ensure that the test is valid with respect to sensitivity; the values obtained should not vary by more than 20% from the SRH values assigned in an international collaborative study (12). Due to poor repeatability and reproducibility of the HI test, no HI titre could be assigned to these sera.

2 National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK.
3 Serum to A/eq/Newmarket/1/77 (Catalogue number E0850010), A/eq/Newmarket/1/93 (E0850021) and A/eq/Newmarket/2/93 (E0850022).
The antibody value measured by SRH should not be less than 150 mm\(^2\). This is higher than the titre required in the European Pharmacopoeia Monograph for inactivated equine influenza vaccines (85 mm\(^2\)) as this value is not considered to be protective. If the titre found for any horse after the first vaccination indicates that there has been an anamnestic response, the result is not taken into account. A supplementary test is carried out, as described above, replacing the horses that showed an anamnestic response with an equal number of new animals.

If the HI test is used, the antibody titre of each serum taken after the second vaccination in each test should not be less than 1/64 (calculated for the original serum, taking into account the predilution of 1/8). Alternatively, the antibody levels stimulated by the vaccine under test should be shown to be at least equal to the antibody levels stimulated by a standard vaccine tested in parallel that has been shown previously to protect horses against challenge infection.

ii) **Challenge studies in horses**

It may be desirable in certain cases to undertake challenge studies in horses to demonstrate potency, particularly if vaccines are being assessed for their ability to protect against antigenically dissimilar viruses. Challenge studies may be carried out by exposing six vaccinated horses/ponies to an aerosol of virulent influenza virus no fewer than 2 weeks after the second dose of vaccine. Comparisons of clinical signs, virus excretion and serological responses are made with a group of no fewer than four unvaccinated control animals challenged at the same time (13, 14). The timing of the challenge procedure will reflect the claims to be made on the data sheet regarding duration of immunity.

If tests for potency in horses have been carried out with satisfactory results on a representative batch of vaccine, these tests may be omitted as a routine control on other batches of vaccine prepared using the same seed-lot system, subject to agreement by the National Control Authority.

iii) **Serological responses of guinea-pigs**

Inject each of no fewer than five guinea-pigs free from specific antibodies with one vaccine dose. Collect blood samples 21 days later, and test the serum by SRH or HI (see Sections B.2.a. and B.2.b.). Perform the tests of each serum using, respectively, the antigen(s) prepared from the strain(s) used in the production of the vaccine. The antibody titre of each serum in each test should not be less than the titre stimulated by a standard vaccine that has been shown to stimulate protective levels of antibody in horses.

d) **Duration of immunity**

Where claims for duration of immunity are made on the data sheet, these should be supported with data on the duration of protective levels of antibody maintained in horses vaccinated according to the recommended schedule. Antibody levels quoted as protective should be validated in challenge studies (see Section C.4.c.ii.) or by comparison with published reports.

e) **Stability**

Vaccines should be stored at 5±3°C and protected from light. The shelf life quoted on the data sheet should be demonstrated by testing the potency of aliquots over time using the guinea-pig potency test (see Section C.4.c.iii.).

f) **Preservatives**

Preservatives are not normally included.

g) **Precautions (hazards)**

The contents of each opened vial should be used within 1 hour of opening. Aseptic precautions should be observed during administration, and only healthy horses should be vaccinated. Occasionally, transient local and or general reactions may occur, and rest may be advisable for 24–48 hours after vaccination.

5. **Tests on the final product**

a) **Safety**

Tests are performed as described in Section C.4.b.i. Once safety has been demonstrated on a prototype batch, safety testing in pregnant mares may be omitted for routine testing of subsequent batches of the final product.

b) **Potency**

See Section C.4.c.iii. As a minimum, serological testing in guinea-pigs should be performed on each batch of the final product.
c) **Maintaining epidemiologically relevant strains in vaccines**

To enable vaccine manufacturers to respond quickly to recommendations from the Expert Surveillance Panel to update vaccine strains, the Committee for Veterinary Medicinal Products for the European Agency for the Evaluation of Medicinal Products has developed a fast-track licensing system to be used when vaccine strains are updated (5).

**REFERENCES**


* * *

NB: There are OIE Reference Laboratories for Equine influenza 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 2.5.6.

EQUINE PIROPLASMOSIS

SUMMARY

Equine piroplasmosis or babesiosis is a tick-borne protozoal disease of horses, mules, donkeys and zebra. The aetiological agents are blood parasites named Theileria equi and Babesia caballi. Theileria equi was previously designated as Babesia equi. Infected animals may remain carriers of these parasites for long periods and act as sources of infection for ticks, which act as vectors. The parasites are found inside the red blood cells of the infected animals.

The introduction of carrier animals into areas where tick vectors are prevalent can lead to an epizootic spread of the disease.

Identification of the agent: Infected horses can be identified by demonstrating the parasites in stained blood or organ smears. Romanovsky-type staining methods, such as Giemsa, give the best results. In carrier animals, low parasitaemias make it extremely difficult to detect parasites, especially in the case of B. caballi infections, although they may sometimes be demonstrated by using a thick blood smear technique.

Paired merozoites joined at their posterior ends are a diagnostic feature of B. caballi infection. The merozoites of T. equi are less than 2–3 µm long, and are round or amoeboid. A characteristic of T. equi is the arrangement of four parasites in a tetrad or ‘Maltese cross’.

When equivocal results are encountered in serological tests, the inoculation of a large quantity of whole blood transfused into a susceptible splenectomised horse will assist diagnosis. The recipient horse is observed for clinical signs of disease and its red blood cells are examined for parasites. Alternatively, a specific tick vector is fed on a suspect animal and Babesia/Theileria may then be identified either in the vector or through transmission by the vector to another susceptible animal.

Serological tests: Infections in carrier animals are best demonstrated by testing their sera for the presence of specific antibodies.

Currently, the complement fixation (CF) test is the primary test used for qualifying horses for importation. Other serological techniques are the indirect fluorescent antibody (IFA) test and enzyme-linked immunosorbent assays (ELISAs). The IFA test can be used to distinguish between T. equi and B. caballi infections. ELISA may be used to detect antibodies to both species in infected horses, although cross-reactions between T. equi and B. caballi occur, so that this cannot yet be recommended as a differential diagnostic test. However, recent advances using a recombinant T. equi and B. caballi merozoite proteins and monoclonal antibodies to these proteins in competitive inhibition ELISA appear to be very promising. The competitive ELISA may be superior to CF tests, especially for detecting long-term infected animals in which the CF titre has waned but which remain IFA seropositive. Unlike the indirect ELISA, the competitive ELISA has been shown to be highly specific for each of the two species of piroplasmosis agent involved.

Requirements for vaccines and diagnostic biologicals: There are no biological products available.

A. INTRODUCTION

Equine piroplasmosis or babesiosis is a tick-borne protozoal disease of horses, mules, donkeys and zebra. The aetiological agents of equine piroplasmosis are Theileria equi and Babesia caballi. Twelve species of ixodid ticks in the genera Dermacentor, Rhipicephalus and Hyalomma have been identified as transstadial vectors of B. caballi and T. equi, while eight of these species were also able to transmit B. caballi infections transovarially
Infected animals may remain carriers of these blood parasites for long periods and will act as sources of infection for tick vectors.

The parasites occur in southern Europe, Asia, countries of the Commonwealth of Independent States, Africa, Cuba, South and Central America, and certain parts of the southern United States of America. *Theileria equi* has also been reported from Australia (but, apparently never established itself in this region), and is now believed to have a wider general distribution than *B. caballi*.

During the life cycle of *Babesia*, the merozoites invade red blood cells (RBCs) where they transform into trophozoites (7, 29). In this situation the trophozoites grow and divide into two round, oval or pear-shaped merozoites. The mature merozoites are now capable of infecting new RBCs and the division process is then repeated.

*Babesia caballi*: the merozoites in the RBCs are pear-shaped, 2–5 µm long and 1.3–3.0 µm in diameter (20). The paired merozoites joined at their posterior ends are considered to be a diagnostic feature of *B. caballi* infection (25).

*Theileria equi*: the merozoites of this organism are relatively small, less than 2–3 µm long (20), and are round or amoeboid. Four parasites are to be found together in the form of a tetrad or so-called ‘Maltese cross’ arrangement. This is a characteristic feature of *T. equi* (10).

It has been shown that sporozoites inoculated into horses via a tick bite invade the lymphocytes (32). The sporozoites undergo development in the cytoplasm of these lymphocytes and eventually form *Theileria*-like schizonts. Merozoites released from these schizonts enter RBCs. The taxonomic position of *T. equi* has been controversial and only relatively recently has it been redescribed as a *Theileria* (26). Further support for the close relation with *Theileria* spp. also comes from the homology found between 30 and 34 kDa *T. equi* surface proteins and similar sized proteins of various *Theileria* spp. (15, 17). However, comparison of the small subunit ribosomal RNA genes of various *Babesia*, *Theileria* and *Cytauxzoon* parasites indicates that *T. equi* falls into a distinct group different from both the *Babesia* and *Theileria* groups (1).

The clinical signs of equine piroplasmosis are often nonspecific, and the disease can easily be confused with other conditions.

Piroplasmosis can occur in peracute, acute and chronic forms. The acute cases are more common, and are characterised by a fever that usually exceeds 40°C, reduced appetite and malaise, elevated respiratory and pulse rates, congestion of mucous membranes, and faecal balls that are smaller and drier than normal.

Clinical signs in subacute cases are similar. In addition, affected animals show loss of weight, and the fever is sometimes intermittent. The mucous membranes vary from pale pink to pink, or pale yellow to bright yellow. Petechiae and/or ecchymoses may also be visible on the mucous membranes. Normal bowel movements may be slightly depressed and the animals may show signs of mild colic. Mild oedematous swelling of the distal part of the limbs sometimes occurs.

Chronic cases usually present nonspecific clinical signs such as mild inappetence, poor performance and a drop in body mass. The spleen is usually found to be enlarged on rectal examination.

A rare peracute form where horses are found either dead or moribund has been reported (21).

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

Horses that are already infected may be identified by demonstrating the parasites in stained blood or organ smears. Romanovsky-type staining methods, such as the Giemsa method, usually give the best results (34).

The low parasitaemias of carrier animals make it extremely difficult to detect the parasites in smears, especially in the case of infections with *B. caballi*. When they occur at such low levels, experienced workers can sometimes detect them by the use of a thick blood smear technique (23). 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 5% Giemsa for 20–30 minutes. An accurate identification of the species of the parasite is sometimes desirable, as mixed infections of *T. equi* and *B. caballi* probably occur frequently.

It is very difficult to diagnose equine piroplasmosis in carrier animals by detection of parasites in the blood and serological methods are preferred for this (see below). However, false-negative or false-positive reactions may
be encountered in the course of serological tests (5, 6, 38). In such cases, the passage of whole blood, although a cumbersome and expensive exercise, is a very useful technique to determine the true position. Large quantities of whole blood (500 ml) are transfused into a susceptible, preferably splenectomised, horse. This animal is then kept under close observation for clinical signs of disease. Diagnosis is confirmed by the presence of parasites in its RBCs.

In an additional technique, a specific clean tick vector is fed on a suspect animal, and the organism can then either be identified in the tick itself, or through the transmission of the organism by the tick vector to another susceptible animal.

Success in the establishment of long-term in-vitro cultures of *T. equi* and *B. caballi* may be one alternative to supplement the methods described above, in order to identify carriers of the parasites (11, 12, 42, 43). *Babesia caballi* parasites were successfully cultured from the blood of two horses that tested negative by the complement fixation (CF) test (12). Similarly, *T. equi* could be cultured from horses that did not show any patent parasitaemias at the time of the initiation of the cultures (42, 43).

Molecular techniques for the detection of *T. equi* and *B. caballi* have been described (2) including a biotin-labelled DNA probe in a polymerase chain reaction-based assay for the detection of *T. equi* (31).

2. **Serological tests**

It is extremely difficult to diagnose the organisms in carrier animals by means of the microscopic examination of blood smears. Furthermore, it is by no means practical on a large scale. The serological testing of animals is therefore recommended as a preferred method of diagnosis, especially when horses are destined to be imported into countries where the disease does not occur, but the vector is present.

Sera should be collected and dispatched to diagnostic laboratories in accordance with the specifications of that laboratory. Horses for export that have been subjected to serological tests and shown to be free from infection, should be kept free of ticks to prevent accidental infections.

A number of serological techniques have been used in the diagnosis of piroplasmosis, such as the CF test, the indirect fluorescent antibody (IFA) test, and the enzyme-linked immunosorbent assay (ELISA).

a) **Complement fixation (a prescribed test for international trade)**

The CF test is the primary test used by some countries to qualify horses for importation (37). A detailed description of antigen production and a test protocol has been given, for example by the United States Department of Agriculture (USDA) (3, 6, 39). Because the CF test may not identify all infected animals, especially those that have been treated, and because of the anti-complementary reactions produced by some sera, and of the inability of IgG(T) (the major immunoglobulin isotype of equids) to fix complement (24), the IFA test has also been accepted for use as a prescribed test for international trade. An example of a CF test protocol is given below.

- **Solutions**
  - *Alsever’s solution*: prepare 1 litre of Alsever’s solution by dissolving 20.5 g glucose; 8.0 g sodium citrate; 4.2 g sodium chloride in sufficient distilled water. Adjust to pH 6.1 using citric acid, and make up the volume to 1 litre with distilled water. Sterilise by filtration.
  - *Stock veronal buffer* (5×): dissolve the following in 1 litre of distilled water: 85.0 g sodium chloride; 3.75 g sodium 5,5 diethyl barbituric; 1.68 g magnesium chloride (MgCl₂·6H₂O); 0.28 g calcium chloride. Dissolve 5.75 g of 5,5 diethyl barbituric acid in 0.5 litre hot (near boiling) distilled water. Cool this acid solution and add to the salt solution. Make up to 2 litres with distilled water and store at 4°C. To prepare a working dilution, add one part stock solution to four parts distilled water. The final pH should be from 7.4 to 7.6.

- **Antigen production**
  - Blood is obtained from horses with a high parasitaemia (e.g. 3–7% parasitaemia for *B. caballi* and 60–85% for *T. equi*), and mixed with equal volumes of Alsever’s solution as an anticoagulant. The plasma/Alsever’s supernatant and buffy coat are removed when the RBCs have settled to the bottom of the flask. The RBCs are washed several times with cold veronal buffer and then disrupted. The antigen is recovered from the lysate by centrifugation at 30,900 *g* for 30 minutes.

  The recovered antigen is washed several times in cold veronal buffer by centrifugation at 20,000 *g* for 15 minutes. Polyvinyl pyrrolidone 40,000 (1–5%[ w/v]) is added as a stabiliser and the preparation is mixed...
on a magnetic stirrer for 30 minutes, strained through two thicknesses of sterile gauze, dispersed into 2 ml volumes and freeze-dried. The antigen can then be stored at below –50°C for several years.

- **Test procedure**
  
i) The specificity and potency of each batch of antigen should be checked against standard antisera of known specificity and potency. Optimal antigen dilutions are also determined in a preliminary checkerboard titration.
  
ii) Test sera are inactivated for 30 minutes at 58°C (donkey and mule sera are inactivated at 62.5°C for 35 minutes) and tested in dilutions of 1/5 to 1/5120. Veronal buffer is used for all dilutions.
  
iii) Complement is prepared and titrated spectrophotometrically to determine the 50% haemolytic dose (C'H50) (36) and used in the test at five times C'H50. The haemolytic system consists of equal parts of a 2% sheep (RBC) suspension and veronal buffer with 5 minimum haemolytic doses (MHDs) of haemolysin (amoceptor) (40). Some laboratories use twice the 100% haemolytic dose, which gives equivalent sensitivity.
  
iv) The test has been adapted to microtitration plates (8). The total volume of the test is 0.125 ml, made up of equal portions (0.025 ml) of antigen, complement (five times C'H50) and diluted serum. Incubation is performed for 1 hour at 37°C.
  
v) A double portion (0.05 ml) of the haemolytic system is added and the plates are incubated for a further 45 minutes at 37°C with shaking after 20 minutes.
  
vi) The plates are centrifuged for 1 minute at 200 g before being read over a mirror.
  
vii) A lysis of 50% is recorded as positive, with the titre being the greatest serum dilution giving 50% lysis. A titre of 1/5 is regarded as positive. A full set of controls (positive and negative sera) must be included in each test as well as control antigen prepared from normal (uninfected) horse RBCs.

Anticomplementary samples are examined by the IFA test. Donkey sera are frequently anticomplementary.

b) **Indirect fluorescent antibody test (a prescribed test for international trade)**

The IFA test has been successfully applied to the differential diagnosis of *T. equi* and *B. caballi* infections (22). The recognition of a strong positive reaction is relatively simple, but any differentiation between weak positive and negative reactions requires considerable experience in interpretation. A detailed description of the protocol of the IFA test has been given (22, 27). An example of an IFA protocol is given below.

- **Antigen production**

Blood for antigen is obtained from horses with a rising parasitaemia, ideally 2–5%. Carrier animals are not suitable for antigen production as they have already produced antibodies. Blood (about 15 ml) is collected into 235 ml of phosphate buffered saline (PBS), pH 7.2. The RBCs are washed three times in cold PBS (1000 g for 10 minutes at 4°C). The supernatant fluid and the white cell layer are removed after each wash. After the last wash, the packed RBCs are reconstituted to normal volume with 4% bovine serum albumin fraction V made up in PBS, i.e. the original packed cell volume = 30%, so that one-third consists of RBCs. If the original RBC volume is 15 ml, then 5 ml of packed RBCs + 10 ml of 4% bovine albumin in PBS constitutes the antigen. After thorough mixing, the antigen is placed in prepared wells on a glass slide using a template or a syringe (27). Alternatively, the cells can be spread smoothly on to microscope slides, covering the entire slide with an even, moderately thick film. These slides are allowed to dry, wrapped in soft paper and sealed in plastic bags or wrapped in aluminium foil, and stored at –20°C for up to 1 year.

- **Test procedure**
  
i) Each sample of serum is tested against an antigen of *B. caballi* and of *T. equi*.
  
ii) Prior to use, the antigen smears are removed from storage at –20°C and incubated at 37°C for 10 minutes.
  
iii) The antigen smears are then removed from their protective covering and fixed in cold dry acetone (–20°C) for 1 minute. Commercially produced slides are available that are pre-fixed.
  
iv) If smears were prepared, squares (14–21 in number, i.e. 2–3 rows of 7 each) are formed on the antigen smears with nail varnish or rapidly drying mounting medium (i.e. Cystoseal).
  
v) Test, positive and negative control sera are diluted from 1/80 to 1/1280 in PBS. Negative and positive control sera are included in each test.
  
vi) Sera are applied (10 µl each) at appropriate dilutions to the different wells or squares on the antigen smear, incubated at 37°C for 30 minutes, and washed several times in PBS and once in water.
c) **Enzyme-linked immunosorbent assay**

At the time of publication, the ELISA had been proposed to replace the CF test as the prescribed test for international trade. Please consult the OIE Web site for the most recent version of this chapter.

The production of recombinant antigens for the use in ELISAs has been described. The recombinant T. equi merozoite protein (EMA-1) has been produced in Escherichia coli (18) and in insect cells by baculovirus (41). Recombinant T. equi Be 82 gene product fused with glutathione S-transferase fusion protein antigen has also been produced in E. coli (9). Recombinant B. caballi rhoptry-associated protein antigen has been produced in E. coli (13, 14). Recombinant antigens produced in E. coli or by baculovirus have the obvious advantage of removing the need to infect horses for antigen production, and they provide a consistent source of antigen for international distribution and standardisation. Recombinant antigens have been used in the indirect ELISA (13) and the competitive inhibition ELISA (C-ELISA) (40). EMA-1 and a specific monoclonal antibody (MAb) that defines this merozoite surface protein epitope, have been used in a C-ELISA for T. equi (18). This C-ELISA overcomes the problem of antigen purity, as the specificity of this test depends only on the MAb used. A 94% correlation was shown between the C-ELISA and the CF test in detecting antibodies to T. equi. Sera that gave discrepant results were evaluated for their ability to immunoprecipitate 35S-methionine-labelled in-vitro translated products of T. equi merozoite mRNA. Samples that were C-ELISA positive and CF test negative clearly precipitated multiple T. equi proteins. However, immunoprecipitation results with serum samples that were C-ELISA negative and CF test positive were inconclusive (19). Limited data at this stage would suggest that the C-ELISA is specific for T. equi (19). This C-ELISA for T. equi was also recently validated in Morocco and Israel, giving a concordance of 91% and 95.7% with the IFA test, respectively (30, 33).

A similar C-ELISA has been developed using the recombinant B. caballi rhoptry-associated protein 1 (RAP-1) and an MAb reactive with a peptide epitope of a 60 kDa B. caballi antigen (14). The results of 302 serum samples tested with this C-ELISA and the CF test showed a 73% concordance. Of the 72 samples that were CF test negative and C-ELISA positive, 48 (67%) were shown to be positive on the IFA test, while four of the five samples that tested CF test positive and C-ELISA negative were positive on the IFA test (14).

A test protocol for an equine piroplasmosis C-ELISA has been described and used for additional validation studies (16, 40). The apparent specificity of the B. equi and B. caballi C-ELISAs lay between 99.2% and 99.5% using sera from 1000 horses presumed to be piroplasmosis-free. One thousand foreign-origin horses of unknown infection status were tested by the C-ELISA and the CF test with an apparent greater sensitivity of the C-ELISA. The results were 1.1% (B. equi) and 1.3% (B. caballi) more seropositive animals detected by C-ELISA than by the CF test; the additional positive results were confirmed by IFA testing. Eight experimentally infected horses (four for B. equi, four for B. caballi) were serially tested from 4 to 90 days post-exposure. Both C-ELISA procedures were again found to be more sensitive than the CF test for the detection of infected animals; the results were confirmed by IFA testing. Seroconversion was detected by C-ELISA as soon as or sooner than by the CF test. Both tests were highly reproducible well-to-well, plate-to-plate, and day-to-day, with overall variances of ± 1.2% and ±1.6% for the B. equi and B. caballi tests, respectively.

An example of a C-ELISA protocol is given below.

- **Solutions**

  **Antigen coating buffer:** prepare the volume of antigen coating buffer required by using the following amounts of ingredients per litre: 2.93 g sodium bicarbonate; 1.59 g sodium carbonate; sufficient ultra-pure water to dissolve, and make up to 1 litre with ultra-pure water. Adjust to pH 9.6.

  **C-ELISA wash solution (high salt diluent):** prepare the volume of C-ELISA wash solution required by using the following amounts of ingredients per litre: 29.5 g sodium chloride; 0.22 g monobasic sodium phosphate; 1.19 g dibasic sodium phosphate; 2.0 ml Tween 20; sufficient ultra-pure water to dissolve, and make up to 1 litre with ultra-pure water. Mix well. Adjust pH to 7.4. Sterilise by autoclaving at 121°C.
Antigen production

Frozen transformed *E. coli* culture is inoculated at a 1/10,000 dilution into any standard non-selective bacterial growth broth (e.g. Luria broth) containing added carbenicillin (100 µg/ml) and isopropylthiogalactoside (IPTG, 1 mM). Cultures are incubated on an orbital shaker set at 200 rpm at 37°C overnight. Cells grown overnight are harvested by centrifugation (5000 g for 10 minutes), washed in 50 mM Tris/HCl and 5 mM ethylene diamine tetra-acetic acid (EDTA) buffer, pH 8.0, and harvested again as before. (Antigen is available from the National Veterinary Services Laboratories, P.O. Box 844, Ames, Iowa 50010, USA.)

Cells are resuspended to 10% of the original volume in the Tris/EDTA buffer to which 1 mg/ml of lysozyme has been added, and incubated on ice for 20 minutes. Nonidet P-40 detergent (NP-40) is then added to a final 1% concentration (v/v), vortexed, and the mixture is incubated on ice for 10 minutes. The material is next sonicated four times for 30 seconds each time at 100 watts, on ice, allowing 2 minutes between sonications for the material to remain cool. The sonicate is centrifuged at 10,000 g for 20 minutes. The resulting supernatant is dispensed in 0.5 ml aliquots in microcentrifuge tubes and may then be stored at −70°C for several years. The presence of heterologous host bacterial antigens does not interfere with the binding of specific equine anti-piroplasma antibodies or the binding of the paired MAbs to their respective expressed recombinant antigen epitopes and is confirmed by the following procedures. The antigen-containing supernatants are quality controlled by titrating them with their paired MAbs and with reference monospecific equine antisera to verify both an adequate level of expression and complete specificity for the homologous species of piroplasmosis agent. Normal serum (negative serum) controls must not interfere with binding of the monoclonals or positive equine reference sera to the expressed antigen preparation.

Test procedure

i) Microtitration plates are prepared by coating the wells with 50 µl of either *B. equi* antigen or *B. caballi* antigen diluted in antigen-coating buffer. The dilution used is determined by standard serological titration techniques. The plate is sealed with sealing tape, stored overnight at 4°C, and frozen at −70°C.

ii) The biotin-labelled anti-murine IgG is diluted in sterile water as directed by the manufacturer, stored at 4°C, and further diluted at the time of use in C-ELISA wash solution to which 2% (v/v) normal equine serum has been added. The avidin–alkaline phosphatase enzyme conjugate is also diluted in C-ELISA wash solution, and the chromogenic enzyme substrate is mixed according to the manufacturer’s instructions.

iii) Plates are thawed at room temperature, the coating solution is decanted, and the plates are washed twice with C-ELISA wash solution.

iv) Undiluted equine sera (50 µl/well) is added to wells. Serum should not be heat-treated. Each serum is tested in duplicate wells. Plates are incubated at 37°C for 40 minutes in a humid chamber.

v) All wells then receive 50 µl/well of diluted anti-*B. equi* or anti-*B. caballi* monoclonal antibody. (The MAb is produced in a cell culture bioreactor and is available from the National Veterinary Services Laboratories, P.O. Box 844, Ames, Iowa 50010, USA.) Plates are incubated for 30 minutes at 37°C in a humid chamber, and then washed three times in C-ELISA wash solution.

vi) Diluted biotinylated anti-murine IgG (50 µl/well) is added to wells. Plates are incubated for 20 minutes at 37°C in a humid chamber, and then washed four times in C-ELISA wash solution.

vii) Avidin–alkaline phosphatase conjugate (50 µl/well) is added to all wells. Plates are incubated, covered, for 15 minutes at room temperature, and then washed three times in C-ELISA wash solution.

viii) Chromogenic enzyme conjugate (50 µl/well) is added to wells, and plates are incubated with shaking at room temperature during colour development.

ix) The colour development is stopped by adding 50 µl EDTA stop solution (2.5% [w/v] solution of EDTA in ultra-pure water) to all wells when the negative serum control wells have an optical density of 0.2–0.4 at 590 nm wavelength (OD590).

x) The plates are read at 590 nm. The average OD590 is calculated for the duplicate wells for all sera. For a valid test, the positive control serum must reduce the amount of the colour by 70–90% compared with the negative control serum and the coefficients of variation of the negative and positive control sera cannot exceed 10%. Duplicate serum sample well values must be within 10% of each other or the sample must be retested, except for strongly positive sera that give OD590 values so close to zero that duplicate numerical precision is unlikely.
If the OD$_{590}$ of the test serum is reduced by 70% or more relative to the negative control serum, the test serum is considered to be positive. For example, if the mean negative control serum OD$_{590}$ is 0.04, then test sera with mean OD$_{590}$ values of 0.12 or less would be positive.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No biological products are available currently.

REFERENCES


40. **United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service, Veterinary Services** (2003). Competitive ELISA for Serodiagnosis of Equine Piroplasmosis (*Babesia equi* and *Babesia caballi*), and Production of Recombinant *Babesia equi* and *Babesia caballi* cELISA Antigens. USDA, National Veterinary Services Laboratories, Ames, Iowa, USA.


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CHAPTER 2.5.7.

EQUINE RHINOPNEUMONITIS

SUMMARY

Equine rhinopneumonitis (ER) is a collective term for any one of several highly contagious, clinical disease entities of equids that may occur as a result of infection by either of two closely related herpesviruses, equid herpesvirus-1 and -4 (EHV-1 and EHV-4).

Infection by either EHV-1 or EHV-4 is characterised by a primary respiratory tract disease of varying severity that is related to age and immunological status of the infected animal. Infections by EHV-1 in particular are capable of progression beyond the respiratory mucosa to cause the more serious disease manifestations of abortion, perinatal foal death, or neurological dysfunction.

Identification of the agent: The standard method of identification of the herpesviral agents of ER continues to be laboratory isolation of the virus from appropriate clinical or necropsy material, followed by seroconfirmation of its identity. The viruses can be readily isolated in equine cell culture from nasopharyngeal samples taken from horses during the febrile stage of respiratory tract infection, from liver, lung, spleen, or thymus of aborted fetuses and early foal deaths, and from the leukocyte fraction of the blood of animals with acute EHV-1 disease. Positive identification of viral isolates as EHV-1 or EHV-4 can be achieved by immunofluorescence with type-specific monoclonal antibodies.

A rapid presumptive diagnosis of rhinopneumonitis abortion can be achieved by direct immunofluorescent detection of viral antigen in cryostat sections of tissues from aborted fetuses, using conjugated polyclonal antiserum.

Sensitive and reliable methods for EHV-1/4 detection by polymerase chain reaction or immunoperoxidase staining have been developed recently and are useful adjuncts to standard virus cultivation techniques for diagnosis of ER.

Post-mortem demonstration of the characteristic histopathological lesions of EHV-1 in tissues from aborted fetuses, cases or perinatal foal death or in the central nervous system of neurologically affected animals complements the laboratory diagnosis of ER.

Serological tests: Because most horses will possess some level of antibody to EHV-1/4, the demonstration of specific antibody in the serum collected from a single blood sample is not sufficient for a positive diagnosis of recent, active ER. Paired, acute and convalescent sera from animals suspected of being infected with EHV-1 or EHV-4 can be tested for a significant rise in virus-specific antibody titre by either complement fixation (CF), virus neutralisation, or enzyme-linked immunosorbent assay. If a single convalescent serum is the only sample available, the CF test is the most informative serological test to perform.

Requirements for vaccines and diagnostic biologicals: Both live attenuated and inactivated viral vaccines of varying composition are commercially available for use in assisting in the control of ER. While vaccination is helpful in reducing the incidence of abortion in mares, and in ameliorating the severity of clinical signs of respiratory infection in young horses, it should not be considered to be a substitute for strict adherence to the well established tenets of sound management practices known to reduce the risk of rhinopneumonitis. Revaccination at frequent intervals is recommended with each of the products, as the duration of vaccine-induced immunity is relatively short.

Standards for production and licensing of both attenuated and inactivated EHV vaccines are established by appropriate veterinary regulatory agencies in the countries of vaccine manufacture and use. A single set of internationally recognised standards for ER vaccines is not available. In
each case, however, vaccine production is based on the system of a detailed outline of production employing a well characterised cell line and a master seed lot of vaccine virus that has been validated with respect to virus identity, safety, virological purity, immunogenicity, and the absence of extraneous microbial agents.

A. INTRODUCTION

Equine rhinopneumonitis (ER) is a historically derived term that describes a constellation of several disease entities of horses that may include respiratory disease, abortion, neonatal foal pneumonitis, or myeloencephalopathy (1, 2, 5, 7). The disease has been recognised for over 60 years as a threat to the international horse industry, and is caused by either of two members of the Herpesviridae family, equid herpesvirus-1 and -4 (EHV-1 and EHV-4). EHV-1 and EHV-4 are closely related alphaherpesviruses of horses with nucleotide sequence identity within individual homologous genes ranging from 55% to 84%, and amino acid sequence identity from 55% to 96% (13, 14). The two herpesviruses are enzootic in all countries in which large populations of horses are maintained as part of the cultural tradition or agricultural economy. There is no recorded evidence that the two herpesviruses of ER pose any health risks to humans working with the agents.

ER is highly contagious among susceptible horses, with viral transmission to cohort animals occurring by inhalation of aerosols of virus-laden respiratory secretions. Extensive use of vaccines has not eliminated EHV infections, and the world-wide annual financial burden from these equine pathogens is immense.

In horses under 3 years of age, clinical ER usually takes the form of an acute, febrile respiratory illness that spreads rapidly through the group of animals. The viruses infect and multiply in epithelial cells of the respiratory mucosa. Signs of infection become apparent 2–8 days after exposure to virus, and are characterised by fever, inappetence, depression, and nasal discharge. The severity of respiratory disease varies with the age of the horse and the level of immunity resulting from previous vaccination or natural exposure. Subclinical infections with EHV-1/4 are common, even in young animals. Although mortality from uncomplicated ER is rare and complete recovery within 1–2 weeks is the normal pattern, the respiratory infection is a frequent and significant cause of interrupted schedules among horses assembled for training, racing, or competitive equestrian events. Fully protective immunity resulting from infection is of short duration, and convalescent animals are susceptible to reinfection by EHV-1/4 after several months. Although reinfections by the two herpesviruses cause less severe or clinically inapparent respiratory disease, the risks of subsequent abortion and/or central nervous system (CNS) disease are not eliminated. The greatest clinical threats to individual breeding, racing, or pleasure horse operations posed by ER are the potential abortigenic and neurological sequelae of EHV-1 respiratory infection.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent (3)

Because ER is a highly contagious disease with the potential for occurring as explosive outbreaks with high mortality from abortigenic or neurological sequelae, rapid diagnostic methods are important. Although several rapid and innovative diagnostic techniques based on enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), immunohistochemical staining with peroxidase, or nucleic acid hybridisation probes have been recently described, their use is often restricted to specialised reference laboratories, and thus the method of choice for diagnosis of ER by diagnostic virology laboratories handling many routine samples continues to be the traditional methodology of cell culture isolation followed by sero-identification of the isolated viruses. Successful laboratory isolation of EHV-1/4 depends on strict adherence to proper methods for both sample collection and laboratory processing.

a) Collection of samples

Samples of nasopharyngeal exudate for virus isolation are best obtained from horses during the very early, febrile stages of the respiratory disease, and are collected via the nares by swabbing the nasopharyngeal area with a 5 × 5 cm gauze sponge attached to the end of a 50 cm length of flexible, stainless steel wire encased in latex rubber tubing. A guarded uterine swab devise can also be used. After collection, the swab should be removed from the wire and transported immediately to the virology laboratory in 3 ml of cold (not frozen) fluid transport medium (serum-free MEM [minimal essential medium] with antibiotics). Virus infectivity can be prolonged by the addition of bovine serum albumin or gelatine to 0.1% (w/v).

Virological examination of fetal tissues from suspect cases of EHV-1 abortion is most successful when performed on aseptically collected samples of liver, lung, thymus, and spleen. The tissue samples should be transported to the laboratory and held at 4°C until inoculated into tissue culture. Samples that cannot be
processed within a few hours should be stored at \(-70^\circ\text{C}\). In ante-mortem cases of EHV-1 neurological disease, the virus can often be isolated from the leukocyte fraction of the blood of acutely infected horses or, less often, from the nasopharynx of the affected animal or cohort animals. For attempts at virus isolation from blood leukocytes, a 20 ml sample of sterile blood, collected in citrate, EDTA (ethylenediamine tetra-acetic acid), or heparin anticoagulant, should be transported without delay to the laboratory on ice, but not frozen. Although the virus has, on occasion, been isolated from post-mortem cases of EHV-1 neurological disease by culture of samples of brain and spinal cord, such attempts to isolate virus are often unsuccessful.

**b) Virus isolation**

For efficient primary isolation of EHV-4 from horses with respiratory disease, equine-derived cell cultures must be used. Both EHV-1 and EHV-4 may be isolated from nasopharyngeal samples using primary equine fetal kidney cells or cell strains of equine fibroblasts derived from dermal (E-Derm) or lung tissue. EHV-1 can be isolated on other cell types, as will be discussed later. The nasopharyngeal swab and its accompanying 3 ml of transport medium are transferred into the barrel of a sterile 10 ml syringe. Using the syringe plunger, the fluid is squeezed from the swab into a sterile tube. A portion of the expressed fluid is then filtered through a sterile, 0.45 µm membrane syringe filter unit into a second sterile tube. Filtration will decrease bacterial contamination, but may also lower virus titre. Recently prepared cell monolayers in 25 cm² tissue culture flasks are inoculated with 0.5 ml of the filtered, as well as the nonfiltered, nasopharyngeal swab extract. Multiwell plates may also be used. Virus is allowed to attach by incubating the inoculated monolayers at 37°C on a platform rocker for 1.5–2 hours. Monolayers of uninoculated control cells should be incubated in parallel with sterile transport medium only.

At the end of the attachment period, the inocula are removed and the monolayers are rinsed twice with phosphate buffered saline (PBS) to remove virus-neutralising antibody that may be present in the nasopharyngeal secretions. After addition of 5 ml of supplemented maintenance medium (MEM containing 2% fetal calf serum [FCS] and twice the standard concentrations of antibiotics [penicillin, streptomycin, gentamicin, and amphotericin B]), the flasks are incubated at 37°C. The use of positive control virus samples to validate the isolation procedure carries the risk that this may lead to eventual contamination of diagnostic specimens. This risk can be minimised by using routine precautions and good laboratory technique, including the use of biosafety cabinets, inoculating positive controls after the diagnostic specimens, decontaminating the surfaces in the hood while the inoculum is absorbing and using a positive control of relatively low titre. Inoculated flasks should be inspected daily by microscopy for the appearance of characteristic herpesvirus cytopathic effect (CPE) (focal rounding, increase in refractility, and detachment of cells). Cultures exhibiting no evidence of viral CPE after 1 week of incubation should be blind-passaged into freshly prepared monolayers of cells, using small aliquots of both media and cells as the inoculum. Further blind passage is usually not productive.

A number of cell types may be used for isolation of EHV-1 from the tissues of aborted fetuses or from post-mortem cases of neurological disease (e.g. rabbit kidney [RK-13], baby hamster kidney [BHK-21], Madin–Darby bovine kidney [MDBK], pig kidney [PK-15], etc.), but equine-derived cell cultures are most sensitive and must be used if the infrequent cases of EHV-4 abortion are to be detected. Around 10% (w/v) pooled specimens, decontaminating the surfaces in the hood while the inoculum is absorbing and using a positive control of relatively low titre. Inoculated flasks should be inspected daily by microscopy for the appearance of characteristic herpesvirus cytopathic effect (CPE) (focal rounding, increase in refractility, and detachment of cells). Cultures exhibiting no evidence of viral CPE after 1 week of incubation should be blind-passaged into freshly prepared monolayers of cells, using small aliquots of both media and cells as the inoculum. Further blind passage is usually not productive.

Culture of peripheral blood leukocytes for the presence of EHV-1 is frequently successful in horses during the early stages of the paralytic disease. On receipt by the diagnostic laboratory, the tube of chilled blood, containing citrate or heparin as anticoagulant, is mixed by inversion and allowed to stand for 1 hour at room temperature. The top layer of leukocyte-rich plasma is removed and centrifuged at 640 g for 15 minutes. After decanting the supernatant, the leukocyte pellet is resuspended in the small volume of residual supernatant by brief vortexing of the tube. The resuspended cells are then rinsed twice with 10 ml of sterile PBS by centrifugation (300 g for 10 minutes) and resuspension. After the last centrifugation, the leukocyte pellet is resuspended in 1 ml of MEM containing 2% FCS. Then, 0.5 ml of the rinsed cell suspension is added to duplicate monolayers of equine fibroblast, equine fetal or RK-13 cell monolayers in 25 cm² flasks containing 8–10 ml freshly added maintenance medium. The flasks are incubated at 37°C for 7 days; the inoculum is not removed. Because CPE may be difficult to detect in the presence of the massive inoculum of leukocytes, each flask of cells is freeze–thawed after 7 days of incubation. Finally, 0.5 ml of the cell-free culture medium from each flask is transferred to freshly made cell monolayers. These are incubated and observed for viral CPE for at least 5–6 days before discarding as negative.
c) Seroconfirmation of virus identity
The basis for identification of any herpesvirus isolate recovered from specimens submitted from suspected cases of ER is its immunoreactivity with specific antisera. Specific identification of an isolate as EHV-1 or EHV-4 can be quickly and simply accomplished by immunofluorescent detection of viral antigen in the infected cell culture using type-specific monoclonal antibodies (MAbs), which are available from OIE Reference Laboratories for equine rhinopneumonitis. The test, which is type-specific and accurate, can be performed on a small aliquot of infected cells from the same container inoculated with clinical or post-mortem material. An isolate made in a laboratory that lacks MAbs can be confirmed as EHV1/4 using a virus-specific polyclonal antiserum or by the PCR (see section B.1.f).

Cell monolayers infected with the isolate are removed by scraping from the flask when at least 75% CPE is evident. The cells are pelleted from the culture medium and resuspended in 0.5 ml of PBS. 50 µl of the cell suspension is placed into two wells of a multiwell microscope slide, air-dried, and fixed for 10 minutes with 100% acetone. Control cell suspensions (uninfected, EHV-1 infected, or EHV-4 infected) are also spotted into each of two wells of the same slide. Control cells may be prepared in advance and stored frozen in small aliquots. A drop of an appropriate dilution of MAb specific for EHV-1 is added to one well of each cell pair, and a drop of MAb specific for EHV-4 is added to each of the other wells. After 30 minutes’ incubation at 37°C in a humid chamber, unreacted antibody is removed by two 10-minute washes with PBS. MAbs bound to viral antigen can be detected with goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC). A drop of diluted conjugate is added to each well and, after 30 minutes at 37°C, the wells are again washed twice with PBS. Cells are examined with a fluorescence microscope, and positive fluorescence with the antibody of appropriate specificity indicates the virus type.

d) Virus detection by direct immunofluorescence
Direct immunofluorescent detection of EHV-1 antigens in samples of post-mortem tissues collected from aborted equine fetuses provides an indispensable method to the veterinary diagnostic laboratory for making a rapid preliminary diagnosis of herpesvirus abortion (9). Side-by-side comparisons of the immunofluorescent and cell culture isolation techniques on more than 100 cases of equine abortion have provided evidence that the diagnostic reliability of direct immunofluorescent staining of fetal tissues obtained at necropsy approaches that of virus isolation attempts from the same tissues. In the United States of America (USA), specific and potent polyclonal antiserum to EHV-1, prepared in swine and conjugated with FITC, is provided to veterinary diagnostic laboratories for this purpose by the National Veterinary Services Laboratories of the United States Department of Agriculture (USDA). The antiserum cross-reacts with EHV-4 and hence is not useful for serotyping. Freshly dissected samples (5 x 5 mm pieces) of fetal tissue (lung, liver, thymus, and spleen) are frozen, sectioned on a cryostat at −20°C, mounted on to microscope slides, and fixed with 100% acetone. After air-drying, the sections are incubated at 37°C in a humid atmosphere for 30 minutes with an appropriate dilution of the conjugated swine antibody to EHV-1. Unreacted antibody is removed by two washes in PBS, and the tissue sections are then covered with aqueous mounting media and a cover-slip, and examined for fluorescent cells indicating the presence of EHV antigen. Each test should include a positive and negative control consisting of sections from known EHV-1 infected and uninfected fetal tissue.

e) Virus detection by immunoperoxidase staining
Enzyme immunohistochemical (IH) staining methods (e.g. immunoperoxidase) have been developed recently as procedures for detecting EHV-1 antigen in paraffin-embedded tissues of aborted equine fetuses or neurologically affected horses (12, 19). Such ancillary IH techniques for antigen detection may facilitate identification of the virus in archival tissue samples or in clinical cases in which traditional laboratory methods for EHV-1 detection have been unsuccessful. Immunoenzymatic staining for EHV-1 is particularly useful for the simultaneous evaluation of morphological lesions and the identification of the infectious agent. Adequate controls must be included with each immunoperoxidase test run for evaluation of both the method specificity and antibody specificity.

f) Virus detection by polymerase chain reaction
The PCR can be used for rapid amplification and diagnostic detection of nucleic acids of EHV-1 and -4 in clinical specimens, paraffin-embedded archival tissue, or inoculated cell cultures (4, 10, 11, 16, 17). A variety of type-specific PCR primers have been designed to distinguish between the presence of EHV-1 and EHV-4. The correlation between PCR and virus isolation techniques for diagnosis of EHV-1 or EHV-4 is high (16). Diagnosis of ER by PCR is rapid, sensitive, and does not depend on the presence of infectious virus in the clinical sample. It now forms an integral part of a range of diagnostic tests currently available for ER, each with its own advantages and limitations.

For diagnosis of active infection by EHV, PCR methods are most reliable with samples from aborted fetuses or from nasopharyngeal swabs of foals and yearlings; they are most useful in explosive epizootics of abortion or respiratory tract disease in which a rapid identification of the virus is critical for guiding
management strategies. The interpretation of the amplification by PCR of genomic fragments of EHV-1 or EHV-4 in tissues (lymph nodes, peripheral blood leukocytes, or CNS) from adult horses is complicated by the high prevalence of latent EHV-1 and EHV-4 DNA in circulating lymphocytes and the trigeminal ganglia of such animals (18).

A simple multiplex PCR assay for simultaneous detection of both EHV-1 and EHV-4 has been described (17). A more sensitive protocol for semi-nested PCR detection of EHV-1 or EHV-4 in clinical or pathological specimens (nasal secretions, blood leukocytes, fetal tissues, etc.) is as follows (16):

i) **Prepare template DNA from test specimens:** Following sample homogenisation and cell (and virion) lysis in the presence of a chaotropic salt, nucleic acids bind selectively to silica or cationic resin substrates. Substrate-bound nucleic acids are purified in a series of rapid wash steps followed by recovery with low-salt elution. The reagents for performing such steps for rapid nucleic acid isolation are available in kit format from a number of commercial sources (e.g. High Pure PCR Template Preparation Kit, Roche Molecular Biochemicals, Indianapolis, USA; QIAamp DNA Kit, Qiagen, Valencia, USA).

ii) **Semi-nested primer sequences specific for EHV-1** (16):

EHV1-gH-F = 5’-AAG-AGG-AGC-ACG-TGT-TGG-AT-3’  
EHV1-gH-R = 5’-TTG-AAG-GAC-GAA-TAG-GAC-GC-3’  
EHV1-gH-RN = 5’-AGT-AGG-TCA-GGC-CGA-TGC-TT-3’

iii) **Semi-nested primer sequences specific for EHV-4** (16):

EHV4-gB-F = 5’-CTG-CTG-TCA-TTA-TGC-AGG-GA-3’  
EHV4-gB-R = 5’-CGT-CTT –CTC-GAA-GAC-GGG-TA-3’  
EHV4-gB-RN = 5’-CGC-TAG-TGT-CAT-CAT-CGT-CG-3’

iv) **PCR conditions for first stage amplification:** Specimen template DNA (5 µl) is added to a PCR mixture (total volume of 25 µl) containing 1× PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, 0.1% Triton X-100), 200 µM of each deoxynucleotide triphosphate (dNTP), 2.5 mM MgCl₂, 2.0 µM of each outer primer (EHV1-gH-F and EHV1-gH-R for EHV-1 detection) and, in a separate reaction mixture, EHV4-gB-F and EHV4-gB-R for EHV-4 detection) and 0.5 u Taq DNA polymerase. Cycling parameters are: initial denaturation at 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute; with a final extension at 72°C for 5 minutes. Separate reaction mixtures containing either known viral DNA or no DNA (water) should be prepared and amplified as positive and negative controls.

v) **PCR conditions for second stage (semi-nested) amplification:** One-half µl of the first amplification product is added to a fresh PCR mixture (total volume of 25 µl) containing 1× PCR buffer, 200 µM of each dNTP, 2.5 mM MgCl₂, 2.0 µM of each primer (EHV1-gH-F and EHV1-gH-RN for EHV-1 detection and, in a separate reaction mixture, EHV4-gB-F and EHV4-gB-RN for EHV-4 detection) and 0.5 u Taq DNA polymerase. Cycling parameters are: initial denaturation at 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute; with a final extension at 72°C for 5 minutes.

vi) **Gel analysis of amplified products:** 5 µl of each final amplified product, including controls, is electrophoresed on a 2.0% agarose gel in Tris/acetate running buffer, along with a 100 base pairs (bp) DNA ladder. The gel is stained with ethidium bromide and viewed by UV transillumination for amplified products of either 287 bp for EHV-1 or 323 bp for EHV-4.

g) **Histopathology**

Histopathological examination of sections of formalin-fixed, paraffin-embedded tissues from aborted fetuses or from neurologically affected horses is an essential part of the laboratory diagnosis of these two clinical manifestations of ER. In aborted fetuses, typical herpetic intranuclear inclusion bodies present within bronchiolar epithelium or in cells at the periphery of areas of hepatic necrosis are pathognomonic lesions for EHV-1. The characteristic, but not pathognomonic, microscopic lesion associated with EHV-1 neuropathy is a degenerative thrombotic vasculitis of small blood vessels in the brain or spinal cord (perivascular cuffing and infiltration by inflammatory cells, endothelial proliferation and necrosis, and thrombus formation).

2. **Serological tests**

Because of the ubiquity of the viral agents of ER and the high seroprevalence among horses in most parts of the world, the demonstration of a negative antibody titre to EHV-1/4 by serological testing of horses designated for
export is not part of present veterinary regulations that seek to prevent international spread of infectious diseases of horses. Serological testing can, however, be a useful adjunct procedure for assisting in the diagnosis of ER in horses. Serodiagnosis of ER is based on the demonstration of significant increases in antibody titres in paired sera taken during the acute and convalescent stages of the disease. The results of tests performed on sera from a single collection date are, in most cases, impossible to interpret with any degree of confidence. The initial (acute phase) serum sample should be taken as soon as possible after the onset of clinical signs, and the second (convalescent phase) serum sample should be taken 3–4 weeks later. Samples of cerebrospinal fluid may also be of value in serodiagnosis of EHV-1 neurological disease. ‘Acute phase’ sera from mares after abortion or from horses with EHV-1 neurological disease may already contain maximal titres of EHV-1 antibody, with no increase in titres detectable in sera collected at later dates. In such cases, serological testing of paired serum samples from clinically unaffected cohort members of the herd for rising antibody titres against EHV-1/4 may provide information useful for retrospective diagnosis of ER within the herd. Finally, the serological detection of antibodies to EHV-1 in heart or umbilical cord blood or other fluids of equine fetuses can be of diagnostic value in rare cases of virologically negative fetuses aborted as a result of EHV-1 infection.

Serum antibody levels to EHV-1/4 may be determined by ELISA (8), virus neutralisation (VN) (15), or complement fixation (CF) tests (15). Because CF antibody titres to EHV-1/4 become negative within a few months following recovery from infection, the CF test is most useful in diagnosing recent infection by the herpesviruses if a convalescent serum is the only sample available for testing. The CF test performed on sera collected from horses with the paralytic form of EHV-1 infection is also useful for making the rapid diagnosis of EHV-1 antibody titre to EHV-1 and EHV-4 was developed and was made commercially available (6). The microneutralisation test is a widely used and sensitive serological assay for detecting EHV-1/4 antibody and will thus be described here.

a) Virus neutralisation test

This serological test is most commonly performed in flat-bottom 96-well microtitre plates (tissue culture grade) using a constant dose of virus and doubling dilutions of equine test sera. At least two replicate wells for each serum dilution are required. Serum-free MEM is used throughout as a diluent. Virus stocks of known titre are diluted just before use to contain 100 TCID\textsubscript{50} (50% tissue culture infective dose) in 25 µl. Monolayers of E-Derm or RK-13 cells are monodispersed with EDTA/trypsin and resuspended at a concentration of 5 x 10\textsuperscript{5}/ml. Note that RK-13 cells can be used with EHV-1 but do not give clear CPE with EHV-4. Antibody positive and negative control equine sera and controls for cell viability, virus infectivity, and test serum cytotoxicity, must be included in each assay. End-point VN titres of antibody are calculated by determining the reciprocal of the highest serum dilution that protects 100% of the cell monolayer from virus destruction in both of the replicate wells.

A suitable test procedure is as follows:

i) Inactivate test and control sera for 30 minutes in a water bath at 56°C.

ii) Add 25 µl of serum-free MEM to all wells of the microtitre assay plates.

iii) Pipette 25 µl of each test serum into duplicate wells of both rows A and B of the plate. The first row serves as the serum toxicity control and the second row as the first dilution of the test. Make doubling dilutions of each serum starting with row B and proceeding to the bottom of the plate by sequential mixing and transfer of 25 µl to each subsequent row of wells. Six sera can be assayed in each plate.

iv) Add 25 µl of the appropriately diluted EHV-1 or EHV-4 virus stock to each well (100 TCID\textsubscript{50}/well) except those of row A, which are the serum control wells for monitoring serum toxicity for the indicator cells. Note that the final serum dilutions, after addition of virus, run from 1/4 to 1/256.

v) A separate control plate should include titration of both a negative and positive horse serum of known titre, cell control (no virus), virus control (no serum), and a virus titration to calculate the actual amount of virus used in the test.

vi) Incubate the plates for 1 hour at 37°C in 5% CO\textsubscript{2} atmosphere.

vii) Add 50 µl of the prepared E-Derm or RK-13 cell suspension (5 x 10\textsuperscript{5} cells/ml) in MEM/10% FCS to each well.

viii) Incubate the plates for 4–5 days at 37°C in an atmosphere of 5% CO\textsubscript{2} in air.
ix) Examine the plates microscopically for CPE and record the results on a worksheet. Alternatively, the cell monolayers can be scored for CPE after fixing and staining as follows: after removal of the culture fluid, immerse the plates for 15 minutes in a solution containing 2 mg/ml crystal violet, 10% formalin, 45% methanol, and 45% water. Then, rinse the plates vigorously under a stream of running tap water.

x) Wells containing intact cell monolayers stain blue, while monolayers destroyed by virus do not stain. Verify that the cell control, positive serum control, and serum cytotoxicity control wells stain blue, that the virus control and negative serum control wells are not stained, and that the actual amount of virus added to each well is between $10^{1.5}$ and $10^{2.5}$ TCID$_{50}$. Wells are scored as positive for neutralisation of virus if 100% of the cell monolayer remains intact. The highest dilution of serum resulting in complete neutralisation of virus (no CPE) in both duplicate wells is the end-point titre for that serum.

xi) Calculate the neutralisation titre for each test serum, and compare acute and convalescent phase serum titres from each animal for a four-fold or greater increase.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Both live attenuated and inactivated vaccines are available as licensed, commercially prepared products for use as prophylactic aids in reducing the burden of disease in horses caused by EHV-1/4 infection. Clinical experience has demonstrated that none of the vaccine preparations should be relied on to provide an absolute degree of protection from ER. Multiple doses, repeated annually, of each of the currently marketed ER vaccines are recommended by their respective manufacturers, with vaccination schedules that vary with the particular vaccine.

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.

At least sixteen vaccine products for ER, each containing different permutations of EHV-1, EHV-4, and the two subtypes of equine influenza virus, are currently marketed by five veterinary biologicals manufacturers.

The clinical indications stated on the product label for use of the several available vaccines for ER are either herpesvirus-associated respiratory disease, abortion, or both. Only four vaccine products have met the regulatory requirements for claiming efficacy in providing protection from herpesvirus abortion as a result of successful vaccination and challenge experiments in pregnant mares. None of the vaccine products has been tested for its ability to prevent the occurrence of neurological disease sometimes associated with EHV-1 infection.

1. Seed management

a) Characteristics and culture

The master seed virus (MSV) for ER vaccines must be prepared from strains of EHV-1 and/or EHV-4 that have been positively and unequivocally identified by both serological and genetic tests. Seed virus must be propagated in a cell line approved for equine vaccine production by the appropriate regulatory agency. A complete record of original source, passage history, medium used for propagation, etc., shall be kept for the master seed preparations of both the virus(es) and cell stock(s) intended for use in vaccine production.

Permanently stored stocks of both MSV and master cell stock (MCS) used for vaccine production must be demonstrated to be pure, safe and, in the case of MSV, also immunogenic. Generally, the fifth passage from the MSV and the twentieth passage from the MCS are the highest allowed for vaccine production. Results of all quality control tests on master seeds must be recorded and made a part of the licensee's permanent records.

b) Validation as a vaccine

i) Purity

Tests for master seed purity include prescribed procedures that demonstrate the virus and cell seed stocks to be free from bacteria, fungi, mycoplasmas, and extraneous viruses. Special tests must be performed to confirm the absence of equine arteritis virus, equine infectious anaemia virus, equine influenza virus, equine herpesvirus-2, --, and -5, equine rhinovirus, the alphaviruses of equine encephalomyelitis, bovine viral diarrhoea virus (BVDV – common contaminant of bovine serum), and porcine parvovirus (PPV – potential contaminant of porcine trypsin). The purity check should also include the exclusion of the presence of EHV-1 from EHV-4 MSV and vice versa.

ii) Safety

Samples of each lot of MSV to be used for preparation of live attenuated ER vaccines must be tested for safety in horses determined to be susceptible to the virulent wild-type virus, including pregnant
mares in the last 4 months of gestation. Vaccine safety must be demonstrated in a ‘safety field trial’ in horses of various ages from three different geographical areas. The safety trial should be conducted by independent veterinarians using a prelicensing batch of vaccine. EHV-1 vaccines making a claim for efficacy in controlling abortion must be tested for safety in a significant number of late gestation pregnant mares, using the vaccination schedule that will be recommended by the manufacturer for the final vaccine product.

iii) Immunogenicity

Tests for immunogenicity of the EHV-1/4 MSV stocks should be performed in horses on an experimental test vaccine prepared from the highest passage level of the MSV allowed for use in vaccine production. The prescribed test for MSV immunogenicity consists of vaccination of horses with low antibody titres to EHV-1/4, with doses of the test vaccine that will be recommended on the final product label. Second serum samples should be obtained and tested for significant increases in neutralising antibody titre against the virus, 21 days after the final dose.

iv) Efficacy

An important part of the validation process is the capacity of a prelicensing lot of the ER vaccine to provide a significant level of clinical protection in horses from the particular disease manifestation of EHV-1/4 infection for which the vaccine is offered, when used under the conditions recommended by the manufacturer’s product label. Serological data are not acceptable for establishing the efficacy of vaccines for ER. Efficacy studies must be designed to ensure appropriate randomisation of test animals to treatment groups, blinding of the recording of clinical observations, and the use of sufficient numbers of animals to permit statistical evaluation for effectiveness in prevention or reduction of the specified clinical disease. The studies should be performed on fully formulated experimental vaccine products (a) produced in accordance with, (b) at or below the minimum antigenic potency specified in, and, (c) produced with the highest passage of MSV and MCS allowed by the approved ‘Outline of Production’ (see Section C.2.). Vaccine efficacy is demonstrated by vaccinating a minimum of 20 EHV-1/4-susceptible horses possessing low serum neutralising antibody titres, followed by challenge of the vaccinates and ten nonvaccinated control horses with virulent virus. A significant difference in the clinical signs of ER must be demonstrated between vaccinates and nonvaccinated control horses. The vaccination and challenge study must be performed on an identical number of pregnant mares and scored for abortion if the vaccine product will make a label usage claim ‘for prevention of’ or ‘as an aid in the prevention of’ abortion caused by EHV-1.

2. Method of manufacture

A detailed protocol of the methods of manufacture to be followed in the preparation of vaccines for ER must be compiled, approved, and filed as an Outline of Production with the appropriate licensing agency. Specifics of the methods of manufacture for ER vaccines will differ with the type (live or inactivated) and composition (EHV-1 only, EHV-1 and EHV-4, EHV-4 and equine influenza viruses, etc.) of each individual product, and also with the manufacturer.

3. In-process control

Cells, virus, culture medium, and medium supplements of animal origin that are used for the preparation of production lots of vaccine must be derived from bulk stocks that have passed the prescribed tests for bacterial, fungal, and mycoplasma sterility; nontumorigenicity; and absence of extraneous viral agents.

4. Batch control

Each bulk production lot of ER vaccine must pass tests for sterility, safety, and immunogenic potency.

a) Sterility

Samples taken from each batch of completed vaccine are tested for bacteria, fungi, and mycoplasma contamination. Procedures to establish that the vaccine is free from extraneous viruses are also required; such tests should include inoculation of cell cultures that allow detection of the common equine viruses, as well as techniques for the detection of BVDV and PPV in ingredients of animal origin used in the production of the batch of vaccine.

b) Safety

Tests to assure safety of each production batch of ER vaccine must demonstrate complete inactivation of virus (for inactivated vaccines) as well as a level of residual virus-killing agent that does not exceed the maximal allowable limit (e.g. 0.2% for formaldehyde). Safety testing in laboratory animals is also required.
Chapter 2.5.7. – Equine rhinopneumonitis

c) Potency

Batch control of antigenic potency for EHV-1 vaccines may be tested by measuring the ability of dilutions of the vaccine to protect hamsters from challenge with a lethal dose of hamster-adapted EHV-1 virus. Although potency testing on production batches of ER vaccine may also be performed by vaccination of susceptible horses followed by either viral challenge or assay for seroconversion, the recent availability of virus type-specific MAb has permitted development of less costly and more rapid in-vitro immunoassays for antigenic potency. The basis for such in-vitro assays for ER vaccine potency is the determination, by use of the specific MAb, of the presence of at least the minimal amount of viral antigen within each batch of vaccine that correlates with the required level of protection (or seroconversion rate) in a standard animal test for potency.

d) Duration of immunity

Tests to establish the duration of immunity to EHV-1/4 achieved by immunisation with each batch of vaccine are not required. The results of many reported observations indicate that vaccination-induced immunity to EHV-1/4 is not more than a few months in duration; these observations are reflected in the frequency of revaccination recommended on ER vaccine product labels.

e) Stability

At least three production batches of vaccine should be tested for shelf life before reaching a conclusion on the vaccine’s stability. When stored at 4°C, inactivated vaccine products generally maintain their original antigenic potency for at least 1 year. Lyophilised preparations of the live virus vaccine are also stable during storage for 1 year at 4°C. Following reconstitution, live virus vaccine is unstable and cannot be stored without loss of potency.

5. Tests on the final product

Before release for labelling, packaging, and commercial distribution, randomly selected filled vials of the final vaccine product must be tested by prescribed methods for freedom from contamination and safety in laboratory test animals.

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

REFERENCES


* *

NB: There are OIE Reference Laboratories for Equine rhinopneumonitis 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 2.5.8.

Glanders

Summary

Glanders is a contagious and fatal disease of horses, donkeys, and mules, and is caused by infection with the bacterium Burkholderia mallei (the name recently changed from Pseudomonas mallei and was previously classified variously as Pfeifferella, Loefflerella, Malleomyces or Actinobacillus). The disease causes nodules and ulcerations in the upper respiratory tract and lungs. A skin form also occurs, known as ‘farcy’. Control of glanders requires testing of suspect clinical cases, screening of apparently normal equids, and elimination of positive reactors. It is transmitted to humans and all infected or potentially infected material must be handled in a laboratory that meets the requirements for Containment Group 3 pathogens.

Identification of the agent: Smears from fresh material may reveal Gram-negative nonsporulating, nonencapsulated rods. The presence of a capsule-like cover has been established by electron microscopy. The bacteria grow aerobically and prefer media that contains glycerol. Unlike the Pseudomonas species, Burkholderia mallei is nonmotile. Guinea-pigs are highly susceptible, and males are used for testing potentially infected material. Intraperitoneal injections are given to attempt to elicit the Strauss reaction (orchitis).

Mallein and serological tests: The mallein test is a sensitive and specific clinical test for glanders. Mallein, a protein fraction of the organism, is injected intradermo-palpebrally or given by eyedrop. In infected animals, the eyelid swells markedly in 1–2 days. A complement fixation test, and an enzyme-linked immunosorbent assay are the most accurate and reliable serological tests for diagnostic use. A rose bengal plate agglutination test has recently been developed in Russia; it has been validated in Russia only.

Requirements for vaccines and diagnostic biologicals: There are no vaccines. Mallein purified protein derivative is currently available commercially from institutes in The Netherlands and Romania (see footnote 1).

A. Introduction

Glanders is a bacterial disease of perissodactyls or odd-toed ungulates with zoonotic potential that has been known since ancient times, and that is caused by infection with the bacterium Burkholderia mallei (the name recently changed from Pseudomonas mallei (25) and was previously classified variously as Pfeifferella, Loefflerella, Malleomyces or Actinobacillus). Outbreaks of the disease may occur in members of the cat family living in the wild or in zoological gardens. Susceptibility to glanders has been proved in camels, bears, wolves and dogs. Carnivores may become infected by eating infected meat, but cattle, sheep and pigs are resistant (16). Glanders in the acute form occurs most frequently in donkeys and mules, which run a high fever and exhibit respiratory signs (swollen nostrils, dyspnoea, and pneumonia); death occurs within a few days. In horses, glanders generally runs a more chronic course and they may survive for several years. Chronic and subclinical ‘occult’ cases are dangerous sources of infection.

In horses, inflammatory nodules and ulcers develop in the nasal passages and give rise to a sticky yellow discharge, accompanied by enlarged firm submaxillary lymph nodes. Stellate scarring follows healing of the ulcers. The formation of nodular abscesses in the lungs is accompanied by progressive debility, febrile episodes, coughing and dyspnoea. Diarrhoea and polyuria can also occur. In the skin form (‘farcy’), the lymphatics are enlarged and nodular abscesses (‘buds’) of 0.5–2.5 cm develop, which ulcerate and discharge yellow oily pus. Discharges from the respiratory tract and skin are infective, and transmission between animals, which is
facilitated by close contact, is by inhalation, ingestion of contaminated material (e.g. from infected feed and water troughs), or by inoculation (e.g. via a harness). The incubation period is from a few days to many months (17).

Glanders is transmissible to humans by direct contact with sick animals or infected materials. In the untreated acute disease, there can be 95% mortality within 3 weeks. However, survival is possible if the infected person is treated early and aggressively with multiple systemic antibiotic therapies (11, 19). A chronic form with abscessation also occurs. When handling suspect or known infected animals or fomites, stringent precautions should be taken to prevent self-infection or transmission of the bacterium to other equids. Laboratory samples should be securely packaged, kept cool and shipped as outlined in Chapter I.1.1. of this Terrestrial Manual. All manipulations with potentially infected material must be performed in a laboratory that meets the requirements for Containment Group 3 pathogens as outlined in Appendix I.1.6.1 of Chapter I.1.6 of this Terrestrial Manual.

Glanders has been eradicated from many countries by statutory testing, elimination of infected animals, and import restrictions. It persists in some Asian, African and South American countries.

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

Cases for specific glanders investigation should be differentiated on clinical grounds from other chronic infections of the nasal mucosae or sinuses, and from strangles (Streptococcus equi infection), ulcerative lymphangitis (Corynebacterium pseudotuberculosis), pseudotuberculosis (Yersinia pseudotuberculosis) and sporotrichosis (Sporotrichium spp.). Glanders should be excluded positively from suspected cases of epizootic lymphangitis (caused by Histoplasma farciminosum), with which it has many clinical similarities. In humans, in particular, glanders should be distinguished from melioidosis (B. pseudomallei infection), which is caused by an organism with close similarities to B. mallei (16).

a) **Morphology of Burkholderia mallei**

The organisms are fairly numerous in smears from fresh lesions, but in older lesions they are scanty (22). They should be stained by methylene blue or Gram stain. The organisms are mainly extracellular, fairly straight Gram-negative rods with rounded ends, 2–5 µm long and 0.3–0.8 µm wide with granular inclusions of various size, often stain irregularly and do not have capsules or form spores. The presence of a capsule-like cover has been established by electron microscopy. This capsule is composed of neutral carbohydrates and serves to protect the cell from unfavourable environmental factors. Unlike other organisms in the Pseudomonas group, Burkholderia mallei have no flagellae and are therefore nonmotile (13). The organisms are difficult to demonstrate in tissue sections, where they may have a beaded appearance (15). In culture media, they vary in appearance depending on the age of the culture and type of medium. In older cultures, there is much pleomorphism. Branching filaments form on the surface of broth cultures.

b) **Cultural characteristics**

It is preferable to attempt isolation from unopened uncontaminated lesions (15). The organism is aerobic and facultatively anaerobic only in the presence of nitrate (6, 13), growing optimally at 37°C (14). It grows well, but slowly, on ordinary culture media, 48-hour incubation of cultures is recommended; glycerol enrichment is particularly useful. After a few days on glycerol agar, there is a confluent, slightly cream-coloured growth that is smooth, moist, and viscid. With continued incubation, the growth thickens and becomes dark brown and tough. It also grows well on glycerol potato agar and in glycerol broth, on which a slimy pellicle forms. On plain nutrient agar, the growth is much less luxuriant, and growth is poor on gelatin (20).

Alterations to characteristics may occur in vitro, so fresh isolates should be used for identification reactions. Litmus milk is slightly acidified by B. mallei, and coagulation may occur after long incubation. The organism reduces nitrates. Although some workers have claimed that glucose is the only carbohydrate that is fermented (slowly and inconstantly), other workers have shown that if an appropriate medium and indicator are used, glucose and other carbohydrates, such as arabinose, fructose, galactose and mannose, are consistently fermented by B. mallei (5). Indole is not produced, horse blood is not haemolysed and no diffusible pigments are produced in cultures (13). A commercial laboratory test kit (e.g. API [Analytical Profile Index] system: Analytab Products, BioMerieux or Biolog [Hayward, California]) can be used for easy confirmation that an organism belongs to the Pseudomonas group. Lack of motility is then of special relevance. A bacteriophage specific for B. mallei is available (23).

All culture media prepared should be subjected to quality control and must support growth of the suspect organism from a small inoculum. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are working correctly.
In contaminated samples, supplementation of media with substances that inhibit the growth of Gram-positive organisms (e.g., crystal violet, proflavine) has proved useful, as has pretreatment with penicillin (1000 units/ml for 3 hours at 37°C) (22). A selective medium has been developed (24) composed of polymyxin E (1000 units), bacitracin (250 units), and actidione (0.25 mg) incorporated into nutrient agar (100 ml) containing glycerine (4%), donkey or horse serum (10%), and ovine haemoglobin or trypton agar (0.1%).

Outside the body, the organism has little resistance to drying, heat, light or chemicals, so that survival beyond 2 weeks is unlikely. Under favourable conditions, however, it can probably survive a few months. *Burkholderia mallei* can remain viable in tap water for at least 1 month (20). For disinfection, benzalkonium chloride or ‘roccal’ (1/2000), sodium hypochlorite (500 ppm available chlorine), iodine, mercuric chloride in alcohol, and potassium permanganate have been shown to be highly effective against *B. mallei* (14). Phenolic disinfectants are less effective.

c) Laboratory animal inoculation

Guinea-pigs, hamsters and cats have been used for diagnosis when necessary. If isolation in a laboratory animal is considered unavoidable, suspected material is inoculated intraperitoneally into a male guinea-pig. Positive material will cause a severe localised peritonitis and orchitis (the Strauss reaction). The number of organisms and their virulence determines the severity of the lesions. Additional steps are used when the test material is heavily contaminated (7). The Strauss reaction is not specific for glanders, and other organisms can elicit it. Bacteriological examination of infected testes should confirm the specificity of the response obtained.

d) Other methods

There have been recent advances in the application of molecular biological techniques to the detection of glanders. A polymerase chain reaction (PCR) for the specific detection of *B. mallei* DNA has been developed (2) that allows differentiation between *B. mallei* and *B. pseudomallei*. The technique has not yet been fully validated or gained wide acceptance. However, PCR has the potential to be a safe, fast method to confirm infection. Methods to differentiate between *B.mallei* and *B. pseudomallei* are important as this cannot be reliably accomplished by the current serological tests.

2. Mallein and serological tests

a) The mallein test (a prescribed test for international trade)

The mallein purified protein derivative (PPD), which is available commercially, is a solution of water-soluble protein fractions of heat-treated *B. mallei*. The test depends on infected horses being hypersensitive to mallein. Advanced clinical cases in horses and acute cases in donkeys and mules may give inconclusive results requiring additional methods of diagnosis to be employed (1).

- **The intradermo-palpebral test**

This is the most sensitive, reliable and specific test for detecting infected perissodactyls or odd-toed ungulates, and has largely displaced the ophthalmic and subcutaneous tests (3): 0.1 ml of concentrated mallein PPD is injected intradermally into the lower eyelid and the test is read at 24 and 48 hours. A positive reaction is characterised by marked oedematous swelling of the eyelid, and there may be a purulent discharge from the inner canthus or conjunctiva. This is usually accompanied by a rise in temperature. With a negative response, there is usually no reaction or only a little swelling of the lower lid.

- **The ophthalmic test**

This is less reliable than the intradermo-palpebral test. A few drops of mallein are instilled into the eye at the canthus. In an infected animal, the eyelids, and sometimes the side of the face, become swollen and there may be a little discharge from the eye. The reaction may also occur to a lesser extent in the opposite eye.

- **The subcutaneous test**

This test interferes with subsequent serological diagnosis and so the other two mallein tests are preferred. Also, the test may not be acceptable in some countries. The horse’s temperature has to be under 102°F (38.8°C) on the day before the test, at the time of the injection, and at 9, 12 and 15 hours after the injection. A 10 cm square skin patch in the middle of the neck is clipped and disinfected; 2.5 ml of dilute mallein are injected subcutaneously into the centre of the patch. With a positive test, the horse develops a pyrexia of 104°F (40.0°C) or over during the first 15 hours, and a firm painful swelling with raised edges develops within 24 hours at the injection site. In nonglandered horses, there is no, or minimal, transient local swelling. Doubtful reactors may be retested after 14 days using a double dose of mallein.
b) Complement fixation test (a prescribed test for international trade)

Although not as sensitive as the mallein test, the CF test is an accurate serological test that has been used for glanders diagnosis for many years (3). It is reported to be 90–95% accurate, serum being positive within 1 week of infection and remaining positive in the case of exacerbation of the chronic process (20).

- Antigen preparation (10)
  - i) Flasks of beef infusion broth with 3% glycerol are inoculated with log-phase growth *B. mallei* and incubated at 37°C for 8–12 weeks.
  - ii) The cultures are inactivated by exposing the flasks to flowing steam (100°C) for 60 minutes.
  - iii) The clear supernatant is decanted and filtered. The filtrate is heated again by exposure to live steam for 75 minutes on 3 consecutive days, and clarified by centrifugation.
  - iv) The clarified product is concentrated to one-tenth the original volume by evaporation on a steam or hot water bath.
  - v) Concentrated antigen is bottled in brown-glass bottles to protect from light and stored at 4°C. Antigen has been shown to be stable for at least 10 years in this concentrated state.
  - vi) Lots of antigen are prepared by diluting the concentrated antigen 1/20 with sterile physiological saline with 0.5% phenol. The diluted antigen is dispensed into brown-glass vials and stored at 4°C. The final working dilution is determined by a block titration. The final working dilution for CF test use is made at the time the CF test is performed.

The resulting antigen is primarily lipopolysaccharide. An alternative procedure is to use young cultures by growing the organism on glycerol–agar slopes for 12 hours and washing off with normal saline. A suspension of the culture is heated for 1 hour at 70°C and the heat-treated bacterial suspension is used as antigen. The disadvantage of this antigen preparation method is that the antigen contains all the bacterial cell components. The antigen should be safety tested by inoculating blood agar plates.

- Test procedure (18)
  - i) Serum is diluted 1/5 in veronal (barbiturate) buffered saline containing 0.1% gelatin (VBSG) or CFD (complement fixation diluent – available as tablets) without gelatine.
  - ii) Diluted serum is inactivated for 30 minutes at 56°C. (Serum of equidae other than horses should be inactivated at 63°C for 30 minutes.)
  - iii) Twofold dilutions of the sera are prepared in 96-well round-bottom microtitre plates.
  - iv) Guinea-pig complement is diluted in the chosen buffer and 5 (or optionally 4) complement haemolytic units-50% (CH50) are used.
  - v) Sera, complement and antigen are reacted in the plates and incubated for 1 hour at 37°C. (An alternate acceptable procedure is overnight incubation at 4°C.)
  - vi) A 2% suspension of sensitised washed sheep red blood cells is added.
  - vii) Plates are incubated for 45 minutes at 37°C, and then centrifuged for 5 minutes at 600 g.

A sample that produces 100% haemolysis at the 1/5 dilution is negative, 25–75% haemolysis is suspicious, and no haemolysis (100% fixation) is positive. Unfortunately, false-positive results can occur, and *B. pseudomallei* and *B. mallei* cross react and cannot be differentiated by serology (3). Also healthy horses can have a false positive CF reaction for a variable period following a mallein intradermal test.

c) Enzyme-linked immunosorbent assays

Both plate and membrane (blot) enzyme-linked immunosorbent assays (ELISAs) have been reported for the serodiagnosis of glanders, but none of these procedures has been shown to differentiate serologically between *B. mallei* and *B. pseudomallei*. Blotting approaches have involved both dipstick dot-blot and electrophoretically separated and transferred western blot methods (8, 21). A competitive ELISA that uses an anti-lipopolysaccharide monoclonal antibody has also been developed and found to be similar to the CF test in performance (9). Continuing development of monoclonal antibody reagents specific for *B. mallei* antigenic components offers the potential for more specific ELISAs in the foreseeable future that will help resolve questionable test results of quarantined imported horses (4, 12). At this time, none of these tests has been validated.

d) Other serological tests

The avidin–biotin dot ELISA has been described (21), but has not yet been widely used or validated. The antigen is heat-inactivated bacterial culture that has been concentrated and purified. A dot of this antigen is
placed on a nitrocellulose dipstick that is then used to test for antibody against \textit{B. mallei} in equine serum. Using antigen-dotted, preblocked dipsticks, the test can be completed in approximately 1 hour. Serum or whole blood can be used for the test, and partial haemolysis does not impart any background colour to the antigen-coated area on the nitrocellulose.

The rose bengal plate agglutination test (RBT) has been described for the diagnosis of glanders in horses and other susceptible animals; the test has been validated in Russia only. The antigen is a heat-inactivated bacterial suspension coloured with rose bengal, which is used in a plate agglutination test.

The accuracy of other agglutination tests and precipitin is unsatisfactory for use in control programmes. Horses with chronic glanders and those in a debilitated condition give negative or inconclusive results.

\section*{C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS}

No vaccines are available.

Mallein PPD for use in performing the intradermo-palpebral and ophthalmic tests is produced commercially by two institutes\(^1\).

The ID-Lelystad has provided the following information on requirements for mallein PPD.

\subsection*{1. Seed management}

Three strains of \textit{Burkholderia mallei} are employed in the production of mallein PPD, namely Bogor strain (originating from Indonesia), Mukteswar strain (India) and Zagreb strain (Yugoslavia). The seed material is kept as a stock of freeze-dried cultures. The strains are subcultured on to glycerol agar at 37°C for 1–2 days. For maintaining virulence and antigenicity, the strains may be passaged through guinea-pigs.

\subsection*{2. Method of manufacture}

Dorset–Henley medium, enriched by the addition of trace elements, is used for production of mallein PPD. The liquid medium is inoculated with a thick saline suspension of \textit{B. mallei}, grown on glycerol agar. The production medium is incubated at 37°C for about 10 weeks. The bacteria are then killed by steaming for 3 hours in a Koch’s steriliser. The fluid is then passed through a layer of cotton wool to remove coarse bacterial clumps. The resulting turbid fluid is cleared by membrane filtration, and one part trichloroacetic acid 40\% is immediately added to nine parts culture filtrate. The mixture is allowed to stand overnight and a light brownish to greyish precipitate settles.

The supernatant fluid is pipetted off and discarded. The precipitate is centrifuged for 15 minutes at 2500 \(g\) and the layer of precipitate is washed three or more times in a solution of 5\% NaCl, pH 3, until the pH is 2.7. The washed precipitate is dissolved by stirring with a minimum of an alkaline solvent. The fluid is dark brown and a pH of 6.7 will be obtained. This mallein concentrate has to be centrifuged thoroughly and the supernatant is diluted with an equal amount of a glucose buffer solution. The protein content of this product is estimated by the Kjeldahl method and freeze-dried after it has been dispensed into ampoules.

\subsection*{3. In-process control}

During the period of incubation, the flasks are inspected frequently for any signs of contamination, and suspect flasks are discarded. A typical growth of the \textit{B. mallei} cultures comprises turbidity, sedimentation, some surface growth with a tendency towards sinking, and the formation of a conspicuous slightly orange-coloured ring along the margin of the surface of the medium.

\subsection*{4. Batch control}

Each batch of mallein PPD is tested for sterility, safety, preservatives, protein content and potency. Sterility testing is performed according to the European Pharmacopoeia guidelines.

\footnote{Institute for Animal Science and Health (ID-Lelystad), Edelhertweg 15, 8219 PH, Lelystad, The Netherlands, and Min Agr Si Industr. Institut Die Cercertari Si Biopreparate ‘Pasteur’, R-7000 - Bucuresti, 77.826 SOS Giulesti Nr 333, Romania.}
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The examination for safety is conducted on from five to ten normal healthy horses by carrying out the intradermo-palpebral test. The resulting swelling should be, at most, barely detectable and transient, without any signs of conjunctival discharge.

Preparations containing phenol as a preservative should not contain more than 0.5% (w/v) phenol. The protein content should be not less than 0.95 mg/ml and not more than 1.05 mg/ml.

Potency testing is performed in guinea-pigs and horses. The animals are sensitised by subcutaneous inoculation with a concentrated suspension of heat-killed *B. mallei* in paraffin oil or incomplete Freund’s adjuvant. Cattle can also be used instead of horses. The production batch is bioassayed against a standard mallein PPD by intradermal injection in 0.1 ml doses in such a way that complete randomisation is obtained.

In guinea-pigs, the different areas of erythema are measured after 24 hours, and in horses the increase in skin thickness is measured by calipers. The results are statistically evaluated, using standard statistical methods for parallel-line assays.

REFERENCES


* * *
There is no chapter on horse pox currently available.
CHAPTER 2.5.10.

EQUINE VIRAL ARTERITIS

SUMMARY

Equine viral arteritis (EVA) is a contagious viral disease of equids caused by equine arteritis virus (EAV), an RNA virus classified in the family Arteriviridae. The virus is found in horse populations in many countries world-wide. Although infrequently reported in the past, confirmed outbreaks of EVA appear to be on the increase.

The majority of naturally acquired infections with EAV are subclinical. Where present, clinical signs of EVA can vary in range and severity. The disease is characterised principally by fever, depression, anorexia, dependent oedema, especially of the limbs, scrotum and prepuce in the stallion, conjunctivitis, an urticarial-type skin reaction, abortion and, rarely, a fulminating pneumonia or pneumo-enteritis in young foals. Apart from mortality in young foals, the case-fatality rate in outbreaks of EVA is very low. Affected horses almost invariably make complete clinical recoveries. A long-term carrier state can occur in a high percentage of infected stallions, but not in mares or nonbreeding horses.

Identification of the agent: EVA cannot be differentiated clinically from a number of other respiratory and systemic equine diseases. Diagnosis of EAV infection is based on virus isolation, detection of viral antigen or nucleic acid, or demonstration of a specific antibody response. Virus isolation should be attempted from appropriate clinical or post-mortem specimens in rabbit, equine, or monkey kidney cell culture. The identity of isolates of EAV should be confirmed by neutralisation test, reverse-transcription polymerase chain reaction (RT-PCR) assay, or by immunocytochemical methods, namely indirect immunofluorescence or avidin–biotin–peroxidase techniques.

Detection and identification of EAV in suspect cases of the disease can also be attempted using the RT-PCR assay and appropriate viral RNA primers.

Where mortality is associated with a suspected outbreak of EVA, a wide range of tissues should be examined for histological evidence of panvasculitis that is especially pronounced in the small arteries throughout the body. The characteristic vascular lesions present in the mature animal are not a notable feature in EVA-related abortions.

Serological tests: A variety of serological tests, including virus neutralisation (VN), complement fixation (CF), indirect fluorescent antibody, agar gel immunodiffusion and the enzyme-linked immunosorbent assay (ELISA), have been used for the detection of antibody to EAV. The tests currently in widest use are the complement-enhanced VN test and the ELISA. The VN test is a very sensitive and highly specific assay of proven value in diagnosing acute infection and in seroprevalence studies. Several ELISAs have been developed, none of which have been as extensively validated as the VN test though some appear to offer comparable specificity and sensitivity. The CF test is less sensitive than either procedure, but it can be used for diagnosing recent infection.

Requirements for vaccines and diagnostic biologicals: Two commercial tissue culture vaccines are currently available against EVA. One is a modified live virus (MLV) vaccine prepared from virus that has been attenuated for horses by multiple serial transfers in primary equine and rabbit cell cultures. It has been shown to be safe and protective for stallions and nonpregnant mares. Vaccination of foals under 6 weeks of age and of pregnant mares in the final 2 months of gestation is contraindicated. There is no evidence of back reversion to virulence of the vaccine virus following its use in the field over a significant number of years. The second vaccine is an inactivated, adjuvanted product prepared from virus grown in equine cell culture that can be used
in nonbreeding and breeding horses. In the absence of appropriate safety data, the vaccine is not currently recommended for use in pregnant mares.

A. INTRODUCTION

Equine viral arteritis (EVA) is a contagious viral disease of equids caused by equine arteritis virus (EAV), a positive-sense, single-stranded RNA virus, and the prototype member of the genus Arterivirus, family Arteriviridae, order Nidovirales (6). Epizootic lymphangitis pinkeye, fièvre typhoïde and rotlaufseuche are some of the descriptive terms used in the past to refer to a disease that clinically resembled EVA. The natural host range of EAV would appear to be restricted to equids and the virus does not present a human health hazard (40). EAV is present in the horse population of many countries world-wide (40). There has been an increase in the incidence of EVA in recent years associated with the greater frequency of movement of horses and use of transported semen (2, 40).

While the majority of cases of acute infection with EAV are subclinical, certain strains of the virus can cause disease of varying severity (40). Typical cases of EVA can present with any combination of the following clinical signs: fever, depression, anorexia, leukopenia, dependent oedema, especially of the limbs, scrotum and prepuce of the stallion, conjunctivitis, ocular discharge, supra or periorbital oedema, rhinitis, nasal discharge, a local or generalised urticarial skin reaction, abortion and, rarely, a fulminating pneumonia or pneuomo-enteritis in young foals. Regardless of the severity of clinical signs, affected horses almost invariably make complete recoveries. The case-fatality rate in outbreaks of EVA is very low; mortality is usually only seen in very young foals, especially those congenitally infected with the virus (29, 40).

EVA cannot be differentiated clinically from a number of other respiratory and systemic equine diseases, the most common of which are equine influenza, equine herpesvirus 1 and 4 infections, infection with equine rhinitis A and B viruses, equine encephalomyelitis and streptococcal infections, with particular reference to purpura haemorrhagica. The disease also has clinical similarities to equine infectious anaemia, cases of infection with Getah virus, African horse sickness fever, and toxicosis caused by hoary alyssum (Berteroa incana). EAV replicates and is shed in large quantities from the respiratory tract of acutely infected animals (39). A variable percentage of acutely infected stallions become long-term carriers in the reproductive tract and constant semen shedders of the virus (40, 41). The carrier state has only been found in the stallion, not in the mare, gelding or sexually immature colt (40).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Virus isolation

Where an outbreak of EVA is suspected, or when attempting to confirm a case of subclinical EAV infection, virus isolation should be attempted from nasopharyngeal and conjunctival swabs, unclotted blood samples, and semen from stallions considered to be possible carriers of the virus (40). To optimise the chances of virus isolation, the relevant specimens should be obtained as soon as possible after the onset of fever in affected horses. There is evidence that heparin can inhibit the growth of EAV in RK-13 cells (1), and therefore, its use as an anticoagulant may interfere with isolation of the virus from whole blood. Where EVA is suspected in cases of mortality in young foals or older animals, isolation of EAV can be attempted from a variety of tissues, especially the lymphatic glands associated with the alimentary tract and related organs, and also the lungs, liver and spleen (31). In outbreaks of EVA-related abortion, placental and fetal fluids and a wide range of placental, lymphoreticular and other fetal tissues can be productive sources of virus (40).

Swabs for attempted isolation should be immersed in a suitable viral transport medium and these, together with any fluids or tissues collected for either virus isolation or reverse-transcription polymerase chain reaction (RT-PCR) testing should be shipped either refrigerated or frozen in an insulated container to the laboratory, preferably using an overnight delivery service. Unclotted blood samples must be transported refrigerated but not frozen.

Although reportedly not always successful in natural cases of EAV infection (32, 40), virus isolation should be attempted from clinical specimens or necropsied tissues using rabbit, equine or monkey kidney cell culture (32, 40). Selected cell lines, e.g. RK-13 (ATCC CCL-37), LLC-MK2 (ATCC CCL-7), and African green monkey kidney (Vero) (ATCC CCL-81) cells or primary horse or rabbit kidney cell culture can be used, with early passage RK-13 cells being the cell system of choice. Several factors have been shown to influence primary isolation of EAV from semen in RK-13 cells (37). Higher isolation rates have been
obtained using 3–5-day-old monolayers, a large inoculum size in relation to the cell surface area in the inoculated flasks or multiwell plates, and most importantly, the incorporation of carboxymethyl cellulose in the overlay medium. It should be noted that most RK-13 cells, including ATCC CCL-37, are contaminated with bovine viral diarrhoea virus, the presence of which appears to enhance sensitivity of this cell system for the primary isolation of EAV. There is some evidence to suggest that primary isolation rates of EAV, particularly from semen, may be increased in RK-13 cells of high passage history.

Inoculated cultures are examined daily for the appearance of viral cytopathic effect (CPE), which is usually evident within 2–6 days. In the absence of visible CPE, culture supernatants should be subinoculated on to fresh cell monolayers after 5–7 days. The majority of isolations of EAV are made in the first or second passage in cell culture (40, 41). The identity of isolates of EAV can be confirmed in a one-way neutralisation test, by RT-PCR assay (2) or by an immunocytochemical method, namely indirect immunofluorescence (11) or the avidin–biotin–peroxidase (ABC) technique (28). A polyclonal rabbit antisera has been used to identify EAV in infected cell cultures. Mouse monoclonal antibodies (MAbs) to the nucleocapsid (N) protein and envelope G protein of EAV (12) and a monospecific polyclonal rabbit antisera to the envelope (M) protein have also been developed and these can detect various strains of the virus in RK-13 cells as early as 12–24 hours after infection (2, 28).

**Virus isolation from semen (the prescribed test for international trade)**

There is considerable evidence that short- and long-term carrier stallions shed EAV constantly in the semen, but not in respiratory secretions or urine; nor has it been demonstrated in the buffy coat of the blood of such animals (40, 41). Stallions should first be blood tested using the virus neutralisation (VN) test or an appropriately validated enzyme-linked immunosorbent assay (ELISA). Virus isolation should be attempted from the semen of stallions serologically positive for antibodies to EAV that do not have a certified history of vaccination against EVA. Virus isolation is also indicated in the case of shipped semen where a blood sample from the donor stallion is not available. It is recommended that virus isolation from semen be attempted from two samples, which can be collected on the same day, on consecutive days or after an interval of several days or weeks. There is no evidence that the outcome of attempted virus isolation from particular stallions is influenced by the interval between collections or time of the year. Isolation of EAV should be carried out preferably on a portion of an entire ejaculate collected using an artificial vagina or a condom and a teaser or phantom mare. When it is not possible to obtain semen by this means, a less preferable alternative is to collect a dismount sample at the time of breeding. Care should be taken to ensure that no antiseptics/disinfectants are used in the cleansing of the external genitalia of the stallion prior to collection. Samples should contain the sperm-rich fraction of the ejaculate with which EAV is associated. The virus is not present in the pre-sperm fraction of semen (40, 41). Immediately following collection, the semen should be refrigerated on crushed ice or on freezer packs for transport to the laboratory with a minimum of delay. Where there is likely to be a delay in submitting a specimen for testing, the semen can be frozen at or below –20°C for a varying period of days or weeks before being dispatched to the laboratory. Freezing a sample has not been found to militate against isolation of EAV from the semen of a carrier stallion.

**Test procedure**

i) Semen samples are pretreated before inoculation into cell culture by short-term sonication (for three 15-second cycles) followed by centrifugation at 1000 g for 10 minutes at 4°C to sediment the spermatozoa.

ii) After removal of culture medium, 3–5-day-old confluent monolayer cultures of RK-13 cells, either in 25 cm² tissue culture flasks or multiwell plates, are inoculated with serial decimal dilutions (10⁻¹–10⁻³) of seminal plasma in tissue culture maintenance medium containing 2% fetal bovine serum and antibiotics. An inoculum of 1 ml per 25 cm² flask is used and no fewer than two flasks per dilution of seminal plasma are inoculated. Inoculum size and number of wells inoculated per dilution of a specimen should be pre-rated where multwell plates are used. Appropriate dilutions of a virus positive control semen sample or virus control of known titre diluted in culture medium should be included in each test.

iii) The flasks or cover plates are closed and gently rotated to disperse the inoculum over the cell monolayers.

iv) Inoculated cultures are then incubated for 1 hour at 37°C either in an aerobic incubator or an incubator containing a humidified atmosphere of 5% CO₂ in air, depending on whether flasks or multiwell plates are used.

v) Without removing any of the inoculum or washing the cell monolayers, the latter are overlaid with 0.75% carboxymethyl cellulose containing medium with antibiotics.

vi) The flasks or plates are reincubated at 37°C and checked microscopically for viral CPE, which is usually evident within 2–6 days.
The identity of any virus isolates should be confirmed by VN, immunofluorescence or ABC technique, using a monospecific polyvalent antiserum to EAV or MAbs to the N or G proteins of the virus (11, 28, 40, 41), or by RT-PCR assay and appropriate viral RNA primers (2).

In the one-way neutralisation test, serial decimal dilutions of the virus isolate are tested against an MAb or monospecific antiserum prepared against the prototype Bucyrus strain of EAV (ATCC VR 796) and also a serum negative for neutralising antibodies to the virus. Corresponding titrations of the prototype Bucyrus virus with the same reference antibody reagents are included as test controls. The test is performed in either 25 cm² tissue culture flasks or multiwell plates. Appropriate quantities of the known EAV positive and negative antibody reagents are inactivated for 30 minutes in a water bath at 56°C and diluted 1/4 in phosphate buffered saline, pH 7.2; then 0.3 ml of diluted antibody reagent is dispensed into five tubes for each virus isolate to be tested. Serial decimal dilutions (10⁻¹–10⁻⁵) of each virus are made in Eagle’s Minimal Essential Medium containing 10% fetal bovine serum, antibiotics and 10% freshly diluted guinea-pig complement. Then, 0.3 ml of each virus dilution is added to the tubes containing positive and negative antibody reagents. The tubes are shaken and the virus/antibody mixtures are incubated for 1 hour at 37°C. The mixtures are then inoculated on to 3–5-day-old confluent monolayer cultures of RK-13 cells, either in 25 cm² flasks or multiwell plates, using two flasks or wells per virus dilution. Each flask is inoculated with 0.25 ml of virus/antibody mixture; the inoculum size is pro-rated where multiwell plates are used. Inoculated flasks or plates are incubated for 2 hours at 37°C, gently rocking after 1 hour to disperse the inoculum over the cell monolayers. Without removing any of the inoculum or washing the cell monolayers, the latter are overlaid with 0.75% carboxymethyl cellulose containing medium and incubated for 4–5 days at 37°C, either in an aerobic incubator or an incubator containing a humidified atmosphere of 5% CO₂ in air. After removal of the medium, monolayers are stained with 0.1% formalin-buffered crystal violet solution. Plaques are counted and the virus infectivity titre is determined both in the presence and absence of EAV antibodies using the Spearman–Kärber method. Identification of a virus isolate is based on the extent of plaque reduction compared with that of the prototype Bucyrus strain of EAV.

Most EAV isolates from carrier stallions are made in the first passage in cell culture using the described test procedure (40, 41). The occurrence of nonviral cytotoxicity or bacterial contamination of specimens are not significant problems when attempting isolation of this virus from stallion semen. Nonviral cytotoxicity, if observed, usually affects monolayers inoculated with the 10⁻¹ and, much less frequently, the 10⁻² dilution of seminal plasma. Treatment of seminal plasma with polyethylene glycol (Mol. wt 6000) prior to inoculation has been used with success in overcoming this problem (19). The method described involves the addition of polyethylene glycol to the 10⁻¹–10⁻³ dilutions of seminal plasma to give a final concentration of 10% in each dilution. The mixtures are held overnight at 4°C with gentle stirring, after which they are centrifuged at 2000 g for 30 minutes and the supernatants are discarded. The precipitates are suspended in cell culture maintenance medium to one-tenth the volume of the original dilutions and the mixtures are homogenised. They are then centrifuged at 2000 g for 30 minutes and the supernatants are taken off and used for inoculation. There is no evidence to indicate that pretreatment of seminal plasma in this manner reduces sensitivity of the virus isolation procedure.

The presence of anti-EAV antibody in the seminal plasma of certain virus-shedding stallions has not been found to prevent the carrier state in these animals.

b) Nucleic acid recognition methods

The RT-PCR assay is an additional method (to virus isolation in cell culture) for EAV detection. Single, nested, and one-tube real-time TaqMan® RT-PCR assays have been developed and evaluated for detection of various strains of the virus in cell culture, semen and nasal secretions (3, 5, 21). Single-step extraction of RNA followed by reverse transcription and amplification has been described (5, 21). Detection of amplified products can be performed either by agarose gel electrophoresis or by an ELISA–PCR assay using commercial reagents. The RT-PCR assay provides a means of identifying virus-specific RNA in clinical specimens, namely nasopharyngeal swab filtrates, buffy coats, semen and urine and in post-mortem tissue samples (2, 3, 5, 21). Comparable results to virus isolation have been obtained with both a nested RT-PCR assay that takes only 2 days to complete and a real-time TaqMan® assay (2, 3, 5, 21). The assay has the advantage of not requiring viable virus for performance of the test. Because of the high sensitivity of the procedure, however, there is the potential for cross-contamination between samples in the laboratory, giving rise to a false-positive result. The risk of cross-contamination is greater in conventional RT-PCR because of the separate reverse transcription step and the second PCR step in nested PCR assays (3). To minimise the risk of this occurring, considerable care needs to be taken, especially during the steps of RNA extraction and amplification, and relevant EAV positive and negative controls and, where appropriate, RNA extracted from the tissue culture fluid of uninfected cells, need to be included in each PCR assay.
Primer selection is critical to the sensitivity of the RT-PCR assay with primers preferably designed from the most conserved regions of the viral genome. Sequences chosen from the viral polymerase, nucleocapsid (N) or envelope (M) protein genes are reportedly effective in a single, nested or real-time TaqMan® RT-PCR assay (3, 5, 21). There is growing evidence that open reading frame (ORF) 7 is the most conserved gene among different strains of EAV (4) and ORF-7-specific primers have detected a diversity of strains of the virus of European and North American origin (3). However, until such time as a complete consensus or universal primer set for EAV has been agreed upon, where feasible, it is advisable to use the RT-PCR assay in conjunction with, and not as an alternative to, virus isolation for the identification of virus in clinical or post-mortem specimens. When virus isolation is attempted in full accordance with OIE recommended procedures, it has been found to be comparable to the RT-PCR assay for the detection of EAV (2, 3, 5, 21).

It should be emphasised that as the RT-PCR assay will not distinguish between infectious and noninfectious or incomplete virus and, consequently, it cannot establish the current infectivity status of a particular animal or sample of semen, a factor of considerable importance with respect to the international trade in horses and equine semen.

Strains of EAV isolated from different regions of the world have been classified into different phylogenetic groups by sequence analysis of the envelope (G_L, G_S, M) protein genes and the nucleocapsid (N) protein gene (4, 8, 39). The relationships between strains demonstrated by nucleotide sequencing is a useful molecular epidemiological tool for tracing the origin of outbreaks of EVA (2).

c) Histopathological and immunohistochemical methods

Where mortality is associated with a suspected outbreak of EVA, a wide range of tissues should be examined for histological evidence of panvasculitis that is especially pronounced in the small arteries throughout the body, particularly in the caecum, colon, spleen, associated lymphatic glands and adrenal cortex (11, 26). The presence of a disseminated necrotising arteritis involving endothelial and medial cells of affected vessels is considered to be pathognomonic of EVA. The characteristic vascular lesions present in the mature animal are not, however, as prominent a feature in EAV-related abortions (25).

EAV antigen can be identified in various tissues of EVA-affected animals either in the presence or absence of lesions (11). It can be detected within the cytoplasm of infected cells by immunofluorescence using conjugated equine polyclonal anti-EAV serum (11), or by the ABC technique using mouse MAbs to the N or G_L proteins of the virus (29).

2. Serological tests

A variety of serological tests including neutralisation (microneutralisation [38] and plaque reduction [30]), the complement fixation (CF) test (16), the indirect fluorescent antibody test (11), the agar gel immunodiffusion (11), and the ELISA (7, 9, 23, 24, 27, 36) have been used to detect antibody to EAV.

The test currently in widest use to diagnose infection, carry out seroprevalence studies, and test horses for export, is a microneutralisation test in the presence of complement. Apart from the VN test, the CF test has been used for diagnosing recent EAV infection as complement-fixing antibodies are relatively short-lived in duration (16). In contrast, neutralising antibody titres to EAV can persist for several years after natural infection (40). Although a number of ELISAs have been developed (7, 9, 27), none has as yet been as extensively validated as the VN, though some appear to offer comparable sensitivity and specificity (7, 23, 24, 36). Unlike the VN test, a positive reaction in the ELISA is not necessarily reflective of the protective immune status of an individual horse to EAV as both non-neutralising and neutralising antibodies are involved.

Antiserum to unpurified EAV has been prepared in horses and in rabbits using conventional immunisation protocols. Also, mouse monoclonal and monospecific rabbit polyclonal antibodies have been developed to the nucleocapsid (N) protein envelope (G_L), protein, envelope (M and G_S) proteins and other proteins of EAV (12).

OIE Standard Sera for EAV are available1 and these can facilitate international standardisation of the microneutralisation test and ELISA.

Only one major serotype of EAV has been recognised so far (30, 40). This is represented by the prototype Bucyrus strain (ATCC VR 796), from which the reference virus used in all EAV serological assays has been derived. Virus stock is grown in the RK-13 cell line, clarified of cellular debris by low-speed centrifugation and stored in aliquots at –70°C. Several frozen aliquots are thawed and the infectivity of the stock virus is determined by titration in RK-13 cells.

1 Available from Dr P.J. Timoney, Director, Maxwell H. Gluck Equine Research Center, Dept of Veterinary Science, University of Kentucky, Lexington, Kentucky 40546-0099, United States of America.
a) **Virus neutralisation (the prescribed test for international trade)**

The VN test is used to screen stallions for evidence of EAV infection and to determine whether there is a need to attempt virus detection in semen using cell culture or RT-PCR assay. It can also be used for diagnostic purposes to confirm infection in suspect cases of EVA. The VN test procedure in current widest use is that developed by the National Veterinary Service Laboratories of the United States Department of Agriculture (38). It is recommended that the test be carried out in RK-13 cells using the approved CVL-Bucyrus (Weybridge) strain of EAV as reference virus. Originally derived from the prototype Bucyrus virus, the passage history of the CVL (Weybridge) strain is not fully documented. The sensitivity of the VN test for detection of antibodies to EAV can be significantly influenced by several factors, especially the source and passage history of the strain of virus used (14, 15). The CVL-Bucyrus strain and the highly attenuated vaccine strain of EAV are of comparable sensitivity for detecting low-titred positive sera, especially from EVA-vaccinated horses. For this test, it is important to obtain a sterile blood sample as contamination of serum can interfere with the result. Efforts are continuing to bring about greater uniformity in the testing protocol and comparability in serological results among laboratories providing the VN test.

### Test procedure

- **Sera are inactivated for 30 minutes in a water bath at 56°C (control sera, only once).**
- **Serial twofold dilutions of the inactivated test sera in serum-free cell culture medium (25 µl volumes) are made in a 96-well, flat-bottomed, cell-culture grade microtitre plate starting at a 1/2 serum dilution and using duplicate rows of wells for each serum to be tested.** Most sera are screened initially at a 1/4 and 1/8 serum dilution (i.e. final serum dilution after addition of an equal volume of the appropriate dilution of stock virus to each well). Positive samples at the 1/8 dilution can, if desired, be retested and titrated out for end-point determination. Individual serum controls, together with negative and known low- and high-titred positive control sera must also be included in each test.
- **A dilution of stock virus to contain from 100 to 300 TCID\(_{50}\) (50% tissue culture infective dose) per 25 µl is prepared using as diluent, serum-free cell culture medium containing antibiotics and fresh guinea-pig or rabbit complement at a final concentration of 10%.**
- **25 µl of the appropriate dilution of stock virus is added to every well containing 25 µl of each serum dilution, except the test serum control wells.**
- **A virus back titration of the working dilution of stock virus is included, using four wells per tenfold dilution, to confirm the validity of the test results.**
- **The plates are covered and shaken gently to facilitate mixing of the serum/virus mixtures.**
- **The plates are incubated for 1 hour at 37°C in a humid atmosphere of 5% CO\(_2\) in air.**
- **A suspension of cells from 3–5-day-old cultures of RK-13 cells are prepared using a concentration that will ensure confluent monolayers in the microtitre plate wells within 18–24 hours after seeding.**
- **100 µl of cell suspension is added to every well, the plates covered with plate lids or sealed with tape and shaken gently.**
- **The plates are incubated at 37°C in a humid atmosphere of 5% CO\(_2\) in air.**
- **The plates are read microscopically for nonviral CPE after 12–18 hours and again for viral CPE after 48–72 hours' incubation. The validity of the test is confirmed by establishing that the working dilution of stock virus contained 30–300 TCID\(_{50}\) virus and that the titres of the positive serum controls are within 0.3 log\(_{10}\) units of their predetermined titres.**

A serum dilution is considered to be positive if there is an estimated 75% or preferably 100% reduction in the amount of viral CPE in the serum test wells compared with that present in the wells of the lowest virus control dilution. End-points are then calculated using the Spearman–Kärber method. A titre of 1/4 or greater is considered to be positive. A negative serum should only have a trace (less than 25%) or no virus neutralisation at the lowest dilution tested. Antibody titres may, on occasion, be difficult to define as partial neutralisation may be observed over a range of several serum dilutions. Infrequently, sera will be encountered that cause toxic changes in the lower dilutions tested. In such cases it may not be possible to establish whether the sample is negative or a low-titred positive. The problem may be overcome by testing another serum sample from the animal in question or by retesting the toxic sample using microtitre plates with confluent monolayers of RK-13 cells that had been seeded the previous day. It has been reported that the toxicity of the serum can be reduced or eliminated if adsorbed with packed RK-13 cells. Vaccination status for equine herpesviruses should be considered when evaluating sera causing non-viral cytotoxicity. One of the equine herpesvirus vaccines currently available in Europe can stimulate antibodies to rabbit kidney cells which, in turn, can interfere with interpretation of the test results.

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2 Available from: Virology Department, Veterinary Laboratories Agency, Weybridge, New Haw, Addlestone KT15 3NB, United Kingdom.
b) Enzyme-linked immunosorbent assay

A number of direct or indirect ELISAs have been developed for the detection of antibodies to EAV (7, 9, 23, 24, 27, 36). These have been based on the use of purified virus or recombinant-derived viral antigens. The usefulness of earlier assays was compromised by the frequency of false-positive reactions (10). The latter were associated with the presence of antibodies to various tissue culture antigens in the sera of horses that had been vaccinated with tissue-culture-derived antigens (10). Identification of the importance of the viral G₂ protein in stimulation of the humoral antibody response to EAV led to the development of several ELISAs that employ a portion of, or the entire recombinant protein produced in a baculovirus expression system (9, 12, 23). Most recently, an ovalbumin-conjugated synthetic peptide representing amino acids 81–106 of the G₂ protein has been used (36). Some of these assays appear to offer comparable sensitivity and specificity to the VN test and may detect EAV-specific antibodies prior to a positive reaction being obtainable in the VN test (7). False-negative reactions can occur, however, with some of these assays. Screening a random peptide-phage library with polyclonal sera from EAV-infected horses led to the identification of ligands, which were purified and used as antigen in an ELISA for EAV (24). No correlation was found, however, between absorbency values obtained with this assay and neutralising antibody titres, indicating that the antibodies being detected were largely against nonsurface epitopes of the virus. An ELISA based on the use of a combination of the G₂ M or N structural proteins of EAV expressed from recombinant baculoviruses successfully detected viral antibody in naturally or experimentally infected horses but not in EVA-vaccinated animals (23). Of major importance with respect to any G₂ protein-based ELISA for EAV is the fact that test sensitivity will vary depending on the ectodomain sequence(s) of this viral protein used in the assay. Considerable amino acid sequence variation within this domain has been found between isolates of EAV (4). To maximise sensitivity of a G₂-based ELISA, it may be necessary to include multiple ectodomain sequences representative of known phylogenetically different isolates of EAV rather than depend on a single ectodomain sequence. Two more recently described ELISAs appear to offer most promise as reliable serodiagnostic tests for EAV infection (9, 36). A blocking ELISA involving MAb produced against the G₂ protein was reported to have a sensitivity of 99.4% and a specificity of 97.7% compared with the VN test (9). Another assay, a G₂ ovalbumin-conjugated synthetic peptide ELISA was shown to have a sensitivity and specificity of 96.75% and 95.6%, respectively, using a panel of 400 VN positive sera and 400 VN negative samples (36). It is expected that an ELISA will be available soon having very similar if not equivalent sensitivity and specificity to the VN test.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

A number of experimental and commercial vaccines have been developed against EVA. Currently, there are two commercially available vaccines, both tissue-culture derived. The first is a modified live virus (MLV) vaccine prepared from virus that has been attenuated for horses by multiple serial transfers in equine and rabbit cell cultures (13, 30). This vaccine is licensed for use in stallions, nonpregnant mares and in nonbreeding horses. Whereas nonbreeding horses can be vaccinated at any time, stallions and mares should be vaccinated not less than 3 weeks prior to breeding. The vaccine is not recommended for use in pregnant mares, especially in the last 2 months of gestation, nor in foals under 6 weeks of age unless in the face of significant risk of exposure to natural infection. The vaccine is commercially available in the USA and Canada. It has also been used in New Zealand, subject to ministerial controls, to aid in that country’s EVA eradication programme.

The second commercially available vaccine against EVA is an inactivated product prepared from virus grown in equine cell culture, which is filtered, chemically inactivated and then combined with a metabolisable adjuvant. This vaccine is licensed for use in nonbreeding and breeding horses. In the absence of appropriate safety data, the vaccine is currently not recommended for use in pregnant mares. The initial vaccination regimen involves two doses of vaccine administered intramuscularly 3–6 weeks apart. Booster vaccination at 6-month intervals is recommended by the manufacturer. The inactivated vaccine is licensed for commercial use in certain European countries, including Denmark, France, Germany, Ireland, Sweden and the United Kingdom.

An additional inactivated vaccine against EVA has been developed in Japan for use should an outbreak of EVA occur in that country (18). It is an aqueous formalin-inactivated vaccine that has been shown to be safe and effective for use in nonbreeding and breeding horses. For optimal immunisation with this vaccine, horses require a primary course of two injections given at an interval of 4 weeks, with a booster dose administered every 6–12 months. As the vaccine is currently not commercially available, no details will be provided on its production.

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.
Chapter 2.5.10. – Equine viral arteritis

1. Seed management

a) Characteristics of the seed

Both MLV and inactivated commercial vaccines are derived from the prototype Bucyrus strain of EAV (ATCC VR 796). Available evidence points to the existence of only one major serotype of the virus, and strain variation is not considered to be of significance in relation to vaccine efficacy (30, 40).

In the case of the MLV vaccine, the prototype virus was attenuated by serial passage in primary cultures of horse kidney (HK-131), rabbit kidney (RK-111), and a diploid equine dermal cell line, ATCC CCL57 (ECID-24) (13, 22, 30). The indications from the use of this vaccine are that the virus is safe and immunogenic between its 80th and 111th passage in primary rabbit kidney (13, 22, 30, 33, 34, 42).

The inactivated adjuvanted vaccine is prepared from the unattenuated prototype Bucyrus strain of EAV (ATCC VR 796) that has been plaque purified and in its fourth serial passage in the diploid equine dermal cell line (ECID-4). After growth in cell culture, the virus is then purified by filtration before being chemically inactivated and adjuvanted.

Suitable lots of master seed virus for each vaccine should be maintained in liquid nitrogen or its equivalent.

b) Method of culture

The virus for both MLV and inactivated vaccines should be grown in a stable cell culture system, such as equine dermal cells, using an appropriate medium supplemented with sterile bovine serum or bovine serum albumin as replacement for bovine serum in the growth medium. Cell monolayers should be washed prior to virus inoculation to remove traces of bovine serum. Extensive virus growth as evidenced by the appearance of cytopathic changes in 80–100% of the cells should be obtained within 2–3 days.

c) Validation as a vaccine

In the case of both MLV and inactivated vaccines, the respective virus strains should be grown in an appropriate cell culture system that has been officially approved for vaccine production and confirmed to be free from extraneous bacteria, fungi, mycoplasmas and viruses (35). The identity of the vaccine virus in the master seed should be confirmed by neutralisation with homologous anti-EAV serum. Incomplete neutralisation of EAV by homologous horse or rabbit antisera has been scientifically documented (38) and is a problem when screening master seed virus for extraneous viruses and when attempting to confirm the identity of the vaccine virus. The problem has been circumvented by reducing the infectivity titre of the master seed virus below that required for seed virus production before conducting a neutralisation test on the diluted virus. Virus/serum mixtures are tested for residual live virus by serial passage in cell culture. No evidence of cytopathic viruses, haemadsorbing viruses, or noncytopathic strains of bovine virus diarrhoea virus should be found, based on attempted virus isolation in cell culture. If cells of equine origin are used, they should be confirmed to be free from equine infectious anaemia virus. The newer technologies of PCR and antigen-capture ELISA may be used in the future as adjuncts to virus isolation in screening for adventitious agents.

The MLV vaccine has been shown to be both safe and effective for use in stallions and nonpregnant mares (33, 34, 42). Vaccination confers a high level of protective immunity that persists for at least several years (22, 30, 40). Based on experimental studies and extensive field use of the vaccine since 1985, there is no evidence of back reversion to virulence of the vaccine virus, nor of recombination of the vaccine virus with naturally occurring strains of EAV. Furthermore, there are ample data to confirm that the attenuated strain of EAV in the current vaccine is not shed in the semen of stallions after vaccination nor does it localise and set up the carrier state in the reproductive tract of the stallion (34, 40, 42).

The commercial inactivated vaccine has been shown to be nonreactive and safe for use in healthy nonbreeding and breeding horses. Transient local reactions may be observed in less than 10% of horses vaccinated with the inactivated vaccine. Limited field studies of this vaccine indicate that it is immunogenic, stimulating a satisfactory degree of immunity, the duration of which has yet to be reported.

Although there are no published reports on the efficacy of either commercial vaccine in preventing establishment of the carrier state in the stallion, an aqueous formalin inactivated vaccine against EVA has been shown to prevent virus persistence in the reproductive tract of vaccinated stallions following subsequent experimental challenge with EAV (17).

2. Method of manufacture

Both the MLV and inactivated vaccines are produced by cultivation of the respective seed viruses in an equine dermal cell system. Cell monolayers should be washed prior to inoculation with seed virus to remove traces of
bovine serum in the growth medium. Inoculated cultures should be maintained on an appropriate maintenance medium. Harvesting of infected cultures should take place when almost the entire cell sheet shows the characteristic CPE. Supernatant fluid and cells are harvested and clarified of cellular debris and unwanted material by filtration. In the case of the inactivated vaccine, the purified virus is then chemically inactivated and adjuvanted with a metabolisable adjuvant.

3. In-process control

The MLV and inactivated vaccines should be produced in a stable cell line that has been tested for identity and confirmed to be free from contamination by bacteria, fungi, mycoplasmas or other adventitious agents. In addition to the preproduction testing of the master seed virus for each vaccine and the cell line for adventitious contaminants, the cell cultures infected with the respective vaccine viruses should be examined macroscopically for evidence of microbial growth or other extraneous contamination during the incubation period. If growth in a culture vessel cannot be reliably determined by visual examination, subculture, microscopic examination, or both should be carried out.

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

In the case of both MLV and inactivated vaccines, each production lot of vaccine should be checked for extraneous bacterial, fungal and mycoplasmal contaminants. The vaccine should be safety tested by the intramuscular inoculation of at least two horses seronegative for neutralising antibodies to EAV with one vaccine dose of lyophilised virus each (35). None of the inoculated horses should develop any clinical signs of disease other than mild pyrexia during the ensuing 2-week observation period. In addition, nasopharyngeal swabs should be collected daily from each horse for attempted virus isolation; white blood cell counts and body temperatures should also be determined on a daily basis. No significant febrile or haematological changes should supervene following vaccination (34, 40, 42). Limited shedding of vaccine virus by the respiratory route for at most 7 days may be demonstrated in the occasional vaccinated horse (42). There is no evidence of virus shedding in the semen of stallions following vaccination (34, 42).

To ensure complete inactivation of the vaccine virus, each serial lot of the inactivated vaccine should be checked for viable virus by three serial passages in equine dermal cells and by direct fluorescent antibody staining with specific EAV conjugate before being combined with adjuvant. This should be followed by a safety test in guinea-pigs and mice.

c) Potency

Potency of the vaccine in the final containers is determined by plaque infectivity assay in monolayer cultures of equine dermal cells and by a vaccination challenge test in horses (35). The vaccine must be tested in triplicate in cell culture, the mean infectivity titre calculated and the dose rate determined on the basis that each dose of vaccine shall contain not less than $3 \times 10^4$ plaque-forming units of attenuated EAV. The in-vivo potency of the MLV and inactivated vaccines is evaluated in a single vaccination challenge test using 17–20 vaccinated and 5–7 control horses or in two tests each comprising ten vaccinates and five controls.

The viral antigen concentration in the inactivated vaccine is over one-thousand times the concentration of viral antigen present in the MLV vaccine.

d) Duration of immunity

Detectable neutralising antibody titres to EAV should develop in the majority of horses within 3 weeks of vaccination with the MLV vaccine (33, 34, 40, 42). Responses to primary vaccination are characterised by a rapid fall in antibody titres with a significant number of animals reverting to seronegativity 1–3 months after vaccination (42). Revaccination with this vaccine results in an excellent anamnestic response, however, with the development of high antibody titres that remain relatively undiminished for 12 months or longer (40).

Experimental studies have shown that most horses vaccinated with the inactivated vaccine develop low to moderate neutralising antibody titres to EAV by day 14 after the second vaccination. There is no published information on the duration of immunity conferred by this vaccine.
Chapter 2.5.10. – Equine viral arteritis

e) Stability

The lyophilised MLV vaccine can be stored for at least 3–4 years at 2–7°C without loss in infectivity, provided it is kept in the dark (22). Infectivity is preserved for much longer periods if vaccine is frozen at −20°C or below. Once rehydrated, however, the vaccine should be used within 1 hour or else destroyed. The inactivated vaccine is stored as a liquid suspension at 2–8°C, with no loss of potency for at least 1 year, provided it is protected from light.

f) Preservatives

The preservatives added to the MLV and inactivated vaccines are neomycin, polymyxin B and amphotericin B.

g) Precautions (hazards)

Pregnant mares should not be vaccinated with the MLV vaccine during the last 2 months of gestation, as there is a risk, albeit minimal, of fetal invasion by the vaccine virus. The possibility of a vaccinally induced anaphylactic reaction, though very rare, could result from the administration of either the MLV or inactivated vaccine. In the absence of appropriate safety data, the inactivated vaccine is currently not recommended for use in pregnant mares.

5. Tests on the final product

a) Safety

With the exception of the inactivated vaccine, which needs to be sterility tested a second time to ensure freedom from contamination, no further safety tests are required on the inactivated or MLV vaccines.

b) Potency

No potency tests additional to those conducted on each production lot of the MLV or inactivated vaccines are required on either final product.

REFERENCES


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NB: There are OIE Reference Laboratories for Equine viral arteritis 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 2.5.11.

HORSE MANGE

See Chapter 2.10.4. Mange

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**
VENEZUELAN EQUINE ENCEPHALOMYELITIS

CHAPTER 2.5.12.

SUMMARY

Venezuelan equine encephalomyelitis (VEE) viruses, of the genus Alphavirus of the family Togaviridae, cause disease ranging from mild febrile reactions to fatal encephalitic zoonoses in Equidae and humans. They are transmitted by haematophagous insects.

High infection rates in humans have been documented following exposure to aerosols from infected laboratory animals or from laboratory accidents. Severe clinical disease or death can occur in humans. Consequently infective VEE viruses or their antigens prepared from infected tissues or cell cultures must never be handled by personnel who do not possess demonstrable immunity in the form of neutralising antibody. All laboratory manipulations must be carried out within certified biological safety cabinets following containment level 3 procedures (see Chapter I.1.6. Human safety in the veterinary microbiology laboratory).

VEE viruses are divided into six antigenic subtypes (I–VI). Within subtype I there are five antigenic variants (variants AB–F). Antigenic variants I-AB and I-C are associated with epizootic activity in equids. The other three variants of subtype I (I-D, I-E, I-F) and the other five subtypes of VEE have been associated with natural enzootic cycles. These variants and subtypes have been considered to be nonpathogenic for equids, although they can cause clinical disease in humans. These are known as enzootic VEE viruses; they cycle between rodents, mosquitoes, and birds. During 1993 and 1996 however, an outbreak of encephalitis in horses in Mexico was shown to be caused by enzootic VEE viruses of subtype I-E.

Identification of the agent: Diagnosis of VEE virus infection can be confirmed by the isolation, identification, and antigenic classification of the isolated virus.

A presumptive diagnosis of equine encephalomyelitis can be made when susceptible animals in tropical or subtropical areas display clinical signs of encephalomyelitis where haematophagous insects are active. VEE virus can be isolated in cell cultures or in laboratory animals using the blood or serum of febrile animals that have been exposed to clinical cases of encephalitis by the bites of infected mosquitoes. It is recovered less frequently from the blood or brains of encephalitic animals.

VEE virus can be identified by complement fixation, haemagglutination inhibition, plaque reduction neutralisation (PRN), or immunofluorescence tests using VEE-specific antibodies. Specific identification of epizootic VEE variants can be made by the indirect fluorescent antibody test, or a differential PRN test using subtype- or variant-specific monoclonal antibody, or by nucleic acid sequencing.

Serological tests: Specific antibodies may be demonstrated by PRN tests against epizootic VEE virus variants or by IgM capture enzyme-linked immunosorbent assay. Antibody can also be demonstrated by the haemagglutination inhibition or the complement fixation tests.

Any diagnosis of VEE in an individual that is based on seroconversion in the absence of an epizootic should be made with care. Although enzootic subtypes and variants are normally of low pathogenicity for equids, subclinical infection will stimulate antibody production to epizootic VEE virus variants.

Infections of equids with enzootic VEE viruses will usually produce a low level viraemia accompanied by antibody development, but without clinical disease in most cases.

Requirements for vaccines and diagnostic biologicals: The only acceptable vaccines against VEE are an attenuated virus vaccine, made with strain TC-83, or inactivated virus preparations.
also made from this strain. Attenuated virus is immunogenic when given by intramuscular injection, but sometimes causes adverse reactions in the recipient.

Formalin-inactivated virulent VEE virus preparations should never be used in equids, as residual virulent virus can remain after formalin treatment, and thereby cause severe illness in both animals and humans. Epizootics of VEE have occurred from the use of such formalin-treated viruses.

A. INTRODUCTION

The Venezuelan equine encephalomyelitis (VEE) complex of viruses, of the genus Alphavirus of the family Togaviridae, are mosquito-transmitted, zoonotic pathogens that produce from mild to severe febrile, occasionally fatal, encephalitic diseases in equines and humans. Infections by aerosols have originated from the cage debris of infected laboratory rodents and from laboratory accidents. Those who handle infectious VEE viruses or their antigens prepared from infected tissues or cell cultures should be vaccinated and shown to have demonstrable immunity in the form of VEE virus-specific neutralising antibody (1, 4). All procedures producing aerosols from VEE virus materials should be conducted in biosafety cabinets at containment level 3 (see chapter I.1.6. Human safety in the veterinary microbiological laboratory) (6, 7).

The VEE virus complex is composed of six subtypes (I–VI). Within subtype I, there are five antigenic variants (AB–F) (2–4, 8, 10). Originally, subtypes I-A and I-B were considered to be distinct variants, but they are now considered to be identical (I-AB). Within subtype III, there are three antigenic variants (A–C). Antigenic variants I-AB and I-C have been associated with epizootic VEE in equids and concurrent epidemics in humans (3, 4, 8–10). These equine pathogens are known as epizootic variants; they have been isolated from equids, humans, and haematophagous insects (primarily mosquitoes) only during equine epizootics. Subtype I-C was isolated in Venezuela in 1993 and Venezuela and Colombia in 1995. Subtype I-D was isolated in Peru in 1994 and 1995. These epizootic isolates appear to have evolved from enzootic I-D subtype (5).

The variants of subtype I (I-D, I-E and I-F) and the other five subtypes (II–VI) have been associated with naturally occurring cycles. Normally, enzootic VEE viruses do not produce clinical encephalomyelitis in the equine species (9), but in 1993 and 1996 in Mexico, an enzootic subtype caused a limited epizootic in horses. These enzootic variants and subtypes can produce clinical disease in humans (3, 4, 8, 10). Infections with both epizootic and enzootic variants and subtypes have been acquired by laboratory workers (6).

Historically, epizootic VEE was limited to northern and western South America (Venezuela, Colombia, Ecuador, Peru and Trinidad) (4). From 1969 to 1972, however, epizootic activity occurred in Guatemala, El Salvador, Nicaragua, Honduras, Costa Rica, Belize, Mexico, and the United States of America (USA) (Texas). Epizootics of VEE caused by I-AB or I-C virus have not occurred in North America and Mexico since 1972. Recent equine and human isolations of epizootic VEE virus were subtype 1-C strains from Venezuela in 1993, 1995 and 1996 and Colombia in 1995. In addition, an enzootic variant (I-E) caused disease in horses in Mexico in 1993. In contrast, enzootic VEE variants and subtypes exist continuously in enzootic foci in the tropical and subtropical Americas including the Florida Everglades (subtype II), Mexico (variant I-E), the Central American countries (variant I-E), Panama (variants I-D and I-E), Venezuela (variant I-D), Colombia (variant I-D), Peru (variant III-C), French Guiana (variant III-B and subtype V), Ecuador (variant I-D), Suriname (variant III-A), Trinidad (variant III-A), Brazil (variants I-F and III-A and subtype IV), and Argentina (subtype VI). In an atypical ecological niche, variant III-B has been isolated in the USA (Colorado and South Dakota) in an unusual association with birds (3, 4, 8, 10).

The foci of enzootic variants and subtypes are found in areas classified as tropical wet forest, i.e. those areas with a high water table or open swampy areas with meandering sunlit streams. These are the areas of the Americas where rainfall is distributed throughout the year or areas permanently supplied with water. Enzootic viruses cycle among rodents, and perhaps birds, by the feeding of mosquitoes (3, 4, 8, 10).

A tentative diagnosis of viral encephalomyelitis in equids can be based on the occurrence of acute neurological disease during the summer in temperate climates or in the wet season in tropical or subtropical climates. These are the seasons of haematophagous insect activity. Virus infection will result in clinical disease in many equids concurrently rather than in isolated cases. Epizootic activity can move vast distances through susceptible populations in a short time (3, 4, 8, 10).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

A confirmatory diagnosis of VEE is based on the isolation and identification of the virus or on the demonstration of seroconversion. The period of viraemia coincides with the onset of pyrexia within 12–24 hours of infection. Viraemia terminates 5–6 days after infection, and coincides with the production of neutralising antibodies and the
appearance of clinical neurological signs. Frequently, VEE viruses cannot be isolated from the brains of infected equids. Blood samples for virus isolation should be collected from febrile animals that are closely associated with clinical encephalitic cases.

Virus may be isolated from the blood or sera of infected animals by inoculating 1–4-day-old mice or hamsters intracerebrally or by the inoculation of other laboratory animals, such as guinea-pigs and weaned mice. It may also be isolated by the inoculation of various cell cultures including African green monkey kidney (Vero), rabbit kidney (RK-13), baby hamster kidney (BHK-21), or duck or chicken embryo fibroblasts, or by inoculation of embryonated chicken eggs. Details of virus identification techniques are described in Chapter 2.5.3. Equine encephalomyelitis (Eastern and Western).

Isolates can be identified as VEE virus by complement fixation (CF), haemagglutination inhibition (HI), or plaque reduction neutralisation (PRN) tests, or by immunofluorescence as described in Chapter 2.5.3. The VEE virus isolates can be characterised by the indirect fluorescent antibody or PRN tests using monoclonal antibody or by nucleic acid sequencing. The VEE virus characterisation should be carried out in a reference laboratory (see Table given in Part 3 of this *Terrestrial Manual*).

2. **Serological tests**

Diagnosis of VEE virus infection in equids requires the demonstration of specific antibodies in paired serum samples collected in the acute and convalescent phases. After infection, PRN antibodies appear within 5–7 days, CF antibodies within 6–9 days, and HI antibodies within 6–7 days. The second convalescent phase serum sample should be collected 4–7 days after the collection of the first acute phase sample or at the time of death. The serological procedures are described in detail in Chapter 2.5.3. Vaccination history must be taken into account when interpreting any of the VEE serological test results. In horses not recently vaccinated with an attenuated live virus strain, demonstration of VEE-specific serum IgM antibodies in a single serum sample supports recent virus exposure.

Any diagnosis of VEE in an individual that is based on seroconversion in the absence of an epizootic should be made with care. Although enzootic subtypes and variants are nonpathogenic for equids, infection will stimulate antibody production to epizootic VEE virus variants.

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

The acceptable vaccines against VEE infection are an attenuated virus vaccine, strain TC-83, and an inactivated virus preparation made from that strain (3, 4, 8, 10). The inactivated vaccine is now the most widely used, and is marketed in EEE/VEE and EEE/WEE/VEE combinations.

Inactivated vaccine should be administered in two doses with an interval of 2–4 weeks between doses. Annual revaccination is recommended.

Attenuated vaccine should be reconstituted with physiological saline and used immediately. Multidose vials are kept on ice while the vaccine is being used. Any vaccine not used within 4 hours of reconstitution should be safely discarded. Foals under 2 weeks of age and pregnant mares should not be vaccinated. Animals are vaccinated subcutaneously in the cervical region with a single dose. Revaccination is not recommended.

**NOTE:** Formalin-treated preparations of virulent epizootic VEE virus should never be used in equids. Residual virulent virus can remain after formalin treatment, and result in severe illness. Epizootics of VEE have occurred in Central and Southern America from the use of such preparations (8, 11).

**1. Seed management**

**a) Characteristics of the seed**

Attenuated VEE virus vaccine strain TC-83 originated from the Trinidad donkey strain (a variant of I-AB) of epizootic VEE virus isolated in 1944. This strain was derived by serial passage of the Trinidad donkey strain in fetal guinea-pig heart cells. It is safe and immunogenic at the established passage levels, and induces protective immunity in vaccinated equids, although adverse reactions can sometimes occur. The vaccine was originally developed for use in personnel involved in high-risk VEE virus research. Suitable seed lots should be maintained at −70°C in a lyophilised state.

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1 Sections on Seed management, Manufacture, In-process control, and Batch control are taken from the Biotechnology, Biologics, and Environmental Protection Division of the United States Department of Agriculture's Animal and Plant Health Inspection Service (APHIS).
Chapter 2.5.12. — Venezuelan equine encephalomyelitis

b) **Method of culture**

The virus is grown in fetal guinea-pig heart cell cultures in a suitable medium.

c) **Validation as a vaccine**

The cells used for vaccine production must be free from bacterial, fungal, mycoplasmal, and viral contamination. VEE virus is identified in batches of vaccine by PRN tests against hyperimmune serum. For inactivated vaccines of cell culture origin, strain TC-83 virus is treated with formaldehyde.

2. **Method of manufacture**

Vaccine is produced by harvesting supernatant fluid from fetal guinea-pig heart monolayers in which the replication of attenuated VEE virus has occurred. The monolayers are maintained at approximately 37°C. The time of harvesting is determined by the occurrence of characteristic cytopathic changes when approximately 70–100% of the cell sheet is affected, that is 1–3 days after infection. The supernatant fluid is clarified by low speed centrifugation and suitable stabilisers are added to protect the virus during freezing and lyophilisation.

3. **In-process control**

Cultures should be examined daily for cytopathic changes. After harvesting, the virus suspension should be tested for the presence of microbial contaminants.

Inactivated vaccines derived from attenuated strain TC-83 virus should be checked to exclude the presence of viable virus after formalin treatment.

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 of the inactivated vaccine are described in Chapter 2.5.3.

Safety tests of the attenuated vaccine are conducted in mice. A 0.5 ml dose is injected intraperitoneally or subcutaneously into each of eight mice, and the animals are kept under observation for 7 days. If adverse reactions attributable to the product occur during this period, the product is considered to be unsatisfactory.

c) **Potency**

Potency tests of the inactivated vaccine are described in Chapter 2.5.3, except that antibody titre in inoculated guinea-pigs will be ≥1/4.

Potency of the attenuated vaccine can be determined by testing in horses. Each of 20 susceptible horses is inoculated subcutaneously with 1 ml of lyophilised vaccine that has a reconstituted virus titre of at least $2.5 \log_{10} \text{TCID}_{50}$ (50% tissue culture infective dose) per ml. For a valid test, at least 19 of 20 vaccinated horses must have HI antibody titres of at least 1/20 or serum neutralising antibody titres of at least 1/40 within 21–28 days of vaccination.

When tested at any time within the expiration period following lyophilisation, the product must have a virus titre of 0.7 $\log_{10}$ greater than that used to test horses as described above, but no less than 2.5 $\log_{10}$ TCID$_{50}$/dose.

The final product must be free from bacterial, fungal, mycoplasmal, or extraneous viral contaminants.

d) **Duration of immunity**

Comprehensive studies on duration of immunity are not available. An annual revaccination is recommended for the inactivated vaccine. Foals that are vaccinated at under 1 year of age should be revaccinated before the next vector season. Revaccination with the attenuated vaccine is not recommended.

e) **Stability**

The lyophilised vaccine is stable and immunogenic for 3 years if kept refrigerated at 2–7°C. After 3 years, vaccine should be discarded. The vaccines should be used immediately after reconstitution. Multidose vials of the attenuated vaccine should be kept on ice while being used. All unused vaccine should be safely discarded 4 hours after reconstitution.
f) **Preservatives**

The preservatives used are thimerosal at a 1/1000 dilution and antibiotics (neomycin, polymyxin, amphotericin B, and gentamicin).

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g) **Precautions (hazards)**

Pregnant mares and foals under 2 weeks of age should not be vaccinated.

All personnel handling infectious VEE viruses or their antigens prepared from infected tissues or cell cultures should be vaccinated and shown to have demonstrable immunity in the form of VEE virus-specific neutralising antibody. All procedures producing aerosols from VEE virus materials should be conducted in biosafety cabinets with biocontainment and efficient filtration of the exhaust air from the laboratory (6, 7).

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5. **Tests on the final product**

a) **Safety and potency**

Safety and potency tests are as outlined above under Batch control (Sections C.4.b. and C.4.c.). The attenuated vaccine must have a virus titre of no less than $2.5 \log_{10} \text{TCID}_{50}$/dose.

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**REFERENCES**


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**NB:** There is an OIE Reference Laboratory for Venezuelan equine encephalomyelitis (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 2.5.13.

EPIZOOTIC LYMPHANGITIS

SUMMARY

Epizootic lymphangitis is a contagious, chronic disease of horses and other Equidae characterised clinically by a spreading, suppurative, ulcerating pyogranulomatous dermatitis and lymphangitis. This is seen particularly in the neck, legs and chest. It can also present as an ulcerating conjunctivitis, or multifocal pneumonia. Transmission is by contact of infected material with traumatised skin, biting flies, or inhalation. The causative agent, Histoplasma capsulatum var. farciminosum, is a dimorphic fungal soil saprophyte. Differential diagnoses include glanders (farcy), caused by Burkholderia mallei, ulcerative lymphangitis due to Corynebacterium pseudotuberculosis, sporotrichosis caused by Sporothrix schenckii, and the skin lesions of histoplasmosis caused by Histoplasma capsulatum var. capsulatum. Amphotericin B is the drug of choice for the treatment of clinical cases of epizootic lymphangitis.

Identification of the agent: Identification of the agent is made by its appearance in smears of the exudate or in histological sections of the lesion material. The yeast form of the organism is present in large numbers in well established lesions, and appears as pleomorphic ovoid to globose structures, approximately 2–5 µm in diameter, located both extracellularly and intracellularly in macrophages and giant cells. Organisms are usually surrounded by a ‘halo’ when stained with Gram stain, haematoxylin and eosin, Periodic acid–Schiff reaction or Gomori methenamine–silver stain. The mycelial form of the organism grows slowly under aerobic conditions at 25–30°C on a variety of media, including Mycobiotic agar, Sabouraud's dextrose agar, brain–heart infusion agar, and pleuropneumonia-like organism nutrient agar.

Serological and other tests: Antibodies to H. capsulatum var. farciminosum develop at or before the onset of clinical signs. Assays reported for detection of antibody include fluorescent antibody, enzyme-linked immunosorbent assay, and passive haemagglutination tests. In addition, a skin hypersensitivity test has been described.

Requirements for vaccines and diagnostic biologicals: Killed and live vaccines have been used on a limited scale in endemic areas, but they are not readily available.

A. INTRODUCTION

Epizootic lymphangitis is a contagious, chronic disease of horses and other Equidae. The disease is characterised clinically by a suppurative, ulcerating, and spreading pyogranulomatous, multifocal dermatitis and lymphangitis. It is seen most commonly in the extremities, chest wall and the neck, but it can also present as an ulcerating conjunctivitis of the palpebral conjunctiva, or as a multifocal pneumonia. It has also been called pseudofarcy or pseudoglanders. Another synonym is equine histoplasmosis, which may be a more accurate name for the disease, as not all clinical cases present obvious lymphangitis. The form that the disease takes seems to depend primarily on the route of entry (14). The skin form of the disease occurs when contaminated soil contacts traumatised skin. The conjunctival form of the disease is believed to be spread by biting flies of the Musca or Stomoxys genera. The pulmonary form of the disease is infrequent and is presumed to occur after inhalation of the organism. In all cases, the lesions are nodular and granulomatous in character, and the organism, once established, spreads locally by invasion and then via the lymphatics. There is often thickening, or ‘cording’, of lymphatics, with the formation of pyogranulomatous nodules. Regional lymph nodes may be enlarged and inflamed. The causative agent, Histoplasma capsulatum var. farciminosum, is a dimorphic fungus. The mycelial form is present in soil; the yeast form is most commonly found in lesions. Histoplasma farciminosum was formerly described as an independent species, but this assessment has been changed and it is now considered to be a variety of H. capsulatum due to the close morphological similarities of both the mycelial and yeast forms (17). Antigenically, H. capsulatum var. farciminosum and H. capsulatum var. capsulatum are indistinguishable (16). DNA sequences of four protein-coding genes have been analysed to elucidate the
evolutionary relationships of H. capsulatum varieties. This indicated that H. capsulatum var. farcininosum is deeply buried in the branch of SAm Hcc group A, (H60 to -64, -67, -71, -74 and -76), looking as if it were an isolate of South American H. capsulatum var. capsulatum (10).

The cutaneous form of the disease could be confused with glanders, which is caused by Burkholderia mallei, ulcerative lymphangitis, which is caused by Corynebacterium pseudotuberculosis, sporotrichosis caused by Sporothrix schenckii, and histoplasmosis caused by H. capsulatum var. capsulatum (9, 11).

The disease is endemic in parts of Africa, the Middle East, and Asia. It is most frequently reported from North Africa, but has also been described in other parts of Africa. The disease has been well documented in India. Reports from other parts of the world are sporadic and need to be carefully examined before the existence of the disease elsewhere can be validated. The prevalence of the disease increases with assembling of animals; it was much more common, historically, when large numbers of horses were stabled together for cavalry and other transportation needs. Mainly, it is horses, mules, and donkeys that are affected by the disease, although infection may occur in camels and cattle. Experimentally, other animals are refractory to infection subsequent to inoculation, with the exception of certain laboratory animal species such as mice and rabbits (14). Infection in humans has also been reported (2, 3, 8). Amphotericin B is the drug of choice for the treatment of clinical cases of epizootic lymphangitis.

As the clinical signs of epizootic lymphangitis can be confused with those of other diseases in the field, definitive diagnosis rests on laboratory confirmation.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Material should be collected directly from the suppurative and nodular lesions. For microbiological isolation, the material should be placed in a liquid nutrient medium with antibacterials and kept refrigerated until culturing, which should be attempted as soon as possible. For direct examination, swabs of lesion material can be smeared on glass slides and fixed immediately. For histopathology, sections of lesion material, including both viable and nonviable tissue, should be placed in 10% neutral buffered formalin. Confirmation of the disease is dependent on the demonstration of H. capsulatum var. farcininosum.

a) Direct microscopic examination

• Gram-stained smears

Smears can be stained directly with Gram stain and examined for the typical yeast form of the organism, which will appear as Gram-positive, pleomorphic, ovoid to globose structures, approximately 2–5 µm in diameter (2). They may occur singly or in groups, and may be found within macrophages or extracellularly. A halo around the organisms (unstained capsule) is frequently observed.

• Histopathology

In haematoxylin and eosin (H&E)-stained histological sections, the appearance of the lesion is quite characteristic and consists of pyogranulomatous inflammation with fibroplasia. Langhans giant cells are common. The presence of numerous organisms, both intra- and extracellularly in tissue sections stained with H&E, Periodic acid–Schiff reaction and Gomori methenamine–silver stain is of diagnostic value (12). There is some indication that the number of organisms increases with chronicity. The organisms are pleomorphic, often described as slightly lemon-shaped basophilic masses, varying from 2 to 5 µm in diameter. Budding organisms are often seen intracellularly within macrophages or giant cells.

• Electron microscopy

Electron microscopy has been applied to skin biopsy samples of 1.5–2.0 mm immediately prefixed in phosphate buffered 2% glutaraldehyde solution at 4°C and post-fixed in 1% osmium tetroxide. Ultra-thin sections were cut and stained with uranyl acetate and lead citrate. Examination demonstrated the fine internal structure of the organism, H. capsulatum var. farcininosum, including the cell envelope, plasma membrane, cell wall, capsule and inner cell structures (1).

b) Culture

The mycelial form of H. capsulatum var. farcininosum grows slowly on laboratory media (2–8 weeks at 26°C). The recommended medium is Mycobiotic agar (2). Other media include Sabouraud’s dextrose agar medium enriched with 2.5% glycerol, brain–heart infusion agar supplemented with 10% horse blood, and pleuropneumonia-like organism (PPLO) nutrient agar enriched with 2% dextrose and 2.5% glycerol, pH 7.8 (8, 13). Addition of antibiotics to the media is recommended: cycloheximide (0.5 g/litre) and chlor-
amphenicol (0.5 g/litre). Colonies appear in 2–8 weeks as dry, yellow to dark brown, granular, wrinkled mycelia. Aerial forms occur, but are rare. Microscopically, hyphae from cultured colonies are septate, branched, pleomorphic and stain variable with Gram stain.

As a confirmatory test the yeast form of *H. capsulatum var. farciminosum* can be induced by subculturing some of the mycelium into brain–heart infusion agar containing 5% horse blood or by using Pine's medium alone at 35–37°C. Yeast colonies are flat, raised, wrinkled, white to greyish brown, and pasty in consistency (13).

c) Animal inoculation

Experimental transmission of *H. capsulatum var. farciminosum* has been attempted in mice, guinea-pigs and rabbits. Immunosuppressed mice are highly susceptible to experimental infection and can be used for diagnostic purposes (1).

2. Serological tests

There are published reports of various tests to detect antibodies as well as a skin hypersensitivity test for detection of cell-mediated immunity.

a) Fluorescent antibody tests

   • Indirect fluorescent antibody test

   The following procedure is as described by Fawi (4).

   i) Slides containing the organisms are made by smearing the lesion contents on to a glass slide or by emulsifying the cultured yeast phase of the organism in a saline solution and creating a thin film on a glass slide.

   ii) The slides are heat-fixed by passing the slide through a flame.

   iii) The slides are then washed in phosphate buffered saline (PBS) for 1 minute.

   iv) Undiluted test sera are placed on the slides, which are then incubated for 30 minutes at 37°C.

   v) The slides are washed in PBS three times for 10 minutes each.

   vi) Fluorescein isothiocyanate (FITC)-conjugated anti-horse antibody at an appropriate dilution is flooded over the slides, which are then incubated for 30 minutes at 37°C.

   vii) Washing in PBS is repeated three times for 10 minutes each.

   viii) The slides are examined using fluorescence microscopy.

   • Direct fluorescent antibody test

   The following procedure is as described by Gabal et al. (5).

   i) The globulin fraction of the test serum is precipitated, and then resuspended to its original serum volume in saline. The serum is then conjugated to FITC.

   ii) Small colony particles of the cultured mycelial form of the organism are suspended in 1–2 drops of saline on a glass slide. With a second slide, the colony particles are crushed and the solution is dragged across the slide to create a thin film.

   iii) The smears are heat-fixed.

   iv) The slides are washed in PBS.

   v) The slides are incubated with dilutions of conjugated serum for 60 minutes at 37°C.

   vi) The slides are washed in PBS three times for 5 minutes each.

   vii) The slides are examined using fluorescence microscopy.

b) Enzyme-linked immunosorbent assay

The following procedure is as described by Gabal & Mohammed (7).

i) The mycelial form of the organism is produced on Sabouraud’s dextrose agar in tubes 20 × 125 mm, and incubated for 4 weeks at 26°C. Three colonies are ground in 50 ml of sterile PBS. The suspension is diluted 1/100 and the microtitre plates are coated with 100 µl/well.
ii) The plates are incubated at 4°C overnight.
iii) The plates are washed with PBS containing Tween 20 (0.5 ml/litre) three times for 3 minutes each.
iv) The plates are incubated with 5% bovine serum albumin, 100 µl/well, at 23–25°C for 30 minutes, with shaking.
v) The plates are washed with PBS containing Tween 20 (0.5 ml/litre) three times for 3 minutes each.
v) Peroxidase-labelled goat anti-horse IgG is diluted 1/800 and used at 100 µl/well, with incubation for 30 minutes at 23–25°C, with shaking.
vii) The plates are washed with PBS containing Tween 20 (0.5 ml/litre) three times for 3 minutes each.
viii) Finally, 100 µl/well of hydrogen peroxide and ABTS (2,2'-Azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) in a citric acid buffer, pH 4, is added.
v) The plates are read at 60 minutes in a spectrophotometer at wavelength 405 nm.
x) The absorbance values are obtained twice from each serum dilution and the standard deviation and average percentage of the absorbance values of the different serum samples are considered in the interpretation of the results.

c) Passive haemagglutination test
The following procedure is as described by Gabal & Khalifa (6).

i) The organism is propagated for 8 weeks on Sabouraud’s dextrose agar. Five colonies are scraped, ground, suspended in 200 ml of saline, and sonicated for 20 minutes. The remaining mycelial elements are filtered out, and the filtrate is diluted 1/160.
ii) Normal sheep red blood cells (RBCs) are washed, treated with tannic acid, washed, and resuspended as a 1% cell suspension.
iii) Different dilutions of the antigen preparation are mixed with the tanned RBCs and incubated in a water bath at 37°C for 1 hour. The RBCs are collected by centrifugation, washed three times in buffered saline and resuspended to make a 1% cell suspension.
iv) Test sera are inactivated by heating at 56°C for 30 minutes and then absorbed with an equal volume of washed RBCs.
v) Dilutions of serum (0.5 ml) are placed in test tubes with 0.05 ml of antigen-coated tanned RBCs.
vi) Agglutination is recorded at 2 and 12 hours.
vii) Agglutination is detected when the RBCs form a uniform mat on the bottom of the tube. A negative test is indicated by the formation of a ‘button’ of RBCs at the bottom of the tube.

d) Skin hypersensitivity tests
Two skin hypersensitivity tests for the diagnosis of epizootic lymphangitis have been described. The first test was described by Gabal & Khalifa (6).

i) The organism is propagated for 8 weeks on Sabouraud’s dextrose agar. Five colonies are scraped, ground, suspended in 200 ml of saline, and sonicated for 20 minutes. The remaining mycelial elements are filtered out and the filtrate is diluted 1/100. Sterility of the preparation is verified by incubating an aliquot on Sabouraud’s dextrose agar at 26°C for 4 weeks.
ii) Animals are inoculated intradermally with 0.2 ml in the neck.
iii) The inoculation site is examined for the presence of a local indurated and elevated area at 48–72 hours post-injection.

Alternatively, a ‘histofarcin’ test has been described by Soliman et al. (15).

i) The mycelial form of the organism is grown on polystyrene discs floating on 250 ml of PPLO media containing 2% glucose and 2.5% glycerine at 23–25°C for 4 months.
ii) The fungus-free culture filtrate is mixed with acetone (2/1) and held at 4°C for 48 hours.
iii) The supernatant is decanted and the acetone is allowed to evaporate.
iv) Precipitate is suspended to 1/10 original volume in distilled water.
v) Animals are inoculated intradermally with 0.1 ml of antigen in the neck.
vi) The inoculation site is examined for the presence of a local indurated and elevated area at 24, 48 and 72 hours post-injection.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Control of the disease is usually through elimination of the infection. This is achieved by culling infected horses and application of strict hygiene practices to prevent spread of the organism. There are published reports on the use of killed (2) and live attenuated vaccines (17) in areas where epizootic lymphangitis is endemic, apparently with relatively good results.

The antigens used for skin hypersensitivity testing are described in the previous section.

REFERENCES


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CHAPTER 2.5.14.

JAPANESE ENCEPHALITIS

SUMMARY

Japanese encephalitis virus is a mosquito-borne flavivirus that causes encephalitis, principally in horses. It also infects humans, and causes abortions in pigs. Pigs act as amplifiers of the virus, and birds can also be involved in its spread.

A definitive diagnosis of Japanese encephalitis in horses depends on the isolation of virus from affected or dead animals. As the isolation rate of virus is usually very low, clinical, serological and pathological findings are useful in diagnosis.

Identification of the agent: For virus isolation, brain material is collected from affected or dead horses that showed the clinical signs of encephalitis. Isolation procedures include the inoculation of unweaned mice and cell cultures. A suspension of brain material in buffered saline containing calf serum (or bovine serum albumin) and antibiotics is inoculated intracerebrally into 2–4-day-old mice. If the mice show neurological signs followed by death within 14 days, then virus identification can be carried out by cell culture. The virus is also isolated in cell cultures made from chicken embryos, in porcine or hamster kidney cells, African green monkey kidney (Vero) cells, the MD-BK cell line, and mosquito cell lines.

A cytopathic effect appears in some cell cultures, but it is usually unclear. Identification of the virus in mice or in tissue cultures is confirmed by serological methods.

Serological tests: Antibody assay is a useful technique for determining the prevalence of infection in a horse population, and also for diagnosing Japanese encephalitis in diseased individuals. The assay methods include virus neutralisation (VN), haemagglutination inhibition, and complement fixation tests. The VN test is the most specific because of its ability to differentiate Japanese encephalitis virus infection from other flavivirus infections. It is best performed as a plaque reduction test.

Requirements for vaccines and diagnostic biologicals: There is an inactivated vaccine prepared from a virus suspension derived from infected mouse brains or infected cell cultures.

A. INTRODUCTION

Japanese encephalitis is a disease of horses caused by a mosquito-borne flavivirus that elicits clinical signs of encephalitis in infected animals, and can be fatal (3, 4). It also infects humans, and causes abortions in pigs. Pigs act as amplifiers of the virus, and birds can also be involved in its spread. Only a single serotype has been identified among Japanese encephalitis virus isolates, although strain variation was recognised by serological and molecular studies. Haemagglutination inhibition and neutralisation tests combined with antigen absorption procedures could distinguish at least two subtypes, Nakayama and JaGAr-01, among Japanese encephalitis virus (5).

B. DIAGNOSTIC TECHNIQUES

The definitive diagnosis of Japanese encephalitis in horses depends on the isolation of the causal virus. The isolation rate of virus from diseased or dead horses is usually very low, which may be due to the instability of the virus under certain environmental conditions, and also to the presence of antibody in infected animals. Clinical, serological and pathological findings are of assistance in diagnosis. Diagnosis is also possible by the detection of specific IgM and IgG antibodies in cerebrospinal fluid by enzyme immunoassay methods (1). Viral nucleic acid has been detected in the brain of infected horses by reverse transcription polymerase chain reaction (7).
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The materials required for virus isolation are obtained from a portion of the corpus striatum, cortex or thalamus of the brains of affected horses. Blood and spinal cord samples are also used for isolation. All materials should be kept cool immediately after they are collected, and frozen to –80°C if virus is to be isolated at a later date. Any potentially infected materials must be handled following containment level 3 procedures (see Chapter I.1.6. Human safety in the veterinary microbiological laboratory), to prevent the risk of human infection. Humans may be infected by direct contact of infectious material with broken skin or mucous membranes, accidental parenteral inoculation or aerosol. Diagnosticians collecting samples should also take the appropriate precautions. A human vaccine is available and at risk field veterinarians and laboratory workers should be vaccinated.

1. Identification of the agent

Samples of brain and spinal cord are homogenised in a 10% suspension in buffered saline, pH 7.4, containing calf serum (2%) or bovine serum albumin (0.75%), streptomycin (100 µg/ml) and penicillin (100 units/ml). The calf serum should be free from Japanese encephalitis antibodies. The suspension is centrifuged at 1500 g for 15 minutes, and the supernatant fluid is removed for inoculation: 0.02 ml is inoculated intracerebrally into 2–4-day-old mice. The inoculated mice are kept under clinical observation for 14 days. No clear clinical signs may develop, but anorexia becomes evident by the disappearance of the white milk spot on the abdomen. The skin then changes colour from pinkish to dark red, and convulsions develop immediately before the mice die. Brains of dead or moribund mice are collected and stored at –80°C for a further passage.

To identify the virus, sucrose/acetone-extracted antigen is prepared from the infected mouse brains of a second passage in mice as described in Section B.2.b.1. This antigen is checked for its ability to agglutinate the red blood cells (RBCs) of 1-day-old chickens or of geese at different pH levels between pH 6.0 and 7.0, at intervals of pH 0.2, according to the method described (2). Briefly, RBC suspensions of 1/24 dilution are prepared in the diluent with different pH values. In a 96-well plate with a U-shaped bottom, the extracted antigen is diluted serially with 25 µl volumes. Then, 25 µl of the diluted RBCs is added to each well. The plate is incubated at 37°C for 1 hour, and the haemagglutination result is read. If the antigen is able to haemagglutinate, it is used in a haemagglutination inhibition (HI) test using a Japanese encephalitis antiserum.

Primary cultures of chicken embryo or hamster kidney cells, or a mosquito cell line C6/36 (a cloned cell line from Aedes albopictus) may be used for virus isolation. The specimens, such as brain and blood taken from animals suspected of being infected, and the brain suspension from mice after inoculation, are inoculated into the cultures; monoclonal antibodies specific to flavivirus and Japanese encephalitis virus may be used to identify the virus by the indirect fluorescent antibody test (6).

2. Serological tests

Serological tests are useful to determine the prevalence of infection in an animal population, the geographical distribution of the virus, and the degree of antibody production in vaccinated horses. To apply the tests to diagnosis in individual horses, it should be remembered that horses in an endemic area may have been inapparently infected with the virus for some time or may have been immunised with a vaccine. Valid data depend on a significant rise in antibody titre within paired sera collected in the acute and convalescent phases. The specificity of each serological test should also be considered. A latex agglutination test to detect swine antibodies to Japanese encephalitis has recently been described (8).

In some regions of the world, there is a need to perform additional tests on related viruses before an unequivocal diagnosis of Japanese encephalitis can be made. For example, in Australia antigenically closely related viruses of Murray Valley encephalitis and Kunjin virus occur. Recent expansion of the distribution of West Nile virus in North America, where St Louis was known to be endemic, further illustrates the flexibility of flaviviruses to adapt to new environments.

a) Virus neutralisation

The plaque reduction test using chicken embryo primary cultures, African green monkey kidney (Vero) cells or baby hamster kidney (BHK) cells is sensitive and reliable.

Japanese encephalitis virus (Nakayama strain or JaGA-01 strain) is propagated by intracerebral inoculation in 1-day-old mice. Moribund or dead mice are obtained, from which brain contents are collected and made into a 10% suspension in phosphate buffered saline (PBS), pH 7.2, containing 10% fetal calf serum. The suspension is centrifuged at 5000 g for 20 minutes at 4°C. The supernatant is stored in aliquots at –80°C.

- Test procedure
  i) Inactivate sera for 30 minutes in a water bath at 56°C.
  ii) Make serial dilutions of the sera in cell culture medium from 1/10 to 1/160 using a 24-well (17 mm in diameter) flat-bottomed microplate (or test tubes).
iii) Dilute stock virus to make 100 plaque-forming units (PFU)/0.2 ml in cell culture medium.
iv) Mix one volume of each diluted serum with an equal volume of diluted virus. Include virus control with culture medium, negative serum control and positive serum control in each plate.
v) Incubate for 90 minutes at 37°C.
vi) Add 200 µl of the virus/serum mixture to wells of BHK-21 cell monolayer formed on 24-well culture plates.
vii) Incubate the plates in a CO₂ atmosphere for 90 minutes at 37°C.
viii) Remove the inoculum and add 1 ml of overlay medium (1.5% carboxymethyl cellulose, 1% fetal calf serum in Eagle's medium).
ix) Incubate the plates in a CO₂ atmosphere for 4 days at 37°C.
x) After removing the culture fluid, fix the plates in a solution containing 2.5% potassium dichromate, 5% glacial acetic acid and 5% formalin for 30 minutes at room temperature. Wear rubber gloves when handling the fixing solution.
xi) Stain the plate in 0.1% crystal violet solution for 30 minutes at room temperature.
xii) Discard the stain and rinse the cells with tap water.
xiii) Air dry the cells and count the plaques.
xiv) Estimate the serum dilution that reduces the number of plaques by 50% or more of the control without serum.

b) Haemagglutination inhibition

The HI test is widely used for the diagnosis of Japanese encephalitis, but has cross-reactivity with other flaviviruses. For this test, the sera must first be treated with acetone or kaolin, and then absorbed with homotypic RBCs to remove any nonspecific haemagglutinins. The RBCs of geese or of 1-day-old chickens are used at the optimum pH (6.6–7.0). The test should be conducted with the treated sera and 8 units of standard antigen; this is commercially available in some countries.

• Haemagglutination (HA)

• Preparation of virus antigen

1. Sucrose–acetone extraction of antigen from infected suckling mouse brains (SMB)
   i) Homogenise infected SMB with 4 volumes of 8.5% sucrose.
   ii) Add the homogenate drop-wise to 20 times its volume of cold acetone.
   iii) Centrifuge (500 g for 5 minutes), then remove the supernatant.
   iv) Resuspend the sediment with the same volume as above of cold acetone, and keep in an ice bath for 1 hour.
   v) Centrifuge (500 g for 5 minutes), then remove the supernatant.
   vi) Pool the sediment with cold acetone in a single tube.
   vii) Centrifuge (500 g for 5 minutes), then remove the supernatant.
   viii) Spread the sediment inside the tube and vacuum dry for 1–2 hours.
   ix) Dissolve the dry sediment with saline: 0.4 volume of original homogenate.
   x) Centrifuge (8000 g for 1 hour, 4°C). The supernatant is ready for use.

2. Infected fluid of Aedes albopictus, clone C6/36, cells
   i) Harvest the infected fluid 1 week after incubation at 28°C.
   ii) Centrifuge (1000 g for 15 minutes). The supernatant is ready for use.

• Preparation of goose red blood cells

1. Solutions

   Acid-citrate-dextrose (ACD): 11.26 g sodium citrate (Na₃C₆H₅O₇.2H₂O); 4.0 g citric acid (H₃C₆H₅O₇.H₂O); 11.0 g dextrose (C₆H₁₂O₆); distilled water to a final volume of 500 ml. Autoclave at 10 lb (1.7 unit pressure) for 10 minutes.
Dextrose-gelatine-veronal (DGV): 0.58 g veronal (Barbital); 0.60 g gelatine; 0.38 g sodium veronal (sodium barbital); 0.02 g (0.026 g) CaCl₂ (for CaCl₂.2H₂O); 0.12 g MgSO₄.7H₂O; 8.50 g NaCl; 10.0 g dextrose; distilled water to a final volume of 1000 ml. Autoclave at 10 lb (1.7 unit pressure) for 10 minutes (five times stock volume is easier to prepare).

2. **Bleeding**
1.5 ml of ACD + 8.5 ml of blood (0.5 ml of ACD + 2.8 ml of blood).

3. **Washing (sterile)**
   i) Total blood + 2.5 volume of DGV. Centrifuge (500 g for 15 minutes), then remove the supernatant.
   ii) Resuspend the sedimented RBCs in three volumes (total blood) of DGV.
   iii) Centrifuge (500 g for 15 minutes), then remove the supernatant. Repeat steps 2 and 3 twice more (total four spin cycles).
   iv) Transfer the final RBC suspension to a flask with aluminium foil cover.

4. **Adjusting the RBC concentration**
   i) 0.2 ml of the RBC suspension + 7.8 ml of 0.9% NaCl (1/40 dilution).
   ii) Read the optical density (OD)₄₉₀ in a spectrophotometer with 10 mm tube.
   iii) Adjust the RBC stock so that 1/40 dilution gives 0.450 of OD₄₉₀. (Final volume = Initial volume × absorbance OD₄₉₀/0.450.)
   iv) Store the RBC stock in a refrigerator for up to 3 weeks.
   v) Before use, resuspend the RBCs gently and dilute 1/24 in VAD.

• **Antigen dilution**
1. Stock solutions (should be kept at 4°C): 1.5 M NaCl: 87.7 g NaCl and distilled water to a final volume of 1000 ml; 0.5 M boric acid: 30.92 g H₃BO₃ and hot distilled water to a final volume of 700 ml (dissolve boric acid and cool down); 1 N NaOH: 40.0 g NaOH and distilled water to a final volume of 1000 ml; borate saline (BS), pH 9.0: 80 ml 1.5 M NaCl, 100 ml 0.5 M H₃BO₃, 24 ml 1.0 N NaOH, and distilled water to a final volume of 1000 ml; 4% bovine albumin: 4 g bovine albumin fraction V (Armour), 90 ml BS, pH 9.0, adjust pH to 9.0 with 1 N NaOH, and BS, pH 9.0, to make a final volume of 1000 ml.

2. **Antigen diluent:** 0.4% bovine albumin/borate saline (BABS): 10 ml 4% bovine albumin, pH 9.0, and 90 ml BS, pH 9.0.

3. Twofold serial dilution of antigen with BABS on microtitre (U-shaped microtray).

• **Addition of goose red blood cells**
1. **Stock solutions**
   1.5 M NaCl

0.5 M Na₂HPO₄: 70.99 g Na₂HPO₄ (for Na₂HPO₄·12 H₂O: 179.08 g), and distilled water to a final volume of 1000 ml.

1.0 M NaH₂PO₄: 138.01 g NaH₂PO₄·H₂O (for Na₂HPO₄·2H₂O: 156.01 g), and distilled water to a final volume of 1000 ml.

2. Working solution: virus adjusting diluent (VAD)

<table>
<thead>
<tr>
<th>VAD</th>
<th>1.5 M NaCl</th>
<th>0.5 M Na₂HPO₄</th>
<th>1.0 M NaH₂PO₄</th>
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<tbody>
<tr>
<td>6.0</td>
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<td>192</td>
<td>104</td>
</tr>
<tr>
<td>7.0</td>
<td>100</td>
<td>240</td>
<td>80</td>
</tr>
</tbody>
</table>

Values of VADs are not the pH of each VAD, but the pH after each VAD is mixed with an equal volume of BABS, pH 9.0.
3. **Procedures**
   
i) 1 volume of stock goose RBCs + 23 volumes of VAD (1/24 dilution).
   
ii) Add 25 µl of diluted RBCs to each well on microtitre tray containing diluted antigen (25 µl/well).
   
iii) Incubate at 37°C for 1 hour, then read the result.
       
+ + Complete agglutination (uniformly thin pellicle of RBCs following the curvature of the well bottom)

+ Partial agglutination (a ring associated with a rough or thinner pellicle)

± Minimal agglutination (a button on a thin or scattered pellicle)

− Negative agglutination (clearly defined button with no RBC film)

End point is the last dilution (highest dilution) in which ++ or + is observed.

Titre: the reciprocal of the end point dilution.

- **Haemagglutination inhibition**

- **Preparation of test sera**

1. **Bleeding and separation of the sera**
   
i) Incubate blood specimen at 37°C for 1 hour and then at 4°C overnight. If the test must be performed immediately, incubating the sample for 2–3 hours at 37°C can replace the overnight incubation.
   
ii) Centrifuge (2000 g for 15 minutes) to separate the serum from the clot.
   
iii) Heat inactivate at 56°C for 30 minutes.
   
iv) Store at −20°C if not processed immediately.

2. **2-mercaptoethanol treatment**
   
i) Place 50 µl of the sera into two small test tubes.
   
ii) Add 150 µl of 0.13 M 2-mercaptoethanol in PBS into one test tube, and 15 µl PBS into another tube.
   
iii) Incubate at 37°C for 1 hour, then cool in an ice bath.

3. **Acetone extraction**
   
i) Place 2.5 ml of cold acetone into each tube. Apply rubber stoppers and extract for 5 minutes in an ice bath.
   
ii) Centrifuge cold (1500 g for 5 minutes), then remove the supernatant.
   
iii) Repeat steps i and ii once more.
   
iv) Spread the sediment inside tubes and vacuum dry at room temperature for 1 hour.
   
v) Add 0.5 ml of BS, pH 9.0, to each tube. Apply rubber stoppers. Dissolve the sediment overnight at 4°C to make 1/10 dilution of the sera.

4. **Kaolin extraction as an alternative to acetone extraction**
   
i) 25% acid-washed kaolin (Fischer) in BS, pH 9.0.
   
ii) 1 volume of sera + 4 volumes of BS + 5 volumes of 25 % kaolin.
   
iii) Extract at room temperature for 20 minutes with occasional shaking.
   
iv) Centrifuge (1000 g for 30 minutes). The supernatant is 1/10 dilution of the sera.

5. **Absorption with goose RBCs**
   
i) To each treated serum add 1/50 volume of packed goose RBCs.
   
ii) Absorb for 20 minutes in an ice bath.
   
iii) Centrifuge (800 g for 10 minutes). The supernatant is ready for the HI test (1/10 dilution).
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- **Haemagglutination inhibition test**
  1. *Primary haemagglutination titration of antigen*
     Dilute the antigen to make 4–8 units/0.05 ml.
  2. *Serial twofold dilution of test sera on microtray*
     - **Serum–antigen reaction**
       Add 25 µl of diluted antigen into each well containing diluted test sera. Place the remainder of the antigen in empty wells and incubate at 4°C overnight.
  3. *Secondary haemagglutination titration of the antigen*
     - i) Collect antigen distributed in empty wells and HA test by serial twofold dilution in 25 µl system.
     - ii) Add 25 µl of BABS to each well to make 50 µl/well.
  4. *Addition of goose RBCs*
     - i) Dilute RBC stock (1/24) in VAD.
     - ii) Distribute 50 µl into each well containing 50 µl of serum antigen mixture or secondary titration of antigen.
     - iii) Incubate at 37°C for 1 hour then read the result.
     - Serum HI titre: the reciprocal of the highest dilution of the test sera showing complete inhibition of HA.
  5. *Interpretation of the results*
     Four-fold difference between the titre in the acute and convalescent sera is considered to be a significant rise or fall and is diagnostic of infection with a virus antigenically related to that used in the test.

c) **Complement fixation**

Complement fixation (CF) is sometimes used for serological diagnosis. The antigen for this test is extracted with acetone/ether from the brains of inoculated mice.

- **Antigen preparation**
  - i) Extract and weigh the brains of the inoculated dead mice.
  - ii) Add to the brains, 20 volumes of cold acetone, kept at −20°C, and homogenise.
  - iii) Centrifuge the suspension at 5000 g for 5 minutes at 4°C, and remove the supernatant.
  - iv) Add to the pellet the same volume of cold acetone as used in step ii above, and mix well.
  - v) Extract with acetone by keeping the pellet at −20°C for 20 minutes, and repeat the centrifugation described in step iii above.
  - vi) Repeat steps iv and v.
  - vii) Repeat steps iv and v, but this time use cold acetone/ether (equal volume mixture).
  - viii) Repeat steps iv and v twice using cold ether.
  - ix) Remove the supernatant by aspirator and spread the pellet over the centrifuge tube.
  - x) Vacuum dry for 1–2 hours.
  - xi) Dissolve the pellet in cold saline (2 ml/g of brain) and keep at 4°C overnight.
  - xii) Centrifuge at 5000 g for 1 hour. The supernatant is the antigen.

- **Test procedure**
  - i) Heat-inactivate the test sera at 1/4 dilution in gelatin–veronal buffer.
  - ii) Serially dilute the sera twofold in a 96-well microtray (25 µl).
  - iii) Add 25 µl of 4 units of antigen and mix by vibration.
  - iv) Add 50 µl of 2 units of complement (pooled fresh guinea-pig serum).
  - v) Mix by vibration and incubate at 4°C for 18 hours.
vi) Leave the microtray at room temperature for 15 minutes.

vii) Add 25 µl of sensitised sheep RBCs to each well.

viii) Mix by vibration and incubate at 37°C for 30 minutes, then read the result.

ix) The highest dilution of test sera showing no haemolysis is the titre of the sera by CF test. A rise or drop of four-fold or more in the titre is considered to be significant.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

The vaccine for Japanese encephalitis in horses is prepared by the inactivation of a virus suspension derived from infected mouse brains or cell cultures.

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 Beijing-1 strain of Japanese encephalitis virus is used for vaccine production in Japan. The strain must be lethal for mice when inoculated intracerebrally, and must be able to grow in a primary culture of porcine kidney. This strain has the capacity to haemagglutinate the RBCs of geese, 1-day-old chickens or pigeons. The virus must be able to be neutralised by a standard antiserum to Japanese encephalitis virus.

b) Method of culture

The original and seed viruses should be grown in mouse brains or cell cultures. The passage levels should not exceed three times that of the original virus and twice that of the seed virus.

c) Validation as a vaccine

The vaccine product from this strain provides immunity to encephalitis in equines and prevents stillbirths in pregnant sows.

It is recommended that the original and seed viruses be maintained below –70°C, or below 5°C after lyophilisation.

2. Method of manufacture

The virus is grown in the brains of mice aged 3–4 weeks or in a monolayer culture. Uninoculated control cultures should not show any cytopathic effects caused by any other virus. Seed virus is inoculated intracerebrally into mice. The brains of those mice that show severe clinical signs of encephalitis are collected. These brains are homogenised in PBS, centrifuged at 1500 g for 30 minutes, and the supernatant fluid is processed as the virus suspension.

Seed virus is inoculated into cell cultures and the fluids are later harvested separately from each batch when virus replication is at its maximum. This fluid is filtered, or centrifuged at 1500 g for 30 minutes, and the supernatant fluid is processed as the virus suspension.

Formalin (0.5%) is added to the suspension to inactivate any live virus; this is considered to be the ‘undiluted virus suspension’. Adjuvant may be added to enhance its immunogenicity.

3. In-process control

The virus suspension should be examined for bacterial contamination by culture techniques and for virus infectivity by intracerebral mouse inoculation or inoculation into cell cultures. The inactivated undiluted virus suspension should be re-examined for contamination by culture and by microscopy after staining, and should be checked by intracerebral mouse inoculation to ensure complete inactivation by the formalin.
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
Ten 3-day-old mice are inoculated intracerebrally with 0.02 ml of the final product, and observed for 14 days to ensure (by the absence of any deaths) the complete inactivation of live virus.

5. Tests on the final product

a) Sterility
Tests for sterility and freedom from contamination of biological materials may be found in Chapter I.1.5.

b) Safety
See Section C.4.b.

c) Formalin determination
The formalin concentration should be less than 0.2% (v/v) by general quantification procedures.

d) Potency
The final product must be checked for immunogenicity by mouse protection tests. The product is diluted one part to ten parts of PBS; 30 mice aged 2–3 weeks are inoculated intraperitoneally with 0.1 ml of the diluted product twice at 3-day intervals. There should be an equivalent uninoculated control group. All mice are challenged intraperitoneally with graded doses of live virus 8 days following the first inoculation, and observed for 14 days. The survival rate should be more than 40% in the immunised group and the mortality rate in the control group should be more than 90%. The titre of challenge virus should be not less than \(10^3\) LD50 (50% lethal dose) per 0.2 ml.

e) Stability
The final product must be shown to be fully effective for 12 months when stored at 4°C.

REFERENCES


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CHAPTER 2.5.15.

SURRA
(Trypanosoma evansi)

SUMMARY

Trypanosomosis¹ caused by Trypanosoma evansi (‘surra’) is the most widely distributed of the pathogenic animal trypanosomoses, affecting domesticated livestock in Asia, Africa and Central and South America. The principal host species affected varies geographically, but buffalo, cattle, camels and horses are particularly affected, although other animals are also susceptible. It is an arthropod-borne disease, Tabanus spp. being implicated as the main vectors. The diagnosis of surra is usually based on the demonstration of the parasites in the blood, supplemented by haematological, biochemical and serological tests.

The disease in susceptible animals is manifested by pyrexia, directly associated with parasitaemia, together with a progressive anaemia, loss of condition and lassitude. Recurrent episodes of fever and parasitaemia occur during the course of the disease. Oedema, particularly of the lower parts of the body, urticarial plaques and petechial haemorrhages of the serous membranes are often observed. Abortions have been reported in buffaloes in Asia. There are indications that the disease causes immunodeficiencies. The clinical signs of surra, however, although indicative, are not sufficiently pathognomonic, and diagnosis must be confirmed by laboratory methods.

Identification of the agent: As the general clinical signs of the various forms of T. evansi infection are not pathognomonic, laboratory diagnosis is required. When there is a high parasitaemia, the examination of wet blood films, stained blood smears and lymph node material should reveal the trypanosomes. In other more chronic cases, such as the carrier state, the examination of thick blood smears, as well as methods of parasite concentration and the inoculation of laboratory animals, are recommended. An attractive complementary approach now being explored is the application of antigen detection tests. Pilot versions of immunoenzyme and latex agglutination assays already exist. Using the polymerase chain reaction, minute amounts of well defined and highly specific trypanosomal DNA sequences can be detected in blood and other samples from infected hosts.

Haematological and biochemical tests are not specific for T. evansi infection, but they reveal the pathological consequences of infection. In areas where the disease is endemic, such tests help to monitor the results of chemotherapy. The tests include measurements of the packed blood cell volume and immunoglobulin levels.

Serological tests: Infection gives rise to specific antibody responses and a variety of antibody detection tests have been introduced. These have been partially validated, but await large-scale evaluation and standardisation. In the laboratory, immunofluorescence and immunoenzyme assays can be employed. For field use, relatively simple tests have yet to be developed. Obvious candidates are direct agglutination tests with trypanosomes from the bloodstream, and indirect agglutination tests with antigen-coated red blood cells (indirect haemagglutination) or latex particles. Assays for detection of circulating antibodies have high measures of validity. Estimates of predictive values of different serological tests indicate that enzyme linked immunosorbent assays (ELISA) for detecting IgG antibodies are more likely to classify correctly uninfected animals, and card agglutination tests (CATT) are more likely to classify correctly truly infected animals. An IgG ELISA would thus be suitable for verifying that animals are free from infection, prior to movement or during quarantine. In situations where there is overt disease, CATTs can be

¹ Nomenclature of parasitic diseases: see the note in Chapter 2.3.15. Trypanosomosis (Tsetse-borne).
used to target individual animals for treatment with trypanocidal drugs. For declaring a disease-free status, serial testing – ELISA followed by re-testing of suspect samples by CATT – is recommended. It must be stressed however, that there are considerable antigenic similarities among the different species of pathogenic trypanosomes, hence in areas where tsetse-transmitted trypanosomoses occur cross-reactions will occur with any serological test employed.

Requirements for vaccines and diagnostic biologicals: No vaccines are available for the disease.

A. INTRODUCTION

The diagnosis of Trypanosoma evansi infection, an arthropod-borne disease, is based on clinical signs and on the demonstration of the parasites by direct or indirect methods.

The clinical signs of surra, the disease caused by T. evansi, are indicative but are not sufficiently pathognomonic and diagnosis must be confirmed by laboratory methods. The disease in susceptible animals, including cattle, buffalo, camels (dromedary and bactrian), horses, pigs, sheep and goats, is manifested by pyrexia, directly associated with parasitaemia, together with a progressive anaemia, loss of condition and lassitude. Recurrent episodes of fever and parasitaemia occur during the course of the disease. Oedema, particularly of the lower parts of the body, urticarial plaques and petechial haemorrhages of the serous membranes are often observed. Abortions have been reported in buffaloes in Asia (15). There are indications that the disease causes immunodeficiency (26).

There is considerable variation in the pathogenicity of different strains and the susceptibility of different host species to disease. Disease may manifest as an acute or chronic condition, and in the latter case may persist for many months. The disease is often rapidly fatal in camels, buffaloes, horses, cattle, llama and dogs, but mild and subclinical infections can also occur in these species. Wild animals such as deer and capybara can become infected. Animals subjected to stress – malnutrition, pregnancy, work – are more susceptible to disease.

Biologically T. evansi is very similar to T. equiperdum, the causative agent of dourine, and morphologically resembles the slender forms of the tsetse-transmitted trypanosomes, T. brucei, T. gambiense and T. rhodesiensе. Molecular characterisation indicates that various strains of T. evansi isolated from Asia, Africa and South America have a single origin. Moreover, it is also likely that T. evansi and T. equiperdum belong to the same species. Like all pathogenic trypanosomes, T. evansi is covered by a dense protein layer consisting of a single protein called the variable surface glycoprotein. This acts as a major immunogen and elicits the formation of specific antibodies. The parasites are able to evade the consequences of these immune reactions by switching the variant surface glycoprotein, the phenomenon known as antigenic variation.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The classical direct method for the diagnosis of trypanosomosis led to the original discovery of the parasite. It is still employed for examining blood or lymph node material, but rarely with extracts of other tissues. In regions where T. brucei and T. equiperdum occur in addition to T. evansi, a specific identification by microscopy on blood smears is not possible. In the future, specific DNA probes may become available (22, 36) that allow the identification of trypanosome species by nonradioactive DNA hybridisation.

• Direct methods

a) Usual field methods

i) Blood sampling

As with all the other members of the subgenus Trypanozoon, T. evansi is a parasite of the blood and tissues; it particularly inhabits the deep blood vessels in cases of low parasitaemia. For this reason, it is recommended that blood for diagnosis be obtained from both the peripheral and deep blood vessels. However it should be realised that less than 50% of infected animals may be identified by examination of peripheral blood.

Peripheral blood is obtained by puncturing a small vein in the ear or tail. Deeper samples are taken from a larger vein by syringe. An area of the ear margin or tip of the tail is first cleansed with alcohol and, when dry, a vein is punctured with a suitable instrument. It is important to sterilise instruments or
to use disposable instruments between individual animals, so that infection cannot be transmitted by residual blood.

ii) **Wet blood films**

A small drop of blood is placed on to a clean glass slide and covered with a cover-slip to spread the blood as a monolayer of cells. This is examined by light microscopy (×200) to detect any motile trypanosomes.

iii) **Stained thick smears**

A large drop of blood is placed on the centre of a microscope slide and spread with a toothpick or the corner of another slide so that an area of approximately 1.0–1.25 cm in diameter is covered. This is air-dried for 1 hour or longer, while protecting it from insects. The unfixed smear is stained with Giemsa (one drop of commercial Giemsa + 1 ml of phosphate buffered saline [PBS, 2.4 g Na₂HPO₄·2H₂O, 0.54 g NaH₂PO₄·2H₂O, 0.34 g NaCl], pH 7.2), for 25 minutes. After washing, the slides are examined under a light microscope at high magnification (×500–1000). The advantage of the thick smear technique is that it concentrates the drop of blood into a small area, and thus less time is required to detect the parasites. The disadvantage is that the trypanosomes may be damaged in the process, and the method is therefore not suited for species identification in case of mixed infections.

iv) **Stained thin smears**

A drop of blood is placed 20 mm from one end of a clean microscope slide and a thin film is drawn out in the usual way. The film is air-dried briefly, fixed in methyl alcohol for 2 minutes and allowed to dry. The smears are then stained by Giemsa (one drop Giemsa + 1 ml PBS, pH 7.2) for 25 minutes. This preparation is poured off, the slide is washed in tap water and dried. Unfixed smears can be stained by covering them with May–Grünwald stain for 2 minutes, and then adding an equal volume of PBS, pH 7.2, and leaving the slides for a further 3 minutes. This preparation is poured off and diluted Giemsa is added for 25 minutes. This is again poured off, the slides are washed with tap water, and dried. Slides are examined at high magnification (×400–1000). This technique permits detailed morphological studies and identification of the trypanosome species. Rapid staining techniques also exist (Field’s stain, Diff Quick®).

v) **Lymph node biopsies**

Samples are usually obtained from the prescapular or precrural (subiliac) lymph nodes. A suitable node is selected by palpation and the site is cleansed with alcohol. The node is punctured with a suitable gauge needle, and lymph node material is aspirated into the syringe. This material is then expelled on to a slide, covered with a cover-slip and examined as for the fresh blood preparations. Fixed smears can also be stored for later examination.

b) **Concentration methods**

In most hosts *T. evansi* can induce mild clinical or subclinical carrier state infections with low parasitaemia in which it is difficult to demonstrate the parasites. Concentration methods become necessary.

i) **Haematocrit centrifugation**

Blood is collected (70 µl) into heparinised capillary tubes (75 × 1.5 mm), which are then sealed at the dry end and centrifuged, sealed end down, at 3000 g for 10 minutes. Two pieces of glass (25 × 10 × 1.2 mm) are glued to a slide and the capillary tube is placed between them. A cover-slip can be placed on top at the level of the buffy coat junction where the trypanosomes will be concentrated. The space around this part of the tube is flooded with water or immersion oil, and the buffy coat area is examined under the microscope (×100–200). A simpler alternative is to examine the centrifuged capillary tube by placing a drop of immersion oil on the tube and ensuring that there is contact between the objective lens and the immersion oil.

ii) **Dark-ground/phase-contrast buffy coat technique**

Blood is collected into heparinised capillary tubes and centrifuged as above. The tube is scratched and broken 1 mm below the buffy coat layer — the upper part thus contains the top layer of red blood cells (RBCs), the buffy coat (white blood cells) and some plasma. The contents of this piece are partially expelled on to a slide, covered with a cover-slip and examined under dark-ground, phase-contrast or ordinary illumination.

As an alternative to the electrically powered haematocrit centrifuge, hand-powered micro-centrifuges have been successfully used for detection of trypanosomosis in cattle and camels (8, 13).
iii) **Haemolysis techniques**

Sodium dodecyl sulphate (SDS) can be used as a reagent to haemolyse RBCs to facilitate detection of motile trypanosomes in parasitised blood samples. As SDS is toxic, contact with skin, inhalation and ingestion should be avoided. SDS solution can be stored for several months at ambient temperature. Both the SDS solution and the blood samples should be used at a temperature above 15°C. At lower temperatures the trypanosomes may be destroyed.

Two general procedures, namely wet blood film clarification and haemolysis centrifugation, can be applied².

- **Wet blood film clarification method**
  
  This method uses the partial lysis of RBCs to facilitate the detection of motile trypanosomes. The method requires an SDS solution: 1% SDS dissolved in Tris/glucose/saline, pH 7.5, inoculating loops (10 µl), slides and cover-slips (24 × 24 mm), and a drop of fresh neat or heparinised blood. Dissolve 100 mg of SDS in 100 ml of isotonic Tris/NaCl/glucose buffer, pH 7.5 (Trizma base 14.0 g, NaCl 3.8 g, glucose 10.8 g; dissolve chemicals in 750 ml distilled H₂O, add 90–100 ml 1 N HCl and adjust pH to 7.5 then make up to final volume of 1000 ml with distilled H₂O). This buffer can be stored in small vials at ambient temperature for several months. The test does not give a significantly higher sensitivity than wet film technique. In addition, the SDS can cause problems in focusing the microscope and the movements of trypanosomes can be severely curtailed owing to the high viscosity of the SDS.

  Put approximately 10 µl of blood on a slide. Add 10 µl of SDS solution using a dip inoculation loop and mix gently. Apply a cover-slip. Read the entire preparation without delay at low magnification (×100 or ×200).

- **Haemolysis centrifugation technique**
  
  Nearly complete lysis of RBCs is required for this procedure. The materials needed include: SDS solution (0.1% SDS dissolved in Tris/glucose/saline, pH 7.5), conical centrifuge tubes, ordinary test tubes, large and fine plastic tapering pipettes with attached bulb, slides, cover-slips (24 × 24 mm or 24 × 32 mm), and heparinised blood.

  Using a pipette or syringe, transfer nine volumes (maximum 6.3 ml) of SDS solution into an ordinary test tube. Aspirate one volume (maximum 0.7 ml) of heparinised blood into a bulb pipette and expel it just above the surface of the SDS solution; mix quickly and thoroughly. Avoid foam formation, which may result in destruction of the trypanosomes. Wait for 10 minutes so as to achieve complete haemolysis.

  Pour the haemolysed suspension into a conical centrifuge tube and spin at approximately 500 g for 10 minutes. With a clean bulb pipette, remove as much supernatant as possible without disturbing the sediment. Again with a fine tapering bulb pipette, remove more supernatant, leaving 10–20 µl of undisturbed sediment at the bottom. Then very carefully collect the entire sediment and put on to a microscope slide. Apply a cover-slip and examine the entire preparation without delay at low magnification (×100 or ×200).

iv) **Mini-anion exchange centrifugation technique**

When a blood sample from humans or animals infected with salivarian trypanosomes is passed through an appropriate anion-exchange column, the host blood cells, being more negatively charged than trypanosomes, are adsorbed into the anion-exchanger, while the trypanosomes are eluted, retaining viability and infectivity. A simplified field method for detection of low parasitaemia has been developed (14, 17, 31). The sensitivity of this technique can be increased by approximately tenfold by the use of buffy coat preparations rather than whole blood (30).

- **Preparation of phosphate buffered saline glucose, pH 8**

  Na₂HPO₄ (anhydrous) (13.48 g); NaH₂PO₄.2H₂O (0.78 g); NaCl (4.25 g); distilled water (1 litre).

  Solutions of different ionic strength are made by diluting the stock PBS, pH 8, and adding glucose to maintain a suitable concentration. For blood of mice, domestic and wild ruminants and dog, add four parts PBS to six parts distilled water and adjust the final glucose concentration to 1%. For blood of pigs and rabbits, add three parts PBS to seven parts distilled water and adjust the final glucose concentration to 1.5%. The PBS/glucose solution (PSG) must be sterile.

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² All necessary materials and instructions can be obtained from the Institute of Tropical Medicine, Laboratory of Serology, Nationalestraat 155, B-2000 Antwerp, Belgium.
• **Equilibration of DEAE-cellulose**
  Suspend 500 g of DEAE-cellulose (diethylamino-ethylcellulose) in 2 litres of distilled water. Adjust the pH to 8 with phosphoric acid. Allow to settle for 30 minutes. Discard the supernatant fluid containing the fine granules. Repeat the procedure three times. Store the equilibrated concentrated suspension of DEAE-cellulose (slurry) at 4°C or in small aliquots at –20°C.

• **Packing of equilibrated DEAE-cellulose**
  A syringe of 2 ml without the plunger is placed on a test-tube rack complete with needle (20 G × 1.5 inch). A disc of Whatman No. 41 filter paper is placed at the bottom of the syringe and moistened by adding a few drops of PSG. Pour 2–2.5 ml of the slurry of equilibrated cellulose into the syringe and allow to pack by elution of the buffer. The height of the sediment should be approximately 3 cm. Wash and equilibrate the column with 2 ml of PSG without disturbing the surface.

• **Adsorption of blood eluate of the trypanosomes**
  Gently place 100–300 µl of heparinised blood above the surface of the cellulose column. Add ten drops of PSG and discard the first ten drops of the eluate. Progressively add 1.5 ml of PSG and start collecting the eluate into a finely tapered Pasteur pipette with a sealed end. Put the filled pipette, protected by a conical plastic pipette tip, in a tube and centrifuge at 525 g (or up to 1000 g) for 10 minutes. Examine the bottom of the pipette under the microscope (× 100 or ×200) using a special mounting device. Alternatively, the eluate could be collected into 50 ml plastic tubes, with conical bottoms, centrifuged at 1000 g and the sediment examined by dark-ground microscopy.
  The column should remain wet constantly throughout the procedure.

**c) Animal inoculation**
  Laboratory animals may be used to reveal subclinical (non-patent) infections in domesticated animals. *Trypanosoma evansi* has a broad spectrum of infectivity for small rodents, and so rats and mice are often used. In studies of *T. evansi* infections in camels, comparisons have been made between thick blood film examinations and rat or mouse inoculation methods; animal inoculation gave 15.2% and 17%, respectively, more positive results amongst infected animals than thick smears alone (7, 27). However, even though mouse inoculation is not 100% sensitive (19) further improvement can be obtained by the use ofuffy coat material. A modified mouse inoculation procedure was used to detect as few as 1.25 *T. evansi*/ml blood (30).

Blood treated with the anticoagulant sodium heparin is inoculated intraperitoneally into rats (1–2 ml) or mice (0.25–0.5 ml). Inoculation of a minimum of two animals is recommended; animals are bled from the tail three times a week to detect parasitaemia. The incubation period before appearance of the parasites and their virulence depends on the strain of trypanosomes, their concentration in the inoculum, and the strain of laboratory animal used. Sensitivity of this *in-vivo* culture system may perhaps be increased by use of immunosuppressed laboratory animals. Drugs such as cyclophosphamide or hydrocortisone acetate, or X-ray irradiation or splenectomy are used for this purpose.

**d) Recombinant DNA probes**
  Specific DNA probes to detect trypanosomes in infected blood or tissue are being evaluated (22, 36).

• **Indirect methods**
  These methods involve haematological or biochemical tests that demonstrate the effects of the parasite on its host rather than directly detecting the parasite itself.

  **a) Haematology**
  Anaemia is usually a reliable indicator of trypanosome infection, although it is not in itself pathognomonic. However, animals with a mild subclinical infection can have parasitaemia without evidence of anaemia.

  Anaemia can be estimated by measuring the packed cell volume and may be used in surveys of herds at risk. The technique is identical to that of haematocrit centrifugation. The capillary tube is examined and the results are expressed as a percentage of packed RBCs to total blood volume.

  **b) Biochemical tests**
  Biochemical tests include flocculation, formol-gel, mercuric chloride precipitation and thymol turbidity tests. Several older methods considered to be outdated still have some use in the field because they are simple
Chapter 2.5.15. — Surra (Trypanosoma evansi)

to perform. The tests all depend on an increase in serum globulins as the result of infection, but this increase is not specific for *T. evansi* infection. The formol-gel and mercuric chloride tests are the tests of choice. These tests are mainly of use in camels and have rarely been used for other species. There are no data on the levels of globulins that are likely to produce a positive reaction.

For the formol-gel test, approximately 3–5 ml of blood is collected into a dry tube and allowed to clot. Approximately 1 ml of cell-free serum is transferred to a smaller tube and two drops of concentrated formalin solution (40% formaldehyde [w/v]) are added. The test is positive if the serum coagulates immediately and turns white. In negative reactions, the serum remains unchanged or coagulation may take up to 30 minutes to appear.

For the mercuric chloride test, approximately 1–2 ml of venous blood is collected into a dry tube and allowed to clot. To detect early cases of *T. evansi* infection, it is necessary to use the strongest concentration of mercuric chloride that would not precipitate normal serum, i.e. between 1/20,000 and 1/30,000. A solution of diluted mercuric chloride (1 ml) in distilled water is transferred to a small tube, and one drop of serum, free from RBCs, is added with gentle shaking. Note: precautions should be taken when handling mercuric chloride, especially prevention of adsorption through the skin.

A positive reaction consists of the appearance of opacity, whereas the test is negative if there is no change within 15 minutes. Some authorities claim that this test is only effective in the case of camels (6).

c) Antigen detection

Some enzyme-linked immunosorbent assay (ELISA) pilot methods for the detection of circulating invariable trypanosomal antigens have been introduced and evaluated on a relatively large scale (11). In principle, test systems designed for *T. brucei* spp. can be expected to work equally well for all representatives of the subgenus Trypanozoon, including *T. evansi* (4, 5, 18, 25, 37). Recently a commercial latex agglutination antigen test for diagnosis of surra has been developed (23), but requires further evaluation.

d) Detection of trypanosomal DNA

During the past few years, several research centres have been working on the development of polymerase chain reaction (PCR) procedures for the detection of minute amounts of trypanosomal DNA sequences. Recent publications concerning possible applications for *T. evansi* are those of Ventura et al. (33) and Wuyts et al. (38). Species-specific DNA probes for the identification of Trypanozoon organisms, however, are not available yet. Although molecular techniques have a high analytical sensitivity, experimental studies in buffalo (9) showed the diagnostic sensitivity of a PCR was only 78%, similar to that of mouse inoculation.

2. Serological tests

Methods to detect specific humoral antibodies to trypanosome antigens include complement fixation (CF), indirect haemagglutination and precipitation tests. These have not been applied in large-scale surveys. More recently, indirect fluorescent antibody (16, 20, 35), ELISA (2, 5, 16, 24, 28, 32, 40) and card agglutination tests (CATT) (1, 39) have been employed. Extensive evaluation of ELISA and CATT has been carried out in buffaloes in Indonesia and Vietnam (3, 34). The collection of samples can be simplified by using filter paper blood spots for later use in the ELISA, while for the CATT whole blood can be substituted for serum (10). Other innovative modifications that might be developed in the future are the use of a colloidal-dye dipstick test (12) that could enable the tests to be carried out under field conditions.

a) Indirect fluorescent antibody test

The antigen consists of dried blood smears containing from five to ten *T. evansi* trypanosomes per field at x500 magnification, collected from a mouse or rat within 4 days post-infection. Smears are dried at room temperature for 1 hour and fixed in acetone for 15 minutes. When kept dry, the fixed smears may be stored at −20°C for several months.

On testing, the slides are first subdivided into several areas using mounting media or Teflon-coated multispot slides, then washed in PBS, pH 7.2, at room temperature for 10 minutes. Stock PBS contains Na_{2}HPO_{4}·2H_{2}O (9.27 g); KH_{2}PO_{4} (2.15 g); NaCl (36.00 g); NaN_{3} (1.0 g); and distilled water (to 1 litre). This is stored at room temperature and replenished each month. It is diluted five-fold in distilled water before use.

After washing, a positive and a negative control serum and field sera to be tested (diluted from 1/50 to 1/100 or more in PBS), are added and allowed to react for 30 minutes. The slides are washed three successive times in PBS for 5 minutes each. A fluorescein-conjugated antisera specific to the animal species tested is then added at a suitable dilution and left for 30 minutes. The slides are rewashed in PBS,
mounted with immunofluorescence mounting media, and examined by fluorescence microscopy. The glycerol solution should be stored at 4°C and renewed every 2 weeks.

The fluorescein conjugate should be stored at −20°C in small aliquots to avoid repeated freezing and thawing. The tube should be shielded from light in some way, for example by wrapping in aluminium foil. The conjugate is diluted in PBS, pH 7.2, or in PBS containing Evans blue 1/1000 (w/v) as a counterstain to facilitate discrimination between positive (green) and negative (red) fluorescence.

In general, monospecific anti-IgG (gamma-chain) conjugates give the most specific results.

b) Enzyme-linked immunosorbent assay

The principle of this technique is that specific antibodies to trypanosomes can be detected by enzyme-linked anti-immunoglobulins using solid-phase polystyrene plates coated with soluble antigen. The enzyme may be peroxidase, alkaline phosphatase or any other suitable enzyme. The enzyme conjugate binds to the antigen/antibody complex and then reacts with a suitable substrate to yield a characteristic colour change either of the substrate itself or of an added indicator (the chromogen).

The antigen for coating the plates is derived from the blood of a heavily parasitaemic rat. The trypanosomes are separated on a DEAE-cellulose column and washed three times by centrifugation in cold PSG, pH 8 (PBS with 1% glucose). The final pellet is suspended in cold PSG to a concentration of 3–5%, and briefly ultrasonicated on ice for 30–120 seconds until disintegration of the organisms is complete. This preparation is centrifuged at 4°C and 40,000 g for 60 minutes. The supernatant is diluted in water so as to obtain a protein concentration of 1 mg/ml. The reagent thus obtained can be stored in small aliquots at −70°C for several months. It can also be freeze-dried and stored at −20°C. Various treatments of the antigen preparations have been applied to improve the accuracy of antibody detection (29).

- Test procedure
  i) Prior to use, the frozen or freeze-dried antigen is diluted or reconstituted with freshly prepared 0.01 M carbonate/bicarbonate buffer, pH 9.6. The reagent is added (100 µl) to each well of a microtitre plate and the plates are incubated overnight at 4°C or for 1 hour at 37°C.
  ii) Excess antigen is removed, the plates are washed with 0.01 M PBS containing 0.05% Tween 20 (PBST), and serum dilutions in PBST are added (100 µl). Current screening dilutions are between 1/100 and 1/1000.
  iii) The plates are incubated at 37°C for 30 minutes and washed three times with PBST.
  iv) The specific conjugated anti-globulin (100 µl) appropriately diluted in PBST (usually between 1/1000 and 1/50,000) is added.
  v) The plates are reincubated at 37°C for 30 minutes and washed three times with PBST.
  vi) For peroxidase conjugates a number of substrate/chromogen solutions can be used, consisting of hydrogen peroxide with a chromogen, such as tetramethylbenzidine (TMB), 2,2’-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and ortho-diphenylenediamine (OPD). A possible substrate/chromogen solution for peroxidase conjugates is 3% hydrogen peroxide (0.17 ml and 35 mg) in citrate buffer (100 ml), pH 6.0. The citrate buffer is made up as follows: Solution A (36.85 ml): (0.1 M citric acid [21.01 g/litre]); Solution B (65.15 ml): (0.2 M, Na2HPO4 [35.59 g/litre]); and distilled water (100 ml). Dissolve 10 mg TMB in 1 ml dimethyl sulphoxide and add to 99 ml of the citrate buffer. A number of these combinations are available commercially in ready-to-use formulations that remain stable at 4°C for up to 1 year.
  vii) The substrate chromogen is added (100 µl) and the plates are incubated at room temperature for 15–20 minutes.
  viii) The reaction is stopped by the addition of 1 M sulphuric acid (50 µl). The absorbance of each well mixture is read at 450 nm for TMB chromogen. Other chromogens may require the use of a different wavelength. All tests should include known high and medium positive control sera, a negative control serum, and a buffer control.

A large variety of other test procedures exists. For closely related animal species, cross-reacting reagents may often be used (e.g. anti-bovine immunoglobulin for buffaloes). As with immunofluorescence, the use of monospecific anti-IgG conjugates is generally recommended. There are a number of methods that can be used to determine a cut-off point to discriminate between positive and negative results. The simplest method is to base the cut-off on visual inspection of the test results from known positive and negative populations. These results are likely to show some overlap. The operator can choose the most appropriate point to modify the false negative or false positive results depending on the required application of the assay. An alternative is to base the cut-off on the mean ±2 standard deviations (SD) or ±3 SD values from a large sample of negative animals. Finally, if no suitable negative/positive samples are available a cut-off
can be based on the analysis of the data from animals in an endemic situation. If a bimodal distribution separates infected from uninfected animals, then an appropriate value can be selected. The ELISA is likely to correctly identify uninfected animals. Equivocal results can be re-tested using CATT. Currently there are no readily available defined antigens. The Institute for Tropical Medicine in Antwerp has a number of antigens that are still undergoing evaluation. CATT kits are readily available from this institute. Diagnostic assays incorporating antigens derived from a single VAT should be interpreted with caution and fully evaluated in different hosts and different geographical regions to determine if their performance is consistent throughout the range of \textit{T.evansi}.

c) Card agglutination tests

It is well known that certain predominant variable antigen types (VATs) are expressed in common in different strains of salivarian trypanosomes from different areas. On this basis, a field test for the diagnosis of Gambian sleeping sickness, the card agglutination test – CATT/\textit{T.brucei gambiense} – was developed at the Laboratory of Serology, Institute of Tropical Medicine, Antwerp (21). The test makes use of fixed and stained trypanosomes of defined VAT. Both variable and invariable surface antigens take part in the agglutination reaction. For the diagnosis of \textit{T.evansi} infections, a similar test system has been developed (1). CATT/\textit{T.evansi} is based on the use of a widespread VAT of \textit{T.evansi} – RoTat 1/2. This antigen is also currently being evaluated for its use in ELISAs. The CATT requires lyophilised antigen, PBS, pH 7.4, plastic-coated cards, heparinised blood or serum, and a rotator. The lyophilised antigen can be stored at 2–8°C for up to 1 year. Reconstituted antigen can be stored at 2–8°C for 2 days, but preferably should be used within 8 hours.

For screening, put 25 µl of diluted test serum (1/4 or 1/8) and one drop (45 µl) of antigen suspension on one of the circles of the test card. After mixing and spreading the reagents, the card is rotated for 5 minutes. A positive reaction is revealed by blue granular deposits visible to the naked eye\(^3\).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No vaccines are available for this disease.

REFERENCES


\(^3\) Experimental CATT/\textit{T.evansi} kits for evaluation purposes are available at the Laboratory of Serology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium. They are being evaluated in collaboration with the Institute of Molecular Biology of the Free University of Brussels, with financial support from the International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya.
Chapter 2.5.15. — Surra (Trypanosoma evansi)


Chapter 2.5.15. — Surra (Trypanosoma evansi)


* *
**SECTION 2.6.**

**SWINE DISEASES IN LIST B**

**CHAPTER 2.6.1.**

**ATROPHIC RHINITIS OF SWINE**

**SUMMARY**

**Definition of the disease:** Atrophic rhinitis is an infectious disease of swine characterised by purulent nasal discharge, shortening or twisting of the snout, atrophy of the turbinate (conchal) bones and reduced productivity. It may occur enzootically or more sporadically, depending on a variety of factors including herd immunity. The most severe progressive form is caused by infection with toxigenic strains of Pasteurella multocida alone or in combination with Bordetella bronchiseptica. Infections with B. bronchiseptica alone can cause a mild to moderate form with nonprogressive turbinate bone atrophy. Turbinate atrophy may only be obvious at slaughter or may be detected in the live animal by use of radiography or tomography. Environmental and management factors also contribute to the severity and incidence of this disease. A large proportion of apparently normal pig herds are infected with B. bronchiseptica and possibly nontoxigenic P. multocida, some with evidence of turbinate atrophy.

**Identification of the agents:** The diagnosis of atrophic rhinitis depends on clinical and post-mortem observations assisted by the recovery and characterisation of P. multocida and B. bronchiseptica in affected swine. The isolation of both organisms is often complicated by the more profuse growth of other bacteria. Isolation rates are improved by preservation of the nasal or tonsillar swab at 4–8°C in a non-nutritive transport medium and by using a selective culture medium. Pasteurella multocida and B. bronchiseptica can be identified by traditional biochemical tests. Pasteurella multocida isolates may be further characterised by their capsular antigens. Capsular type D is most prevalent in many areas of the world, but in some regions type A predominates. Capsular antigens may be distinguished serologically by indirect haemagglutination or immunofluorescence, chemically by flocculation in acriflavine, or by susceptibility to hyaluronidase. Toxigenicity of P. multocida isolates can be demonstrated by testing for cytotoxicity in cultured cells. A commercially available toxin-specific enzyme-linked immunosorbent assay (ELISA) is now widely used in some areas of the world to differentiate toxigenic from nontoxigenic isolates. In addition, it is suitable for detection of toxin production by bacteria from primary culture plates without the need for prior isolation and identification of individual colonies. This ELISA is increasingly used as the preferred test for control of the progressive form of the disease.

Recently developed assays based on the use of DNA probes or polymerase chain reaction provide sensitive and specific detection methods for those laboratories with the capability to perform them.

**Serological tests:** Detection of antibodies to P. multocida and B. bronchiseptica is of little value as nontoxigenic strains of P. multocida share cross-reactive antigens with toxigenic strains and B. bronchiseptica can be isolated from many swine herds. A test based on detection of antibodies to the P. multocida toxin is commercially available but its usefulness is limited as not all infected swine develop such antibodies. Widespread vaccination with P. multocida toxoid induces antibodies of vaccinal origin, complicating interpretation of results.

**Requirements for vaccines and diagnostic biologicals:** Several vaccines are available commercially that contain bacterins of B. bronchiseptica and a mixture of toxigenic and/or
nontoxigenic strains of P. multocida, or a toxoid derived from P. multocida or from a recombinant Escherichia coli.

A. INTRODUCTION

Atrophic rhinitis is an infectious disease of swine characterised by purulent nasal discharge combined with shortening or twisting of the snout, turbinate (conchal bone) atrophy and reduced productivity. Two forms have been recognised (9):

a) A severe progressive form caused by toxigenic isolates of Pasteurella multocida, most commonly capsular types D or A, alone or in combination with Bordetella bronchiseptica.

b) A less severe form with mild to moderate turbinate atrophy, often without significant snout changes, caused by B. bronchiseptica.

Increased severity of these infections is associated with intensive production, overstocking, and poor management, housing and environmental conditions. Reduced productivity is generally associated with moderate to severe atrophic rhinitis, although the precise relationship between infection with these bacteria and reduced weight gains has not been thoroughly elucidated. Bordetella bronchiseptica and toxigenic P. multocida are commonly found in many domesticated and wild animal species that could potentially transmit the bacteria to swine herds.

Bordetella bronchiseptica or toxigenic P. multocida may be present in a herd with no clinical evidence of disease, especially when other respiratory pathogens are absent and environmental and management conditions are optimal. Such carrier herds pose a risk of transmitting these agents to other herds in which progression to severe disease may occur.

The diagnosis of atrophic rhinitis depends on clinical, pathological and microbiological investigations, with the latter being particularly important for herds infected subclinically. It is now generally accepted that a herd in which toxigenic P. multocida is present be defined as affected with progressive atrophic rhinitis, whether or not clinical signs of the disease are evident (21). Control in many countries has, therefore, centred on detection of infection, even in asymptomatic animals considered to be potential carriers.

Clinical signs include sneezing, nasal discharge, and shortening or twisting of the snout, accompanied by atrophy of the nasal turbinate bones, reduced growth rate and, in severe cases, difficulty in eating. Turbinate atrophy may only be seen at slaughter when snout sections at the level of the second premolar tooth are examined, but radiography (11) and tomography have also been used for detection in live animals. Subjective assessment of turbinate atrophy is convenient and often useful for monitoring herds (9, 27). Objective scales of measure have also been described (14) and are better suited for studies requiring data analysis. Diagnosis is assisted by detection of characteristic histopathological changes including fibrous replacement of the bony plates of the ventral conchae with varying degrees of inflammatory and reparative changes.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agents

a) Culture

As P. multocida preferentially colonises the tonsil, tonsillar swabs or biopsies will provide the highest isolation rates (1). Nasal swabs are preferred for isolation of B. bronchiseptica. When sampling the tonsil is not practical, nasal swabs suffice for isolation of both organisms. A single swab is used to sample both sides of the nasal cavity and should then be placed in a non-nutritive transport medium (e.g. phosphate buffered saline) and kept at 4–8°C during transit to avoid overgrowth by other faster-growing bacteria. Transit time should not exceed 24 hours.

Although both P. multocida and B. bronchiseptica grow readily on blood agar, a selective medium is preferred as overgrowth of other bacteria that are present in higher numbers often interferes with their detection. An additional difficulty related to B. bronchiseptica is that this organism grows more slowly than most other bacteria present in clinical samples. Various formulations of media containing antibiotics have been used for isolation of P. multocida, but comparison of studies in the literature indicates that the highest isolation rates are obtained with modified Knight medium (bovine blood agar containing 5 µg/ml clindamycin, 0.75 µg/ml gentamicin) (18) or KPMD (bovine blood agar containing 3.75 U/ml bacitracin,
b) Biochemical characteristics

*Pasteurella multocida* is a Gram-negative, bipolar, pleomorphic rod and forms nonhaemolytic, greyish colonies on blood agar with a characteristic, ‘sweetish’ odour (24). It fails to grow on MacConkey agar, but yields positive oxidase and catalase reactions and produces indole.

*Bordetella bronchiseptica* is also a Gram-negative rod, forming convex colonies 1–2 mm in size, usually haemolytic, on blood agar or Bordet–Gengou medium after 48 hours of growth (25). It is nonfermentative, but positive for oxidase, catalase, citrate and urea and grows in 6.5% NaCl.

Agglutination tests using specific antisera have been described for confirming the identity of presumptive *B. bronchiseptica* isolates, but appropriate sera are not widely available for use.

- Capsular typing of *Pasteurella multocida*

Capsular typing of *P. multocida* is useful for epidemiological purposes. Serotyping by indirect haemagglutination has been used traditionally (3), but only a few laboratories throughout the world make and maintain the antisera required. However, simpler chemical methods can usually distinguish most swine isolates. Those producing a type D capsule form a heavy flocculate in 1/1000 aqueous acriflavine (5), while capsular type A strains can be identified by inhibition of growth in the presence of hyaluronidase (4). A small proportion of swine isolates are noncapsulated.

- Acriflavine test procedure for capsular type D *Pasteurella multocida*
  i) Inoculate a tube containing 3 ml of brain–heart infusion broth, using a freshly grown blood agar culture, for each *P. multocida* isolate to be tested. Include a known type D strain and a known type A strain as positive and negative controls.
  ii) Incubate inoculated tubes at 37°C for 18–24 hours.
  iii) Pellet bacteria by centrifugation and remove 2.5 ml of the supernatant.
  iv) Add 0.5 ml of a 1/1000 aqueous solution of acriflavine neutral. Acriflavine solution should be freshly prepared each week and stored at 4°C, protected from light.
  v) Mix to resuspend the bacterial pellet and incubate the tube at room temperature, without shaking.
  vi) Observe at 5 minutes for the presence of a heavy flocculent precipitate.

- Hyaluronidase test procedure for capsular type A *Pasteurella multocida*
  i) Prepare fresh bovine blood agar cultures of the isolates to be tested. Include a known type A strain and a known type D strain as positive and negative controls.
  ii) Inoculate each strain to be examined on a separate trypticase soy blood agar plate with 6% bovine blood by streaking several parallel lines of growth, approximately 3–5 mm apart, across the diameter of the plate. For maximum production of hyaluronic acid it is important that the plates be fresh and not dehydrated.
  iii) Heavily streak a hyaluronidase-producing strain of *Staphylococcus aureus* at right angles to the lines of *P. multocida* growth.
  iv) Incubate the plates at 37°C, in a humidified atmosphere, and observe periodically for up to 24 hours. Type A strains will exhibit a marked inhibition of growth in the region adjacent to the growth lines of *S. aureus*.

- Detection of the *Pasteurella multocida* toxin

Diagnosis of progressive atrophic rhinitis depends on characterisation of *P. multocida* isolates as toxigenic. The heat-labile toxin of *P. multocida* produces dermonecrosis in guinea pigs and is lethal in mice following intraperitoneal injection. Toxigenicity can also be demonstrated *in vitro* by testing for cytopathic effects on
monolayers of embryonic bovine lung (EBL) cells (28) or African green monkey kidney (Vero) cells (23). The bacteria are grown in brain–heart infusion broth incubated at 37°C for 24 hours and then pelleted by centrifugation. The supernatant is sterilised by filtration and titrated in monolayer cultures prepared in microtitre plates. Following incubation at 37°C for 2–3 days, the monolayers are stained with crystal violet and examined microscopically to detect cytopathic effects. A rapid cell culture test, in which the suspect colonies are grown on an agar overlay of EBL cells (6), permits more efficient analysis of large numbers of isolates.

An enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies can detect the toxin in mixtures of bacteria recovered from primary isolation media (12, 13). This is an important advantage as swine may be colonised simultaneously with a mixture of toxigenic and nontoxigenic strains (1, 9). Cell culture methods would require every colony of P. multocida in the sample to be tested – which is clearly impractical – to achieve the same level of sensitivity as the ELISA. This ELISA is commercially available throughout Europe and Latin America1 (though not in the United States of America) and has been widely adopted in many areas as the preferred test for identification of carriers and for control of progressive atrophic rhinitis. Though highly specific, a positive result without previous history of disease or suspicious signs should be thoroughly investigated to recover toxigenic isolates from the animals sampled.

### C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are several commercially available vaccines that contain whole-cell bacterins of B. bronchiseptica and a mixture of toxigenic and nontoxigenic P. multocida, or a P. multocida toxoid. Live, attenuated B. bronchiseptica vaccines are also available. Vaccines containing only B. bronchiseptica are not suitable for control of progressive atrophic rhinitis, but may be of benefit in herds with the nonprogressive form. Pasteurella multocida and B. bronchiseptica vaccines appear to reduce the level of colonisation by these bacteria, but do not eliminate them or prevent infection.

The P. multocida toxin is the single most important protective antigen with respect to progressive atrophic rhinitis. Vaccines based on a P. multocida toxoid offer specific protection against the action of the toxin, which, by itself, can be used to reproduce all of the major signs of this disease (see ref. 12 for review). The level of toxin produced by P. multocida is relatively low and the toxin-specific antibody response induced by bacterin-only vaccines may not be optimal. The difficulty and expense of large-scale purification prevent routine incorporation of purified toxoid into vaccines. Recent field studies have shown that a recombinant P. multocida toxin derivative that is non-toxic, but immunogenic, has superior efficacy in swine (2, 22).

Bordetella bronchiseptica produces a variety of toxins and adhesins that are potential virulence factors in swine. Only one, the outer membrane protein pertactin, has been shown to protect against disease in pigs (17). Despite

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1 Available from Dako A/S, Produktionsvej 42, DK-2600 Glostrup, Denmark.
this fact, a dermonecrotic toxin produced by *B. bronchiseptica*, unique from the toxin produced by *P. multocida*, has traditionally been regarded as the primary virulence factor and protective immunogen in swine (9). Several studies strongly implicate the toxin as a virulence factor and it undoubtedly plays some role in pathogenesis and, perhaps, in protection. However, the role of pertactin and several additional virulence factors in protective immunity cannot be excluded.

*Bordetella bronchiseptica* is subject to phenotypic variation under certain growth conditions (e.g. temperatures below 37°C or the presence of chemical modulators such as MgSO₄ or nicotinic acid), in which production of most virulence factors is reversibly turned off. Spontaneously occurring mutants, permanently unable to produce most virulence factors, also arise with a low frequency during culture. Careful attention to colony morphology, on an area of the plate with well-separated colonies, is essential to retain cultures in the phase I (also known as Bvg⁺), or virulent, mode. Phase I colonies are small (1–2 mm in diameter), domed, and haemolytic on blood agar. Loss of haemolysis and the appearance of larger, flat colonies indicate conversion to the avirulent form. Whenever possible, cultures should be propagated using single haemolytic colonies to minimise the slow accumulation of avirulent clones within the culture.

Precise details of standards for the production of effective commercial vaccines are not available, but they are known to contain 10¹⁰ cells of formalin-killed *B. bronchiseptica* and 10 µg of *P. multocida* toxoid per dose. It is also clear that purified toxoid (inactivated by formaldehyde) is more immunogenic than crude toxoid, and that the immunogenicity of the inactivated form is not affected by mixture with a *B. bronchiseptica* bacterin. The recombinant *P. multocida* toxin derivative has been inactivated by deletion of a portion of the gene that does not compromise protective immunogenicity. All commercially available vaccines contain either an oil adjuvant or aluminium hydroxide gel.

*Bordetella bronchiseptica* used for vaccine production should be a phase I virulent culture and the *P. multocida* isolates used should be toxigenic. Seeds of *B. bronchiseptica* and toxigenic *P. multocida* of established identity and passage history should be stored by conventional means. A defined number of passages should be used to give the production culture. *Bordetella bronchiseptica* should be inactivated by formaldehyde. As the toxin of *P. multocida* has an intracellular location and is released on cell lysis during the stationary phase, the culture supernatant should be harvested approximately 48 hours after the end of the exponential phase of growth.

1. **Seed management**

   a) **Characteristics of the seed**

   The seed-lot system should be employed for the bacterial strains used to prepare whole-cell bacterins, as well as for the strains from which purified antigens are derived.

   In the case of whole-cell bacterins, the origin and history of both the *P. multocida* and *B. bronchiseptica* strains should be described and the full characterisation of the master seeds should be laid down in a master seed batch protocol.

   Working seeds used for vaccine production should be derived from the master seed and checked for all relevant properties as described in the master seed batch protocol.

   b) **Method of culture**

   All bacterial strains should be cultivated in suitable media that efficiently support growth and the expression of relevant antigens.

   c) **Validation as a vaccine**

   i) **Purity**

   Both the master seed and the working seed must be pure cultures, free from bacterial, mycotic, mycoplasmal and viral contamination.

   Identity of the bacterial species and the production of relevant antigens should be confirmed.

   ii) **Safety**

   Although inactivation of the bacterial cultures by a validated method is a standard procedure, both bacterial species produce dermonecrotic toxins; detoxification of these toxins should be confirmed when toxoids are used as vaccine components. Standard safety tests for inactivated vaccines should be carried out (7, 8).

   iii) **Efficacy**

   The efficacy of a trial vaccine should be measured by vaccinating groups of pregnant sows. Their progeny should be challenged by virulent cultures of *B. bronchiseptica* and toxin-producing
Chapter 2.6.1. – Atrophic rhinitis of swine

*P. multocida*. Significant protection should be obtained against the clinical signs of the progressive form of atrophic rhinitis, i.e. turbinate atrophy. The clinical signs induced in the controls and vaccinates may be compared according to the scoring system of Done (11).

2. Method of manufacture

Both *B. bronchiseptica* and *P. multocida* cultures should be propagated in media that supports efficient growth and allows optimal expression of the antigens that are relevant for the induction of protective antibodies. *Bordetella bronchiseptica* should be confirmed to be a phase I culture and, for *P. multocida*, it should be confirmed that the culture contains sufficient levels of toxin.

*Bordetella bronchiseptica* cells, and either *P. multocida* cells and/or toxin, are inactivated, detoxified and formulated with an adjuvant. Commonly used adjuvants are aluminium salts or oil emulsions.

3. In-process control

During the manufacturing process, the following in-process controls are carried out.

a) **Purity and identity of the seed cultures**

Cultures are inoculated on blood agar plates and incubated. No nonspecific colonies should grow on these plates.

b) **Purity and identity of the production cultures**

Cultures are inoculated on blood agar plates and incubated. No nonspecific colonies should grow on these plates.

c) **Inactivation of cultures before further processing**

Cultures are inactivated with formaldehyde. Tests are performed to check the effectiveness of the inactivation process and to test for residual formaldehyde.

d) **Quantification of antigens**

This is carried out by performing a total cell count using a bacterial counting chamber for enumerating whole cells or an antigenic mass determination for defined antigens, e.g. *P. multocida* toxin, by quantitative enzyme immunoassay.

4. Batch control

a) **Sterility**

Every batch of vaccine should be tested for sterility according to standard methods (see Chapter I.1.5.) described in the European Pharmacopoeia or the United States Code of Federal Regulations.

b) **Safety**

Every batch of vaccine should be tested for safety in the target animal, by giving a double dose by the recommended route of vaccination, and a second, single dose 2 weeks later. No abnormal local or systemic reactions should occur.

c) **Potency**

Every batch of vaccine should be tested for potency using a validated serological test that correlates with the protection obtained in the efficacy experiment, as described under Section C.1.c.iii. The potency test is not necessarily carried out in the target animal – mice or rabbits can be used. In these latter cases, correlation has to be shown with protective antibody levels in the target animal.

d) **Duration of immunity**

Normally the vaccine is applied during the late stage of pregnancy, so that progeny will be protected by the uptake of colostral antibodies.

When the vaccine may be applied irrespective of the stage of pregnancy, duration of immunity should be at least 6 months, so that booster vaccinations twice a year should maintain effective antibody levels.
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### e) Stability
Every batch of vaccine should be subjected to an accelerated shelf-life test, which has been correlated with real-time shelf-life testing.

### f) Preservatives
When a preservative is used, the concentration should be measured for each batch. It must not exceed the maximum permitted level.

### g) Precautions
When an oil emulsion is used as the adjuvant, accidental injection of the operator can cause a severe local reaction. Medical attention should be sought immediately, treating the wound as a grease-gun injury.

### 5. Tests on the final product

#### a) Safety
Every batch of vaccine should be tested for safety, as described in Section C.4.b.

#### b) Potency
Every batch of vaccine should be tested for potency, as described in Section C.4.c.

### REFERENCES


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CHAPTER 2.6.2.  
PORCINE BRUCELLOSIS

SUMMARY

Brucellosis in pigs is caused by Brucella suis, a bacterial infection that, after an initial bacteraemia, causes chronic inflammatory lesions in the reproductive organs of both sexes, with occasional localisation and lesions in other tissues. The species Brucella suis consists of five biovars, but the infection in pigs is caused by B. suis biovars 1, 2 or 3. The disease caused by biovars 1 and 3 is similar, while that caused by biovar 2 differs from 1 and 3 in its host range, its limited geographical distribution and its pathology. Biovar 2 is rarely pathogenic for humans, whereas biovars 1 and 3 are highly pathogenic, causing severe disease. Porcine brucellosis is of widespread occurrence; generally, however, the prevalence is low, with the exception of South America and South-East Asia where the prevalence is higher. In some areas, B. suis infection has become established in wild or feral pigs – diagnostic methods recommended for wild and feral pigs are the same as for domestic pigs. Various biovars of B. suis cause infections in animals other than pigs, such as reindeer, caribou, hares and various murine species, and occasionally in cattle and dogs. Brucella suis infections in animals other than pigs are reviewed in an Appendix at the end of this chapter.

Signs of disease in sows include abortion at any stage of gestation, and birth of dead or weak piglets. In boars, the most prominent sign is orchitis, and the secondary sex organs may be affected. Brucella suis may be present in the semen, sometimes in the absence of clinical signs. Transmission during copulation is more common than is the case with brucellosis in ruminants. In both sexes, bones and especially joints and tendon sheaths may be affected, causing lameness and sometimes paralysis. Pigs are susceptible to artificial infection with B. abortus and B. melitensis, but reports of natural disease in pigs being caused by either of these organisms are rare. In humans, the infection is usually confined to those who are occupationally exposed to pigs, and to laboratory workers. The capability of B. suis to colonise the bovine udder with subsequent shedding in milk means that it has the potential to be a serious human health risk.

Identification of the agent: Brucella suis is readily isolated from live pigs by culture of birth products, and from carcasses by culture of lymph nodes and organs. Selective media are available for culture of contaminated samples. In nature, B. suis occurs invariably in the smooth phase – the appearance on solid medium is typical of smooth brucellae. Biovars of porcine origin agglutinate with monospecific A antiserum, and not with M antiserum. Definite identification of species and biovars may be effected by phage typing and biochemical tests, preferably carried out in specialised laboratories.

Serological tests: To date, none of the conventional serological tests has been shown to be entirely reliable in routine diagnosis in individual pigs. Their preferred use is for the identification of infected herds. The indirect enzyme-linked immunosorbent assay (ELISA) and competitive ELISA are the prescribed tests for international trade purposes. The buffered Brucella antigen tests (BBATs), i.e. the buffered plate agglutination test (BPAT) and the rose bengal test (RBT), are suggested as alternative tests for screening purposes or complete herd tests. A fluorescence polarisation assay has also been developed. The allergic skin test is also useful for identifying infected herds.

Requirements for vaccines and diagnostic biologicals: Brucella suis strain 2 vaccine has been used for immunising pigs in China (People’s Rep. of). Confirmation of the results obtained in China is required before strain 2 vaccine can be recommended for general use. In other countries, experimental work has shown that B. melitensis Rev.1 vaccine is superior to B. suis strain 2 in protecting sheep against B. melitensis. Sufficient data are not available to conclude if B. abortus strain RB51 vaccine is efficacious in protecting swine against exposure to B. suis. In practice, no
A. INTRODUCTION

Porcine brucellosis is an infection caused by biovar 1, 2 or 3 of *Brucella suis*. It occurs in many countries where pigs are raised. Generally, the prevalence is low, but in some areas, such as South America and South-East Asia, the prevalence is much higher. Porcine brucellosis may be a serious, but presently unrecognised, problem in some countries. *Brucella suis* biovar 1 infections have been reported from feral pigs in some of the southern States of the United States of America (USA), and in Queensland, Australia. In Queensland, a number of human infections have been reported from people who hunt and handle material taken from feral pigs (16).

The disease is generally transmitted by consumption of feed contaminated by birth and/or abortion products and uterine discharges. Pigs will readily eat aborted fetuses and membranes. Transmission during copulation also occurs frequently, and this has implications for those practising artificial insemination.

In pigs, as in ruminants, after the initial bacteraemia, *B. suis* colonises cells of the reproductive tract of either sex. In females, placentas and fetuses are invaded, while in males, invasion occurs in one or more of the following: testis, epididymis, seminal vesicles, and/or bulbo-urethral glands. In males the lesions, which are most often unilateral, start with a hyperplasia that may progress to abscess formation; the final stage is characterised by sclerosis and atrophy. Arthritis may occur in various joints, and sometimes spondylitis occurs.

The most common manifestation of brucellosis in female pigs is abortion, occurring very early or at any time during gestation. Vaginal discharge is not often evident, and the problem may appear to be infertility rather than abortion. In males, brucellosis is more likely to be persistent, with lesions in the genital tract often leading to interference with sexual activity, which can be temporary or permanent. The boar may excrete brucellae in the semen without any apparent abnormality in the sex organs or interference with sexual activity.

In both sexes, there may be swollen joints and tendon sheaths, lameness and, occasionally, posterior paralysis. A significant proportion of both male and female pigs will recover from the infection, often within 6 months, but many will remain permanently infected.

Brucellosis caused by *B. suis* biovar 2 differs from infection caused by biovars 1 and 3 in its host range, its distribution, and in its pathology. In recent outbreaks in Europe, wild pigs have been implicated as the source of transmission of biovar 2 to pigs that are reared outdoors (7). In addition to wild swine, the European hare (*Lepus capensis*) is also a reservoir of *B. suis* biovar 2 and has been implicated as a possible source of transmission to domestic livestock (1, 7). The geographical distribution of biovar 2 has historically been in a broad range between Scandinavia and the Balkans (1). *Brucella suis* biovar 2 causes miliary lesions in tissues, particularly reproductive tissues, that often become purulent. To date, biovar 2 has only once been reported as the cause of human brucellosis.

The common biovars of *B. suis* (1 and 3) are serious human pathogens and precautions are needed when handling and disposing of potentially infective material. This is especially so in the laboratory after culture has greatly increased the number of organisms present. Laboratory manipulation of the cultures or contaminated material from infected animals must be done under strict biosecurity conditions to safely handle this dangerous zoonotic agent. Biosecurity containment level 3 or higher is recommended, as described in Chapter I.1.6. Human safety in the veterinary microbiology laboratory.

B. DIAGNOSTIC TECHNIQUES

In porcine brucellosis, culture methods are at least as sensitive as serology (4). As the produce of almost all pig-raising enterprises passes through abattoirs, surveillance methods (serology and culture) can be applied effectively at this point. In many areas, traditional village pig breeding is being replaced by or accompanied by the development of larger commercial units, thereby increasing the use of artificial insemination. Therefore artificial insemination using brucellosis-free boars can be a valuable aid in the control of porcine brucellosis, the inadvertent use of infected semen could, obviously, cause incalculable damage.

1. Identification of the agent

Optimal samples for bacteriological culture and methods for processing samples are similar to those described for in Chapter 2.3.1. Bovine brucellosis. Standard and selective media used for other species of brucellae are suitable for *B. suis* (see Chapter 2.3.1. Bovine brucellosis). The addition of serum is not essential, but basal medium containing 5% serum is a satisfactory medium, both for isolation, maintenance of cultures and typing. The addition of CO₂ to the atmosphere is not required.
In nature, *B. suis* invariably occurs in the smooth form and colonies are indistinguishable from other smooth *Brucella* species, described in Chapter 2.3.1. Bovine brucellosis.

Biovars 1, 2 and 3 of *B. suis* are all A surface antigen dominant, and growth may be presumptively identified by slide agglutination with monospecific A antisem. Confirmatory identification of species and biovar should be performed in a specialised reference laboratory. The OIE Reference Laboratories for brucellosis are listed in the Table given in Part 3 of this *Terrestrial Manual*.

Confirmation of species and biovar depends on phage tests, production of H₂S (only biovar 1 produces H₂S), and growth in the presence of dyes. Some strains of *B. suis* biovar 1 are atypical in that they grow on media containing 20 µg/ml basic fuchsin. Most strains of *B. suis* are inhibited by safranin O at a concentration of 1/10,000, whereas *B. suis* reacts more rapidly in the urease test than either *B. abortus* or *B. melitensis*. Oxidative metabolic tests are supplemental tests that can be used for distinguishing *B. suis* from other smooth *Brucella* species.

Molecular genetic techniques using the polymerase chain reaction (PCR) and specific primers are available that can distinguish *B. suis* from other smooth species of *Brucella* (3, 18). However, these PCR techniques cannot distinguish biovars within *B. suis*, neither have they been fully evaluated and standardised. The 3.3 Mb complete genomic sequence of *B. suis* strain 1330 has been determined, and has a similar chromosome structure and gene content to that of *B. melitensis* strain 16M (14). The *B. suis* sequence will be beneficial for basic research on taxonomy, virulence, and protective antigens of *B. suis*, and may prove beneficial in developing new diagnostic tests.

## 2. Serological tests

None of the conventional serological tests used for the diagnosis of porcine brucellosis are reliable for diagnosis in individual pigs. A significant problem is the fact that weaners up to 2–3 months of age are susceptible to infection with *B. suis*, but their agglutinating antibody response to the infection is very limited.

These conventional tests use antigens that are dependent on smooth lipopolysaccharide (LPS) for their activity. Due to the sharing of an ‘O’ chain polysaccharide, such antigens react equally with the LPS of *Yersinia enterocolitica* serotype 0.9 and are not, therefore, able to distinguish between antibodies to these two infections. *Yersinia enterocolitica* infection in pigs is not uncommon in some areas (21). Studies have suggested that the sensitivities and specificities of the buffered acidified plate antigen assay, the 2-mercaptopethanol test, the indirect enzyme-linked immunosorbent assay (I-ELISA), a competitive ELISA (C-ELISA), and the fluorescent polarisation assay (FPA) are similar (13). Swine serum may sometimes also contain nonspecific antibody, thought to be IgM, further reducing the specificity of conventional serology, especially the serum agglutination test (SAT). Also, swine complement interacts with guinea-pig complement to produce a pro-complementary activity that reduces the sensitivity of the complement fixation test (CFT). Sensitivity levels as low as 38% (15) and 49% (17) have been reported for the CFT; therefore this test cannot be recommended for the diagnosis of brucellosis in individual pigs. For international and other trade purposes, such as purchasing boars, the disease status of the herd and of the area in which the herd is situated are of more importance than tests on individual animals. In spite of this, the European Union and some other countries still insist that only pigs whose serum shows an agglutination titre less than 30 International Units (IU) per ml and a CFT of less than 20 ICFTU (international CFT units) should be allowed to cross international borders.

### • Reference sera

Primary reference standards are those against which all other standards are compared and calibrated. These reference standards are currently being developed and will be available to national reference laboratories when completed. Biological reagents for the C-ELISA and I-ELISA for the diagnosis of porcine brucellosis are available in small quantities for research or standardisation purposes.

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

- **Indirect ELISA**

Indirect and competitive ELISAs have been developed for the diagnosis of brucellosis in individual pigs and for screening large numbers of sera. These techniques promise to be more efficient than any of the tests mentioned above, and the C-ELISA appears to be better at distinguishing antibody reactions that are due to infection with *Y. enterocolitica* serotype 0.9 from those that are due to infection with *Brucella* sp. The method for the I-ELISA is described in detail in Chapter 2.3.1. Bovine brucellosis, however, monoclonal antibody specific for porcine IgG conjugated with horseradish peroxidase should be used.

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1 Obtainable from the OIE Reference Laboratory for Brucellosis at the Animal Diseases Research Institute, 3851 Fallowfield Road, Nepean, Ontario K2H 8P9, Canada.
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- Competitive ELISA

C-ELISA procedures for detection of porcine antibody to Brucella sp. (11) are identical to the procedures used for bovine antibody to B. abortus described in Chapter 2.3.1. This assay is capable of eliminating some reactions due to Y. enterocolitica serotype 0.9 and other cross-reacting antibody, such as IgM, which have lower affinity for Brucella epitopes as compared with the monoclonal antibody used in the assay. The C-ELISA is recommended as a confirmatory test as its sensitivity and specificity exceeds those of the agglutination tests.

b) Fluorescence polarisation assay (an alternative test for international trade)

The FPA for detection of porcine antibody to Brucella sp. is essentially the same as that described for cattle (for more details see Chapter 2.3.1); the serum dilution can vary depending upon validation with local samples but is often used at 1/25 for the tube test and 1/10 for the plate test (11). It is a simple technique for measuring antigen/antibody interaction and may be performed in the laboratory or in the field. This assay may assist in eliminating some of the reactivity resulting from exposure to Y. enterocolitica serotype 0.9 and other cross-reacting antibody. Lyophilised porcine sera tend to increase background activity in this assay. The FPA may be used as a screening and/or confirmatory test.

c) Buffered Brucella antigen tests (an alternative test for international trade)

For screening purposes or complete herd tests, the buffered Brucella antigen tests (BBAT), i.e. the card test, the rose bengal plate agglutination test (RBT) or the buffered plate agglutination test (BPAT), are recommended as alternative tests. The preparation and standardisation of BBAT antigens and the methods of performing the tests are described in Chapter 2.3.1. Bovine brucellosis. All biovars of B. suis affecting pigs have the same immunodominant A antigen as do most of the B. abortus biovars, which makes the B. abortus antigens appropriate for testing swine sera.

3. Other tests

a) Allergic (hypersensitivity) tests

Allergic tests have been widely used for the diagnosis of brucellosis in herds of pigs in Eastern Europe, in the countries that were formerly in the Union of Soviet Socialist Republics (USSR), and in the People’s Republic of China. The specificity is very high, however, the sensitivity of the allergic test is similar to that of the serological tests (BBATs) and is not reliable for diagnosis in individual pigs. Some infected animals that are negative serologically, react positively to the skin test, and vice versa. Thus, where practicable, it is useful to perform both tests. As the active principle of the allergic test is protein, there should be no cross-reaction with Y. enterocolitica serotype 0.9, at least in theory, and the authors are not aware of any demonstration of this cross-reactivity.

Many different allergens have been used for diagnosis, but one that is simple to prepare, that has been used effectively as a herd test in pigs, and that is still in use in some countries is an acid hydrolysate, sometimes called brucellysate or brucellin fraction F. Despite containing some polysaccharide, this preparation does not stimulate agglutinins or complement-fixing antibodies, nor does it sensitise animals (9). The method for preparing brucellin fraction F (Section C2), acid hydrolysis, will give rise to a substantial quantity of O-polysaccharide in the supernatant fluid, thus not helping in the differentiation of Yersinia from Brucella antibody. A method of preparation is given in Section C2. As a diagnostic agent in pigs, 0.2 ml of the allergen is injected intradermally into the skin at the base of the ear. The reaction is read after 48 hours. A positive reaction shows erythema on nonpigmented skin and an oedematous swelling. In severe reactions there may also be some necrosis.

More recently, brucellin INRA has been developed for use in ruminants; the authors are not aware of it having been used in pigs. A rough strain is used in its preparation, thereby avoiding the presence of smooth LPS. The preparation, testing, and use of brucellin INRA is described in detail in Chapter 2.3.1. Bovine brucellosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Numerous attempts have been made to develop a vaccine to immunise pigs against B. suis. Only one product has found any acceptance for field use — B. suis strain 2 (S2) vaccine has been used extensively in south China (People’s Rep. of). To date, it does not appear to have been used elsewhere in pigs, probably because it has been shown to confer less protection in sheep against B. melitensis than the Rev.1 vaccine (20). Sufficient data are not available to conclude if B. abortus strain RB51 vaccine is efficacious in protecting swine against exposure to B. suis.
C1. *Brucella suis* strain 2 vaccine

S2 is a smooth, stable, naturally attenuated strain of *B. suis* biovar 1. Extensive studies have failed to detect any characteristic to distinguish S2 from field strains of biovar 1, except the level of virulence, which, as judged by its persistence in mice and guinea-pigs, is much lower than that of the average field strain. The low level of virulence and its stability has been demonstrated by passage in pregnant sows and boars (22).

The S2 vaccine may be given orally, for example in the People’s Republic of China it is often mixed in food, although it is warned that fermented food rapidly inactivates the vaccine. Oral vaccination could be an attractive possibility for the control of brucellosis in feral pigs. Challenge experiments in both sexes have shown appreciably lower levels of infection in the vaccinated compared with unvaccinated control animals, even in sows challenged with infected semen. For oral vaccination, doses of around $2 \times 10^{10}$ are required, and usually two doses are given at an interval of approximately 2 months. Antibody produced in response to oral vaccination is said to disappear by 6 months after vaccination. Liang Xingxian (10) reports that in Guangdong Province in south China, porcine brucellosis has been controlled by a combination of annual serological testing of breeding stock with elimination of serologically positive animals and vaccination of serologically negative animals. In 1985, the reactor rate was 10%; in 1987 it had fallen to 1.2%, and no reactive boars had been found in the 4 years to 1991. Before the S2 vaccine is accepted for general use, its safety and immunogenicity should be thoroughly investigated under the conditions pertaining in each country.

1. Seed management

Freeze-dried seed cultures for the experimental production and use of S2 vaccines are available from recognised institutions. This freeze-dried material may be propagated by culture on nutrient agar or trypticase–soy agar for 2–3 days to produce seed lots that should not be more than three subcultures away from the starting material.

Any country interested in introducing this vaccine should organise adequate challenge experiments and field trials in pigs.

2. Method of manufacture

The S2 vaccine is produced in fermenters using the same procedures and media as described for *B. abortus* strain 19 (S19) (see Chapter 2.3.1. Bovine brucellosis). However, for the propagation of S2 vaccine on solid medium, either nutrient agar or trypticase–soy agar is preferred to potato agar.

3. In-process control and further testing

In-process control, batch control and tests on the final product are all the same as for *B. abortus* S19. With regards the length of immunity, annual revaccination is recommended.

C2. Brucellysate (brucellin fraction F)

a) Preparation

i) Grow a smooth culture of *Brucella* (e.g. *B. suis*) on agar medium or in a fermenter.

ii) Harvest cells grown on solid medium in normal saline (0.15 M NaCl).

iii) Deposit by centrifugation and resuspend to 50–100 $\times 10^9$ cells/ml in normal saline.

iv) Add 1 N hydrochloric acid and adjust the pH to 1.2–2.0. Incubate at room temperature for 90 minutes.

v) Autoclave at 120°C for 25 minutes and leave overnight in the autoclave.

vi) Separate the cells by centrifugation.

vii) Adjust the pH of the supernatant hydrolysate to 6.8–7.0. Warm in a water bath at 80°C for 30 minutes.

viii) Pass through a sterilising filter.

ix) Estimate the nitrogen content of the concentrated hydrolysate. Adjust the protein nitrogen content to 25–30 mg/100 ml.

x) Check for sterility. Check for lack of toxicity by injecting 0.5 ml into each of three to five mice.

2 Obtainable from the National Institute for the Control of Veterinary Products and Pharmaceuticals, Ministry of Agriculture, 30 Baishiqiao Road, Beijing 100081, China (People’s Rep. of), or from VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom. Supply from the Weybridge laboratory needs the prior permission of the World Health Organization.
Potency may be tested in sensitised guinea-pigs in comparison with a product of known potency. Guinea-pigs may be sensitised by repeated injections of killed \( B. \) suis in Freund’s incomplete adjuvant (2).

b) Field use

As a diagnostic agent in pigs, 0.2 ml of the allergen is injected intradermally into the skin at the base of the ear. The reaction is read after 48 hours. A positive reaction shows erythema on nonpigmented skin and an oedematous swelling. In severe reactions there may also be some necrosis.

APPENDIX: \textit{BRUCELLA SUIS} INFECTIONS IN ANIMALS OTHER THAN PIGS

1. \textbf{Rangiferine brucellosis}

\textit{Brucella suis} biovar 4 causes serious disease in reindeer or caribou (\textit{Rangifer tarandus} and its various subspecies) throughout the Arctic region, Siberia, Canada and Alaska (12). Some of these animals are domesticated, others are wild and migratory. \textit{Rangifer tarandus} is very susceptible to \( B. \) suis infection, which causes fever, depression and various local signs, such as abortion, retained placentas, metritis, sometimes with blood-stained discharge, mastitis, bursitis and orchitis. In the Arctic region, \( B. \) suis biovar 4 constitutes a serious zoonosis (5). Transmission to humans may be by direct contact or through consumption of milk and other inadequately heated products from reindeer. Bone marrow, which is considered to be a special delicacy in this region, is also a source of human infection.

The methods already described for isolating and identifying \( B. \) suis in samples taken from pigs are equally applicable to \( B. \) suis biovar 4 in samples taken from reindeer. Biovar 4 grows well on all the usual media used for the culture of \textit{Brucella}. It reacts positively with both A and M monospecific sera. For serology, the tube agglutination test has been reported to be satisfactory, with titres from 1/20 being considered diagnostic. The CFT has also been used, but the clinical interpretation of these tests in reindeer has not been established.

Vaccination of reindeer with \( B. \) abortus S19 vaccine, or alternatively with \( B. \) abortus 45/20 adjuvant vaccine, has been tried experimentally without any clear-cut result. In the case of S19, the reaction to vaccination was rather severe and immunity in the vaccinated animals could only be demonstrated against challenge with very small doses of \( B. \) suis biovar 4. Gall \textit{et al.} (6) compared several serological tests and found that the specificity values for the BPAT and CFT using reindeer/caribou sera was lower than the I-ELISA, C-ELISA and the FPA, while sensitivity values were similar for all tests.

2. \textbf{Brucella suis} infection in other nonporcine species

There are two different types of epidemiological situation with regard to \( B. \) suis infection in other nonporcine species. In the first case, \( B. \) suis infection occurs in animals that are not the natural host of the particular infection through the ingestion of contaminated materials or by co-habitation with infected natural hosts. For example, Arctic foxes and wolves may contract \( B. \) suis biovar 4 from reindeer; dogs and rodents, such as rats and mice, may acquire other \( B. \) suis biovars by cohabitation with infected hosts. Cattle may become infected by cohabitation with infected feral swine. The infecting bacteria are invariably the well defined biovars of the natural host species.

In the second case, wildlife species that are natural hosts for \( B. \) suis or \( B. \)-suis-like infections become infected. One example is the so-called murine brucellosis of the former USSR, where small rodents are infected with \( B. \) suis biovar 5. Other similar situations have been reported from Queensland, Australia and from Kenya. In all three cases, \( B. \) suis strains with different characteristics were involved, and at least one of them was difficult to classify.

Brucellosis caused by \( B. \) suis biovar 2 is perhaps a special case. The reservoir of infection is in wild pigs (\textit{Sus scrofa}) living in the same area (7, 8), or in the European hare (\textit{Lepus capensis}) (19), or in both. Biovar 2 infection historically has been confined to an area between Scandinavia and the Balkans. Domestic swine reared outdoors in this area are at highest risk for transmission of biovar 2 from wildlife vectors. After invading domestic pig herds, biovar 2 is likely to spread as rapidly as biovars 1 and 3. The disease in hares is characterised by the formation of nodules, varying in size from that of a millet seed to a cherry or even larger; these often become purulent. Such nodules may occur in almost any location, sometimes subcutaneously or intramuscularly, in the spleen, liver or lung and in the reproductive organs of either sex. The bodily condition of the hare may be surprisingly unaffected.

Serological investigations in nonporcine species are usually carried out for screening purposes. In these particular circumstances, specificity is more important than sensitivity. Here the CFT is recommended, although the buffered \textit{Brucella} plate agglutination test may be useful because of its simplicity. In many previous
investigations, the tube agglutination test was used, apparently with satisfaction. However, in nonporcine species the interpretation of serological results may be problematic. Where supposedly positive samples are encountered, serological screening should be followed by bacteriological investigation.

For bacteriological investigations in situations such as these, where the infecting organisms may have unusual characteristics, it is advisable to duplicate the culture on selective media by culture on plain medium supplemented with 5% serum, and to broaden the investigation by incubating the cultures in an atmosphere containing 10% CO₂. Colonies resembling *Brucella* can be tentatively identified by Gram staining, by slide agglutination tests with monospecific A and M sera, and by anti-rough *Brucella* serum (Chapter 2.3.1 Bovine brucellosis) *Brucella suis* biovar 5 is unusual in that it reacts with monospecific M serum, and not with monospecific A serum. Further identification is best carried out in a specialised laboratory.

**REFERENCES**


* * *

**NB:** There are OIE Reference Laboratories for Porcine 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).
**CHAPTER 2.6.3.**

**ENTEROVIRUS ENCEPHALOMYELITIS**
(previously Teschen/Talfan disease)

**SUMMARY**

Enterovirus encephalomyelitis was first described as a particularly virulent, highly fatal encephalomyelitis of pigs, previously known as Teschen disease. It is caused by strains of porcine enterovirus serotype 1 (PEV-1) of the genus Teschovirus of the family Picornaviridae. Less severe forms of the disease were first recognised in the United Kingdom, where it was called Talfan disease, and in Denmark, where it was called Poliomyelitis suum. In addition to PEV-1 strains, the milder form of the disease is caused by other types of PEV, including PEV-2, -3, -4, -5, -6, -8, -12 and -13.

The disease was first described in Czechoslovakia in 1929. During the 1940s and 1950s it caused serious losses in European countries and was spread to other continents. The clinical disease is now rare, although serological evidence indicates that virus variants that are not pathogenic or of low pathogenicity circulate in pig populations.

**Identification of the agent:** The virus has affinity for the central nervous system and therefore suspensions of brain and spinal cord from affected pigs are used as inocula for virus isolation. The virus propagates successfully on monolayers derived from swine tissue, in particular from kidney. If PEV is present, it gives rise to specific cytopathic effects characterised by round refractile cells.

For PEV identification and serotyping, suitable tests are employed using specific antisera or monoclonal antibodies against standard strains of PEV. Virus neutralisation tests and indirect fluorescent antibody tests are preferred.

**Serological tests:** Because the seroprevalence of PEV-1 may exceed 60% in healthy pig populations in Central Europe, and identical clinical signs may be caused by other viruses, including other serotypes of PEV, a single serological test for PEV-1 giving positive results does not indicate that the neurological signs observed are actually caused by a PEV-1 infection. A fourfold rise in titre together with typical signs should be considered to be an indication that PEV-1 infection caused clinical disease. For screening for specific antibodies in pig populations, it is recommended to use the virus neutralisation test in microtitre plates or the enzyme-linked immunosorbent assay.

**Requirements for vaccines and diagnostic biologicals:** Vaccines are available. Inactivated vaccines produced from virus propagated on cell culture are recommended for active immunisation. The virus is usually inactivated with formaldehyde or other suitable inactivants, and blended with adjuvants.

**A. INTRODUCTION**

Enterovirus encephalomyelitis (previously Teschen/Talfan diseases) is an acute condition of pigs characterised by central nervous system (CNS) disorders. Teschen is the name of the town in the Czech Republic where the disease was first recognised in 1929 (4, 5). In the 1950s, the disease spread throughout Europe and caused huge losses to the pig breeding industry. Less severe forms of the disease were first recognised in the United Kingdom, where it was called Talfan disease, and in Denmark, where it was called Poliomyelitis suum; these were benign enzootics of swine. The disease is now rare. The causal agent of enterovirus encephalomyelitis is porcine enterovirus serotype I (PEV-1) of the Picornaviridae family (2, 5). The 13 serotypes, PEV-1 to PEV-13, of porcine enterovirus were grouped into three groups – I, II and III – on the basis of cytopathic effect (CPE) produced, serological assays and replication in different cell cultures. PEV-1 to PEV-7 and PEV-11 to PEV-13 were identified as group I. Based on nucleotide sequencing and phylogenetic analysis, group I has now been
designated as the genus *Teschovirus*. Group II contains PEV-8 and group III includes PEV-9 and -10, and these two groups belong to the genus *Enterovirus* (15). PEV-2, -3, -4, -5, -6, -8, -12 and -13 have been isolated from pigs with milder forms of the disease (14). PEV infections often do not produce clinical signs. Serotypes may be differentiated using a virus neutralisation (VN) test (2, 10), complement fixation test (6) or indirect fluorescent antibody (IFA) test (1, 12).

The infection only occurs in swine; other animal species are not susceptible.

PEV may be identified and standard antisera have been prepared by hyperimmunisation of guinea-pigs, rabbits, or colostrum-deprived piglets with standard strains of PEV serotypes 1–6, 8, 12, and 13.

The virus enters the animal via the oral or nasal cavity. The incubation period is about 14 days. The main signs of the prodromal stage are fever up to 41.5°C, lassitude, anorexia and locomotor disturbances. This stage is followed by hypersensitivity, tremors, clonic spasms of the legs, opisthotonos and nystagmus. In the final clinical stage, paralysis proceeding from the hind part through the loins to the fore part of the body is observed. Paralysis of the thermoregulatory centre results in hypothermy. When respiratory muscles are paralysed, the animal dies of suffocation.

Laboratory diagnosis of the disease is based on typical clinical signs plus histological lesions of the brain and spinal cord, identification of the virus in the CNS of affected pigs, and on the detection of specific antibodies in the blood of convalescent animals.

**B. DIAGNOSTIC TECHNIQUES**

1. **Histological examination and Immunohistochemistry (IHC)**

For histological diagnosis, samples of cerebrum, cerebellum, diencephalon, medulla oblongata and cervical and lumbar spinal cord are collected. The samples are fixed in formaldehyde and sections are stained using conventional histological methods. The virus multiplies in the CNS causing a nonsuppurative encephalomyelitis with perivascular lymphocyte infiltration (4). Pathological changes are observed in the grey matter of the diencephalon, cerebellum, medulla oblongata and in the ventral horns of the spinal cord, and to a lesser extent in the cerebral hemispheres. Degeneration of neurons and their replacement by glial connective tissue develops in the late stage of the disease.

Identification of infection can be confirmed by IHC on fixed, paraffin-embedded CNS sections using specific antisera or monoclonal antibodies. This technique allows correlation of pathological changes with the location of the agent.

2. **Identification of the agent**

   a) **Isolation of the virus**

   Progress in diagnosis of enterovirus encephalomyelitis and vaccine production has been made possible by the propagation of virus in cell culture (7, 9).

   Samples of brain and spinal cord are collected from pigs slaughtered at an early clinical stage of the disease. When not processed immediately, the samples should be placed in a solution prepared from equal parts of phosphate buffered isotonic saline solution (PBS), pH 7.4, and glycerol. Pieces of tissue are minced to prepare a 10% (w/v) suspension in PBS. The suspension is centrifuged at 800 g for 10 minutes and the supernatant fluid is used for inoculation of cell cultures. Monolayer cultures of primary porcine kidney or established cell lines derived from porcine tissue are suitable for isolation of PEV.

   - **Test procedure**
     i) Test tubes or tissue culture vessels with monolayer cell cultures are used. Growth medium is discarded and tubes or vessels are inoculated with 0.1 ml of suspect tissue homogenate.
     ii) Inoculated test tubes are placed on a roller drum or tissue culture vessels are placed on a tray and incubated for 1 hour at 37°C.
     iii) The inoculum is discarded; the tubes or tissue culture vessels are washed with PBS and replenished with 1–20 ml (depending on the type of tissue culture vessel used) of maintenance medium without calf serum.
     iv) The tubes are examined microscopically each day. If the sample contains PEV, characteristic CPE will be seen after 3–4 days. The CPE is characterised by small foci of rounded refractile cells. After
Chapter 2.6.3. – Enterovirus encephalomyelitis (previously Teschen/Talfan disease)

In several passages the virus grows better and produces complete CPE after 24 hours. The identity of PEV can be confirmed by the use of specific antiserum or monoclonal antibodies. The VN or the IFA test is best suited to this purpose. Once an isolate has been identified serologically as PEV, piglet inoculation is the only certain means of determining that the given isolate is pathogenic.

b) Virus neutralisation test for porcine enterovirus identification

The virus harvested from cell cultures is diluted in cell culture maintenance medium over the range $10^{-1}$ to $10^{-6}$ in tenfold steps. For enterovirus serotyping, 14 rows of each dilution are prepared; 50 µl of standard antisera to PEV-1–13 diluted 1/10 is added to rows 1–13 and 50 µl of negative serum is added to the last row. Mixtures are incubated overnight at 4°C or for 1 hour at 37°C and thereafter inoculated into roller tube cultures or into wells of microtitration plates with confluent monolayer cell cultures. The inoculated cell cultures are incubated at 37°C. Assessment is carried out 72 hours later and every following day up to day 10, depending on when the CPE is seen. The identification of a PEV serotype is confirmed if the titre of the isolated virus in the presence of that antiserum is at least $10^3$ lower than that virus incubated with negative serum.

c) Indirect fluorescent antibody test for the confirmation of porcine enterovirus antigen in cells

The IFA test is based on the reaction of the antigens in infected cells with specific antibodies in positive serum (12). The reaction is visualised by a fluorescein isothiocyanate (FITC)-conjugated antiliglobulin, using a microscope with a UV or a blue light source. The antigen is detectable in cells 12 hours after the infection with PEV, i.e. before the development of CPE. Polyclonal antisera often show cross-reactivity with different PEV types, which can confuse the interpretation of results.

- **Test procedure**
  i) Monolayers of porcine kidney cells on cover-slips are inoculated with the suspected material. Positive and negative controls should be processed in parallel with the test specimens.
  ii) After incubation for 12–16 hours, the cover-slips are removed, washed twice in PBS, air-dried and fixed in cold acetone for 5–15 minutes.
  iii) The cover-slips are placed into a wet box and flooded with rabbit or pig hyperimmune anti-PEV serum optimally diluted 1/10 with PBS or with PEV-specific monoclonal antibody at working dilution.
  iv) The wet box is closed and incubated at 37°C for 60 minutes.
  v) The cover-slips are removed and washed three times in PBS, then flooded with FITC-conjugated anti-rabbit or anti-pig goat serum, at a previously assessed working dilution, and incubated at 37°C for 30 minutes.
  vi) The cover-slips are then washed three times with PBS, air-dried and mounted in 0.1 M Tris-buffered glycerol, pH 8.6.

After processing, the cover-slips are examined microscopically. The control slides are examined first to confirm that the fluorescence observed is specific. The fluorescence is apple green in colour and occurs in the cell cytoplasm and at the periphery of the nucleus. Instead of cover-slips, multis spot slides or multiwell plates can also be used.

d) Reverse-transcription polymerase chain reaction

The reverse-transcription polymerase chain reaction (RT-PCR) provides a method for detection and differentiation of specific gene regions of porcine enteroviruses (11, 16). The nested RT-PCR with specific primer sets has been used to differentiate among group I, II and III viruses (16). PCR is more rapid and less laborious than the virus isolation by tissue culture technique and serotyping. However, the PCR technique is currently restricted to specialised laboratories only.

3. Serological tests

Because the seroprevalence of PEV-1 may exceed 60% in healthy pig populations in some countries of Central Europe, and identical clinical signs may be caused by other viruses – including other serotypes of PEV – a single serological test for PEV-1 giving positive results does not indicate that neurological signs observed are actually caused by PEV-1. A four-fold rise in titre together with typical signs should be considered to be an indication that PEV-1 infection caused clinical disease. Another reason that paired serum samples are needed for confirmation of the significance of titres is that cross-reactions have been reported with orphan enteroviruses.

Pigs that have recovered from disease, or those with inapparent disease, produce specific antibodies. Several serological methods are available for their detection, of which the microtitre VN test using pig kidney cell cultures is the most useful (8). An ELISA has been developed that is more sensitive and rapid (3).
For serological diagnosis it is necessary to have standard strains of PEV serotypes propagated in cell cultures and hyperimmune serum monospecific for PEV types.

- **Standard strain of porcine enteroviruses**

*Characteristics:* Following long experience, the strain 'Zabreh', isolated in Czechoslovakia during the period of peak incidence of the disease, was selected as the standard strain to generate the severe form of enterovirus encephalomyelitis. The pathogenicity of the strain is maintained by intracerebral passages in healthy, colostrum-deprived piglets. The virus produces typical signs of enterovirus encephalomyelitis after an incubation period of 5–7 days. For serological diagnosis, the following strains of PEV serotypes should be used as standard strains: type 1: Talfan, type 2: T80, type 3: O2b, type 4: PS36, type 5: F26, type 6: PS37, type 7: F43, type 8: V13, type 9: UKG/410/73, type 10: LP54/Eng/75, type 11: UKG/173/74, type 12: 2899/84, type 13: 460/88.

*Stock virus:* Standard strains are propagated on monolayers of cell culture either from primary porcine kidney or testes or on an established cell line, for example PK-15. A 10% suspension in PBS, pH 7.4, is prepared from the brain and spinal cord of piglets infected experimentally with PEV. Some types are isolated from faeces. The suspension is centrifuged and the supernatant is used for the inoculation of cell cultures. The procedure for the cultivation of PEV in cell cultures is as follows:

The growth medium is removed from the cell culture and after rinsing with buffered saline, cells are inoculated with the virus suspension at 37°C. The size of the inoculum should be equal to 10% of the growth medium. After 1 hour of incubation at 37°C, the inoculum is decanted, the culture vessel is rinsed with buffered saline, and the cells are overlayed with the appropriate volume of serum-free medium supplemented with antibiotics. CPE is apparent within 48 hours, and the monolayer disintegrates more or less completely during the next 48–72 hours. In the subsequent three to five passages in cell culture, the development of the CPE accelerates and the concentration of virions increases. Titration of the virus is performed in tube cultures or on microtitre plates. A cell-adapted strain usually reaches TCID\(_{50}\) (50% tissue culture infective dose) titres of \(10^6–10^7\) per ml.

The fluid harvest is checked for specificity using known specific hyperimmune antiserum. Treatment with 5% chloroform and cultivation in human and bovine cell cultures and chicken embryos, is used to exclude contamination with other viruses. PEV is chloroform resistant and multiplies only in cultures of swine origin. Immunofluorescent antibody staining is useful to detect possible contaminants that are also chloroform resistant and propagate on cells of swine origin (e.g. parvovirus), or that are noncytopathogenic. The stock virus should be dispensed into small aliquots and preserved at –60°C. Frozen virus retains its properties for several years. For stock virus that is to be used in the neutralisation test, a constant dose of 100 TCID\(_{50}\) is recommended.

- **Specific hyperimmune serum**

Specific hyperimmune serum is obtained by repeated immunisation of guinea-pigs, rabbits or colostrum-deprived piglets with PEV. Although the animals are selected from specific pathogen free breeds, they are nonetheless tested before immunisation for absence of antibodies against PEV. The standard strains should be used. Rabbits are immunised either intravenously, using virus suspension alone, or subcutaneously or intraperitoneally, using the virus suspension with 10% oil adjuvant. Good results may be obtained by administering three doses of 2 ml of virus suspension + 0.2 ml oil adjuvant, at intervals of 2 weeks. The rabbits are bled 10 days after the last immunisation. Piglets are immunised in the same way. The harvested sera are clarified by centrifugation and stored in small aliquots at –20°C. The sera are titrated using a neutralisation test and constant antigen. Only sera with an antibody titre of at least 1/256 can be used for the identification of the virus.

a) **Virus neutralisation test in microtitre plates**

The test is performed in flat-bottomed cell culture microtitre plates, using low passage porcine kidney or testes cells or cell lines derived from porcine cells. Stock virus is grown in cell monolayers. The virus harvested from cell cultures is clarified by centrifugation and stored in aliquots at –20°C. Culture medium, such as Eagle’s complete medium LYH (Hanks balanced salt solution with yeast extract, lactalbumin and antibiotics), is used as diluent.

- **Test procedure**
  i) Inactivate swine sera for 30 minutes at 56°C.
  ii) The sera to be tested are diluted in cell culture medium in twofold steps from 1/2 to 1/64, four wells per dilution and 50 µl volumes per well.
  iii) Controls include positive and negative sera, cells and medium control.
  iv) Add to each well 50 µl of virus stock previously diluted in culture medium to provide 100 TCID\(_{50}\).
  v) Incubate for 1 hour at 37°C with the plates covered. The residual virus stock is also incubated.
vi) Make back titrations of the residual virus stock in four tenfold dilution steps using 50 µl per well and four wells per dilution.

vii) Add 50 µl of porcine kidney cell suspension at 5 × 10^5 cells per ml.

viii) After further shaking, lids are put on and the plates are incubated at 37°C in a 5% CO₂ atmosphere for 2–3 days or longer, to a maximum of 8 days.

ix) Examine the plates microscopically for CPE. The test should be validated by checking the back titration of virus and titration of positive control serum. Virus should give a value of 100 TCID₅₀ with a permissible range of 30–300. The standard positive serum should give a titre within 0.3 log₁₀ units from its predetermined mean. A negative serum should give no neutralisation at the lowest dilution tested, i.e. 1/2.

x) The VN results are determined by the Spearman–Kärber method as the dilution of serum that neutralised the virus in 50% of the wells.

xi) Virus neutralisation titres are regarded as positive if the corresponding serum neutralises the virus at an initial serum dilution of 1/8 or higher.

b) Enzyme-linked immunosorbent assay

An alternative method for the detection and titration of specific antibodies against PEV is the ELISA technique (3). The test is performed in microtitre plates using PEV grown on cell cultures as antigen. The technique can be carried out using the following steps.

- **Antigen preparation**
  i) The stock virus is prepared in established cell lines as described in Section C below.
  ii) The harvested virus is clarified by centrifugation at 200 g for 15 minutes, and then precipitated with a final 50% saturated (NH₄)₂SO₄ for 120 minutes at 4°C.
  iii) After centrifugation at 2000 g, the resulting precipitate is suspended in TEN buffer (Tris-hydroxymethyl-methylamine [0.01 M], ethylene diamine tetra-acetic acid [1 mM] and NaCl [0.15 M]), pH 7.4, to 1/100 of the initial volume.
  iv) The concentrated viral suspension is extracted by shaking with freon 3/1 for 10 minutes at 4°C.
  v) Following further centrifugation, the supernatant is divided into two separate phases. The upper aqueous phase, containing the viral antigen, is desalinated by passage through a 2.5 ‰ 40 cm cylinder packed with sephadex G 25.
  vi) The viral solution is finally concentrated by ultracentrifugation at 160,000 g for 3 hours.
  vii) The pellet is suspended in TEN buffer, pH 7.4, in approximately 1000th the initial volume of virus.
  viii) Insoluble proteins are separated by light centrifugation, and the supernatant is used as the positive antigen in the ELISA.

- **Test procedure**
  i) Plates are sensitised with prediluted antigen in phosphate buffered saline (PBS), pH 7.2, by adding 100 µl to each well. The absorption of antigen to the surface of the plate takes place overnight at 4°C. Parallel rows of the plate should be treated with negative antigen.
  ii) The plate is washed five times in PBS to remove excess antigen.
  iii) Test sera are diluted 1/20 with PBST (PBS solution containing 0.05% Tween 20). 50 µl of the diluted sera is placed into each of two wells with positive antigen and into two wells with negative antigen (negative antigen is prepared as described above except that the tissue culture is not inoculated with virus and cells are disrupted by freezing). The plate is incubated for 1 hour at 37°C.
  iv) The plates are washed five times with PBST.
  v) A predetermined dilution of horseradish peroxidase conjugated with anti-swine immunoglobulin prepared in rabbits is added in 50 µl quantities to each well. The plates are incubated for a further 1 hour at room temperature.
  vi) The plates are washed five times in PBS.
  vii) Substrate solution (0.1% orthophenylendiamine with 0.03% hydrogen peroxide in PBS, pH 6.0) is added in 100 µl quantities to each well.
  viii) After the addition of substrate, positive samples change colour to dark brown. When a sufficient degree of colour reaction is seen in the wells of known positive sera, the reaction is stopped by
addition of 50 µl of 2 M sulphuric acid to each well. The absorbance of the wells is measured at a wavelength of 492 nm, preferably using an automatic multichannel spectrophotometer with print-out mechanism. Positive and negative sera and noninfected cells should be processed as controls in parallel with the test specimens.

ix) The absorbance of a serum is the mean reading of two wells with positive antigen minus the mean reading of two wells with negative antigen. Absorbance readings of test sera that exceed by more than twofold the mean reading of standard negative sera are regarded as positive.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

• Vaccines against enterovirus encephalomyelitis

During the period of highest incidence of the disease in central Europe and Madagascar, active immunoprophylaxis was an important means for the control of this infection (13). As severe clinical disease has disappeared, vaccination has been discontinued and the vaccine is no longer being produced or used anywhere in the world.

REFERENCES


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CHAPTER 2.6.4.

TRANSMISSIBLE GASTROENTERITIS

SUMMARY

Transmissible gastroenteritis (TGE) is an enteric disease of pigs caused by TGE virus (TGEV), a member of the Coronaviridae. Since 1984, a distinct respiratory variant (porcine respiratory coronavirus or PRCV) has spread throughout many parts of the world. This virus is probably a deletion mutant of TGEV. PRCV does not appear to be an important primary pathogen, but it has greatly complicated the diagnosis of TGE, particularly by serological means.

Laboratory diagnosis is made by demonstrating the presence of virus, viral antigens or viral nucleic acid in material from suspected cases, or by demonstrating virus-specific humoral antibodies.

Identification of the agent: Virus may be identified by virus isolation in tissue culture, electron microscopy, various immunodiagnostic assays, and more recently by specific detection of viral RNA. The most commonly employed rapid assays are probably the immunodiagnostic ones, particularly enzyme-linked immunosorbent assays (ELISAs) on faeces and fluorescent antibody tests on cryostat sections of intestine. Another enteric disease, porcine epidemic diarrhoea, is caused by a serologically distinct coronavirus that nevertheless has an identical appearance under the electron microscope. Diagnostically, immune electron microscopy circumvents this problem.

Serological tests: The most widely used methods are virus neutralisation tests and ELISAs. Only in the latter case is differentiation from PRCV possible, as TGEV and PRCV antibodies show complete cross-neutralisation.

Requirements for vaccines and diagnostic biologicals: There are no commercial biological products available internationally. However, several countries practise vaccination, and in the United States of America, licences have been issued authorising the production and distribution of monovalent and combined vaccines.

A. INTRODUCTION

Transmissible gastroenteritis (TGE) is an enteric disease of pigs caused by TGE virus (TGEV), a member of the Coronaviridae. Since 1984, a distinct respiratory variant (porcine respiratory coronavirus or PRCV) has spread throughout many parts of the world and is now found in most countries where surveys for it have been conducted, one exception being Oceania. Occurrences of TGE have become more sporadic. The disease is still reported on an occasional basis from parts of Europe, North America and Asia (30). TGEV multiplies in and damages the enterocytes lining the small intestine, producing villous atrophy and enteritis. Diarrhoea and vomiting occur in pigs of all ages; mortality is highest in neonates. Extra-intestinal sites of virus multiplication include the respiratory tract and mammary tissues (19), but the virus is most readily isolated from the intestinal tract and from faeces. By contrast, PRCV is most readily isolated from the upper respiratory tract, the tonsils or the lungs, and little if any enteric multiplication of virus occurs (9, 31, 38). PRCV is probably a deletion mutant of TGEV (40).

As TGE is a contagious disease that can occur as explosive epizootics, rapid diagnostic methods for its confirmation are particularly important. The disease can also take the form of a low-level endemic problem of post-weaning diarrhoea, which is more difficult to diagnose.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Virus may be identified by virus isolation in tissue culture (11), immunofluorescence, reversed passive hemagglutination, enzyme-linked immunosorbent assays (ELISAs), radioimmunoassay (RIA), hybridisation with
DNA probes, electron microscopy, and, more recently, by specific detection of viral RNA (13, 20, 36, 54, 63). Molecular techniques such as reverse-transcription polymerase chain reaction (RT-PCR) and nested RT-PCR developed in the past few years have increased the sensitivity and specificity of detection and differentiation of TGEV and PRCV directly from field samples (20, 21, 36). An alternative diagnostic method that has been recommended for laboratories lacking facilities for specialised tests is the oral dosing of susceptible piglets with suspect intestinal contents. However, laboratory tests are still required to confirm susceptibility of the pigs prior to inoculation and to show that any illness induced in these animals is due to TGE. The most commonly employed rapid assays are probably the immunodiagnostic ones, particularly enzyme-linked immunosorbent assays (ELISAs) on faeces (4, 24, 61) and fluorescent antibody tests (FAT) on cryostat sections of intestine (39). Detection of virus by reversed passive hemagglutination has also been described (1). Another enteric disease, porcine epidemic diarrhoea, is caused by a serologically distinct coronavirus that nevertheless has an identical appearance under the electron microscope. Diagnostically, immune electron microscopy circumvents this problem (43, 60).

**a) Virus isolation in tissue culture**

Apart from the inoculation of live piglets (11), this is the most definitive method of diagnosis. However, for routine use it is slow and laborious. TGEV does not grow well in cell culture, making this technique impractical as a routine diagnostic procedure.

Isolation is usually attempted from faeces or from carcass material, particularly the small intestine. Loops of affected small intestine, ligated at each end to retain the contents, are the preferred specimens. As the virus is heat labile, all samples should be fresh or chilled.

Sample material is homogenised in cell culture medium or phosphate buffered saline (PBS), pH 7.2, containing antibiotics, e.g. penicillin (1000 U/ml), dihydrostreptomycin (1000 µg/ml), and mycostatin (20 U/ml), to produce a 10% suspension. This is allowed to stand out of direct sunlight for 30 minutes at room temperature. The suspension is then sonicated and clarified by low-speed centrifugation. The supernatant fluid may be mixed with an equal volume of heat-inactivated bovine serum in order to reduce the cytotoxic effect of the material and it is then used to inoculate susceptible tissue cultures, such as 3–4-day-old primary or secondary pig kidney monolayers. Other low passage porcine cultures (such as thyroid or testis) and some cell lines (16, 26) may also be used for primary virus isolation. After incubation at 37°C for 1 hour, the cell sheets are overlaid with a medium, such as Earle’s yeast lactalbumin (EYL) balanced salt solution, containing sodium bicarbonate and antibiotics, e.g. penicillin (100 U/ml), dihydrostreptomycin (100 µg/ml), mycostatin (20 U/ml), and 1% fetal calf serum. Incorporation of trypsin into the culture medium may enhance primary viral recovery (5, 16). Uninoculated control cultures are established concurrently and all cultures are incubated at 37°C.

Viral cytopathic effect (CPE) may be observed after 3–7 days, characterised by cells rounding, enlarging, forming syncytia and detaching into the medium. Plaque formation is sometimes more reliable and easier to recognise. A suitable plaquing overlay is 1.6% noble agar in 2 × minimal essential medium with 1% NaCO₃, antibiotics (as above), 0.7% neutral red and 1% DEAE (diethylaminoethyl) (100 µg/ml). Wild-type TGEV does not grow readily in tissue culture, so several subpassages may be necessary before these distinctive changes become apparent. Cytopathic isolates must be confirmed as TGEV by immunostaining or by in-vitro neutralisation tests using appropriate TGEV-specific antisera (5). If suitable monoclonal antibodies (MAbs) are available they can be used to distinguish between TGEV and PRCV by immunostaining methods (14, 53). Differentiation of TGEV from PRCV can also be accomplished by TGEV-specific cDNA probes (2) or by discriminatory RT-PCR or nested RT-PCR (20, 21, 36).

**b) Fluorescent antibody test for viral antigens**

This is a rapid, sensitive and specific means of identifying TGE viral antigens in cryostat sections of intestine. A freshly dead pig is required, and the ideal animal should be under 4 weeks of age (preferably less than 1 week) and just starting to show clinical signs of the disease (that is, within 24–28 hours of infection). Within 30 minutes of death, 2 cm lengths from four different regions of the posterior part of the small intestine should be removed. Lengths of 5–10 mm are cut from these for snap freezing with solid CO₂. Correct orientation of the material is important to ensure that subsequent cutting by cryostat yields true transverse sections. Sections are cut 6 µm thick, mounted on cover-slips, air-dried and fixed in acetone. Fixed positive and negative control sections are stored at −20°C for staining in parallel. After washing with Tris buffer, pH 8.7, or PBS, the sections are stained with a diluted solution of fluorescein isothiocyanate (FITC)-conjugated TGEV antibody, and placed in a humid incubator at 37°C for 30 minutes. Any unbound stain is removed by washing in Tris buffer. If desired, the sections are counterstained with a 10⁻² dilution of Evans blue in Tris buffer and mounted in glycerol.

Stained sections should be examined by ultraviolet light microscopy as soon as possible. The quality of the staining is assessed by reference to the controls. An accurate interpretation depends on the preservation of the villous architecture, the epithelial cells of which are examined for intracytoplasmic fluorescence.
A peroxidase–antiperoxidase method for the demonstration of TGEV has been developed recently for detection of TGEV and PRCV in both frozen and formalin-fixed, and paraffin-embedded tissues (18, 52).

c) Enzyme-linked immunosorbent assay detection of faecal virus antigens

A double antibody-sandwich system may be used, for instance with a capture MAb and a polyclonal enzyme-linked detector antibody (24, 47). This test is based in the capture of the viral antigen from the faecal sample by three MAb, two specific for the S protein (site A and D) and one for the nucleoprotein N (24, 47). A negative coating is used as control for the specificity of the test, consisting of antibodies purified from the ascitic fluid of mice inoculated with SP2/0 myeloma cells that do not recognise TGEV. MAbs are applied to 96-well microplates in a bicarbonate buffer, pH 9.6, and incubated overnight at 37°C. All samples are tested in duplicate wells, one containing positive coating (TGEV MAbs) and one containing the negative coating. Faecal samples are diluted in cell culture medium (1/10), vortexed and centrifuged at low speed (2000 g) for 15 minutes. Then the supernatant is decanted into sterile tubes and tested or stored frozen. Plates are washed twice with washing buffer (PBS containing 0.05% Tween 20) before adding the prepared faecal samples. The plates are incubated overnight at 37°C. After washing four times, a biotinylated polyclonal anti-TGEV serum is added in PBS buffer containing 0.05% Tween 20. The plates are incubated at 37°C for 1 hour. The plates are washed four times before adding a horseradish peroxidase-labelled streptavidin conjugate and incubated at 37°C for 1 hour. The plates are washed six times before adding the enzyme substrate, which is ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) with 0.03% H₂O₂ in 0.1 M citrate buffer, pH 4.2. The reaction is stopped after 30 minutes at room temperature by the addition of 5% sodium dodecyl sulphate and the absorbance determined in an ELISA reader at 405 nm. TGEV negative and positive faecal samples are included on each plate.

d) Nucleic acid recognition methods

In-situ hybridisation (ISH) and RT-PCR methods have been described for the direct detection of TGEV in clinical samples, with differentiation from PRCV (20, 36, 54). A second round of nested PCR may significantly enhance the sensitivity (20, 21, 35). Differentiation between TGE viruses may be achieved by analysing PCR products with restriction endonuclease enzymes (63) or by sequencing (27, 37). Duplex RT-PCR for the combined detection of TGEV and porcine epidemic diarrhoea virus has been described (22).

2. Serological tests

Serology may be diagnostic if a rising titre of antibody can be demonstrated. In addition, a single seropositive result has diagnostic value if collected from a population previously known to be seronegative. As the possibility of acquiring carrier virus status among pigs can be reduced by accepting only seronegative animals, serological testing is also a common precondition for importation.

Following infection with TGEV or PRCV, viral antibodies can be detected in serum from 6 or 7 days post-infection, and such antibodies persist at least for many months. Although PRCV and TGEV antibodies show complete neutralisation of either virus, there are differences in the specificities of some of the non-neutralising antibodies (7, 14, 53), as PRCV lacks certain epitopes present on the TGEV. However, virus neutralisation (VN) is not a practical method to differentiate PRCV from TGEV infection. MAbs to such regions can be incorporated into competitive ELISAs to detect serum antibody that is entirely TGEV specific. While such tests are reliable in that they do not produce false-positive results with PRCV antisera, false negatives may occur because of a reduced sensitivity compared with neutralisation tests, and because of strain variation among TGE viruses, such that no single TGEV-specific MAb will recognise all strains (6, 53). The problem of insensitivity can be reduced by using the tests on a group or herd basis. These MAB-based ELISAs are the method of choice for differentiating PRCV from TGEV to qualify animals for export.

In addition, using such test for differential diagnosis before 3 weeks after exposure to PRCV produced inconsistent and unreliable results (49). More accurate results were also achieved by testing paired serum samples (acute and convalescent) in the assays and by using the recombinant spike (S) protein of TGEV as the coating antigen in place of TGEV-infected, fixed swine testicular cells (49).

a) Transmissible gastroenteritis virus/porcine respiratory coronavirus tests

These tests detect antibody to both TGEV and PRCV, and include VN tests, indirect ELISAs (15, 17, 25, 28, 41) and competitive ELISAs based on TGEV/PRCV group-specific MAbs (34).

VN tests can be performed with a variety of cell types and viral strains. Commonly used cell lines include swine testes (26) or primary or continuous porcine kidney cells. Such tests have been very widely used for many years and are commonly regarded as standards against which to assess new assays. A modification of the method of Witte (62) uses flat-bottomed tissue-culture grade microtitre plates, a cell line of A72 cells derived from a dog rectal tumour, and a field strain of virus adapted to grow in such cells: 100 TCID₅₀ (50% tissue culture infective dose) of virus is incubated with heat-inactivated test sera, and neutralisation is indicated by absence of CPE after further incubation with A72 cells in Leibovitz 15 medium (Sigma, United
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Kingdom) with added antibiotics, 10% fetal calf serum and 1% L-glutamine. The total volume of reagents in all wells should be 150 µl.

- **Virus neutralisation: test procedure**
  1. Sera are inactivated for 30 minutes in a water bath at 56°C.
  2. Doubling dilutions of test sera are made in cell culture medium beginning with undiluted serum (this gives a neutralisation stage dilution of 1/2 when mixed with an equal volume of virus). The dilutions are prepared in a 96-well flat-bottomed cell-culture grade microtitre plate using, optimally, three 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.
  3. 25 µl TGEV stock is added to each well at a dilution in culture medium calculated to provide 100 TCID$_{50}$ per well. Virus should be added to two out of the three wells containing serum at each dilution. The third well serves as a serum-only control and should receive 25 µl per well of culture medium instead of virus.
  4. The residual virus is back titrated in four tenfold steps using 25 µl per well and at least four wells per dilution; 25 µl of culture medium is added to each of the back-titration wells to compensate for the absence of a test serum.
  5. The plates are agitated briefly and then incubated for 1 hour in a 5% CO$_2$ atmosphere at 37°C.
  6. 100 µl of, for example, A72 cell suspension at 2 x 10$^5$ cells per ml is added to each well.
  7. The plates are incubated for 3–7 days in a 5% CO$_2$ atmosphere at 37°C; the test can be performed successfully, if the plates are incubated without CO$_2$.
  8. The plates are read microscopically for CPE. The test is validated by checking the back titration of virus (which should give a value of 100 TCID$_{50}$ with a permissible range of 50–200 TCID$_{50}$) and the control sera. The standard positive serum should give a value within 0.3 log$_{10}$ units either side of its predetermined mean. Readings of each test serum dilution should be made with reference to the appropriate serum-only control to distinguish viral CPE from serum-induced cytotoxicity or contamination.
  9. The test serum results are determined by the Spearman–Kärber method as the dilution of serum that neutralised the virus in 50% of wells.
  10. A negative serum should give no neutralisation at the lowest dilution tested (i.e. undiluted serum, equivalent to a dilution of 1/2 at the neutralisation stage).

b) Transmissible gastroenteritis virus-specific tests

TGEV-specific tests are ELISAs that use an MAb that recognises TGEV but not PRCV (6, 8, 49, 53, 59) and are the tests of choice for qualifying animals for export. Test sera from pigs previously infected with a strain of TGEV recognised by the MAb will contain antibodies of the same specificity that can compete with it for binding to TGEV antigen-coated ELISA plates. ELISA antigens may be prepared from cell lysates of kidney cell lines that were either inoculated with tissue-culture-adapted strains of TGEV, or uninfected. Alternatively TGEV-infected or uninfected swine testes cells fixed in 80% acetone have been used as an antigen source, or antigens may be prepared from recombinant S (rec-S) protein harvested in soluble form from an insect (SF9) cell line infected with a recombinant baculovirus expressing a TGEV S protein containing the four major antigenic sites (49, 53). Positive and negative antigens are coated to alternate rows of microtitre plates using bicarbonate buffer, pH 9.6. Diluted test sera, including positive and negative controls, are added to appropriate wells and incubated overnight before further addition of diluted MAb to all wells. Bound MAb is detected by a peroxidase-conjugated anti-mouse antibody that induces a colour reaction in the presence of an appropriate substrate. The colour changes are measured using spectrophotometer, and for each test sample the net result is the difference in absorbance between the positive and negative antigen wells, expressed as a percentage of the result obtained with the negative control serum. The negative—positive cut-off value for the test must be determined by previous testing of known negative and positive populations. There are a number of commercial kits available, many are TGEV specific.

Haemagglutination-based tests described to date (23, 29, 50) were validated before the appearance of PRCV. However, they may be TGEV specific as TGEV, but not PRCV, is haemagglutinating (46).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Vaccination against TGE is carried out in several countries.
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.

Information on experimental work or field trials of TGEV vaccines licensed for use in the United States of America (USA) has been reviewed, including possible limitations in their field efficacy and concepts related to the design of optimal TGEV vaccines (42, 44, 45). Several manufacturers are licensed to produce TGEV vaccines in the USA: the vaccines include modified live and inactivated vaccines. The modified live vaccines are used for oral administration to pregnant sows (to induce passive immunity) or have also been licensed for oral administration to nursing or weaned pigs (to induce active immunity). Inactivated TGEV vaccines are licensed for parenteral inoculation of pregnant sows by the intramuscular route or for intraperitoneal administration to nursing or weaned pigs. In general, these vaccines induced marginal passive protective against TGEV challenge of nursing piglets when evaluated under controlled experimental conditions or in the field. Although they fail to adequately protect against epizootic TGE, data suggest that these vaccines may provide some efficacy against enzootic TGEV by stimulating an anamnestic antibody response to TGEV in serum and milk (44, 45).

The main reason proposed for TGEV vaccine failures was their inability to stimulate high levels of secretory IgA (SIgA) antibodies in milk analogous to the SIgA antibody responses found in the milk of sows naturally infected with TGEV (44, 45). Furthermore, these vaccines did not adequately protect the sow against TGE, such that illness in the sow often resulted in anorexia, agalactica and failure to passively protect her piglets. Thus the modified live vaccines may fail to replicate to the extent required to induce protective immunity in the intestine, or if given to seronegative neonatal animals, concerns exist regarding their possible reversion to virulence. Killed vaccines given parenterally do not induce SlgA antibodies; cell-mediated immune responses are often poor and the duration of immunity may be short-lived. Although use of PRCV strains as vaccine candidates for TGE has been proposed, experimental studies regarding their efficacy against TGEV have shown a lack of efficacy (33) or only partial cross-protection (3, 10, 58). However, the widespread prevalence of PRCV infections in the swine population in Europe appears to have dramatically reduced the incidence of epizootic TGE in Europe (38). Newer recombinant DNA strategies for the development of TGEV vaccines include the possible use of an S protein subunit vaccine (contingent upon the development of mucosal delivery systems and adjuvants) (32, 48, 51) or the use of live recombinant viral or bacterial vectors that express TGEV genes important for the induction of immunity (12, 42, 44, 45, 55, 56, 64).

There are a number of general requirements (e.g. produced in a licensed facility, label rules, tracking capability, etc.) that apply to all biological products including vaccines. A set of regulations exist (called standard requirements, or SRs) that describe testing to be done on the vaccine and parent materials. Detailed information on SRs for vaccines in the USA are contained in the Code of Federal Regulations (CFR) Title 9, Volume 1, Part 113 (abbreviated below as 9 CFR, 113) (57). The European Pharmacopoeia also contains information. Unfortunately, there is no specific standard requirement for TGEV vaccines.

1. Seed management

a) Characteristics of the seed

The seed virus must be tested for purity and identity. The purity includes freedom from bacteria and fungi (9 CFR 113:27), mycoplasmas (9 CFR 113:28), and extraneous viruses (9 CFR 113:55) (57). The demonstration of identity is usually accomplished by VN or FAT. Genetically engineered vaccines or naturally selected vaccines with claims of antigen-coding gene deletion/inactivation are required to provide evidence (genotypic and/or phenotypic) of that identity.

b) Method of culture

Culture must be carried out on proven uncontaminated (approved) cells, and the number of cell culture passages is limited (usually to five). It is not required that the species of origin of the cell line be that of the target species.

c) Validation as a vaccine

Vaccine validation takes two forms. The master seed is considered to be immunogenic if a vaccine made at the highest passage, and according to the outline of production, is shown to be protective. The lowest antigenic level (modified live virus titre or inactivated antigen mass) shown to be protective becomes the baseline for all future serials (lots) of the product. In the case of live products, factors for titration variation and the death curve over time would be added. These trial vaccines should be tested for purity, safety, and efficacy by the manufacturer. Protection must be shown against the natural disease with the virulent challenge virus. Virulent challenge virus is defined as the dose that causes disease in 95% of the susceptible controls. Three prelicense serials must subsequently be made and tested by the manufacturer and by the licensing authority, for potency, sterility and safety.
2. Method of manufacture

This is proprietary information for each manufacturer and hence not available.

3. In-process control

This is largely proprietary. Some in-process controls refer directly to production (e.g. O$_2$ concentration in the fermenter). Another category, however, includes tests similar to the final container potency test. For all vaccines, the simpler the final batch or container potency test, the more likely it is that it may be used as a monitoring/blending test: for example, virus titration on sub-batches may be used to predict final blended batch titre. Ingredients of animal origin must be sterilised or shown to be free from contamination.

4. Batch control

Batches must be blended to the final specifications and bottling specifications (e.g. fermentation runs may be pooled, or one run may be split and pooled with each of three others, etc.). In some countries, bulk and process control define the product and are the subject of intense regulation and scrutiny. The emphasis in the USA is on the final product. Batch control techniques must be detailed in the outline of production and must be meaningful, trackable, and the manufacturer must discard product that fails to meet specifications. If a batch is to be exported to another country for bottling or blending, then it is subjected to all the testing as though it were final product.

a) Sterility

All products must be tested for sterility. The manufacturer may also run sterility tests on batches for monitoring. Tests are similar to those described in Section C.1.a.

b) Safety

Safety tests are done before the licence is granted, and then on the final container (Sections C.5.a. and C.5.b.).

c) Potency

Potency would normally only be done if the potency test were a simple test (e.g. ELISA) to confirm the blending calculations before bottling.

d) Duration of immunity

Duration of immunity is tested in the prelicence (efficacy) serial test, not the batch control. New products are required to support label claims for revaccination schedules with efficacy trials (challenge) at the specified time after vaccination.

e) Stability

Stability is established before the licence is granted. Usually accelerated ageing (37°C) is used to estimate the lifetime so that the products do not have to be kept at storage temperature (4°C) for the real-time period. This can be adjusted with real-time data later. The manufacturer is not required to do stability testing. Manufacturers are required to state the amount of antigenic material that will be in their product throughout the shelf life. Samples of product are selected (usually live) and tested within 30 days of expiration to see if, for example, the titre is at the level stated in the manufacturer’s outline. Stability is also affected by moisture. Moisture left in a desiccated product can shorten its life, so this has to be tested in the final product or in-process.

f) Preservatives

There are restrictions on the maximum amounts of antibiotics that can be in a product. Restrictions on some vaccine components are related to their safety and to whether the stated withdrawal period is long enough for the component to have cleared before the animal is slaughtered. Preservatives used are proprietary.

g) Precautions (hazards)

Any risks to vaccinates need to be clearly stated on the label. This usually applies to pregnancy warnings for abortogenic live viruses, and the general anaphylaxis warning, but may also attempt to warn the user about soreness or swelling at the injection site, or transient fever or inappetence in some cases. No unusual label precautions apply to the TGE vaccines currently licensed.
5. Tests on the final product

a) Safety

Usually this will be a mouse and/or a guinea-pig or swine safety test (9 CFR 113:33, and ref. 62). Sterility tests are also carried out on the final product.

b) Potency

There is no single test for release potency. Whatever test is used must be correlated to protection in the host animal (the efficacy tests). The potency of live TGEV vaccines can be evaluated by in-vitro titration of the viral infectious dose in cell culture (42). This titre must be correlated with the minimum viral titre required to induce protective immunity against experimental challenge, and also against natural challenge under field conditions. The potency of killed vaccines is evaluated by vaccination and challenge tests using different doses of the vaccine. Titres of neutralising antibodies induced by inoculation of laboratory animals with the vaccine may be accepted if there is an established correlation with development of protective immunity.

Particular viral antigens associated with the induction of neutralising antibodies and protection against challenge can be quantified in killed vaccines using specific MAbs in ELISA, such as neutralising MAbs to the S protein of TGEV (42).

REFERENCES


Chapter 2.6.4. — Transmissible gastroenteritis


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NB: There is an OIE Reference Laboratory for Transmissible gastroenteritis (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 2.6.5.

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME

SUMMARY

Porcine reproductive and respiratory syndrome (PRRS) is characterised by reproductive failure of sows and respiratory problems of piglets and growing pigs. The disease is caused by the PRRS virus, a virus currently classified as a member of the newly established order of Nidovirales, family Arteriviridae, genus Arterivirus. The primary target cell of the virus is the alveolar macrophage of the pig. Two major antigenic types of the virus exist, the European and the American type.

PRRS occurs in most major pig-producing areas throughout the world. The reproductive failure is characterised by infertility, late fetal mummification, abortions, stillbirths, and the birth of weak piglets that often die soon after birth from respiratory disease and secondary infections. Older pigs may demonstrate mild signs of respiratory disease, sometimes complicated by secondary infections. Animals other than pigs do not seem to be affected by PRRS.

Identification of the agent:
Virological diagnosis of PRRS virus infection is difficult; the virus can be isolated from tissues such as serum, ascitic fluids, or organ samples, such as lungs, tonsil, lymph nodes and spleen of affected pigs. As porcine alveolar macrophages are the most susceptible culture system for virus of both antigenic types, these cells are recommended for virus isolation. MARC-145 (MA-104 clone) cells are also suitable. There is variability between batches of macrophages in their susceptibility to PRRS virus. Thus, it is necessary to identify a batch with high susceptibility, and maintain this stock under liquid nitrogen until required. The virus is identified and characterised by immunostaining with specific antisera. Additional techniques, such as immunohistochemistry and in situ hybridisation on fixed tissues and reverse-transcription polymerase chain reaction, have been developed for laboratory confirmation of PRRS virus infection.

Serological tests:
A wide range of serological tests is currently available for the detection of serum antibodies to PRRS virus. The immunoperoxidase monolayer assay uses alveolar macrophages and the indirect immunofluorescence assay uses MARC-145 cells that are usually infected using either the European or the American antigenic type of the virus, respectively. Both assays can however be designed with both PRRS virus types. Commercial or in-house enzyme linked immunosorbent assays (ELISA) are now often used. One commercial ELISA is specific for both the European and American types of the virus. An indirect ELISA, a blocking ELISA and a double ELISA that can distinguish between serological reactions to the European and the American types have been described.

Requirements for vaccines and diagnostic biologicals:
Vaccines can be of value as an aid in the prevention of reproductive and respiratory forms of PRRS. Modified live vaccines are not suitable for use in pregnant sows and gilts and in boars. Vaccination may result in shedding of vaccinal virus in semen. Modified live virus vaccines can persist in vaccinated animals, and potential transmission to nonvaccinated animals and subsequent vaccine-virus-induced disease have been reported.

A. INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is characterised by reproductive failure of sows and respiratory distress of piglets and growing pigs (3, 17, 37) and is a significant cause of economic loss. The disease was first recognised in 1987 in the United States of America (USA), where it was soon known as mystery
Chapter 2.6.5. — Porcine reproductive and respiratory syndrome

swine disease because of the elusive nature of its causal agent, but later was called swine infertility and respiratory syndrome. During the winter of 1990–1991 the disease emerged in Western Europe, where it spread rapidly and acquired many more names, including Seuchenhafter Spätabort der Schweine, Abortus blauw, blue-ear pig disease, syndrome dysgénésique et respiratoire du porc, and porcine epidemic abortion and respiratory syndrome (10, 13, 48). Throughout this chapter, the name PRRS will be used for the disease; this is the name most generally accepted by the international veterinary community.

The PRRS virus (PRRSV), the causative agent of PRRS, is currently classified as a member of the order Nidovirales, family Arteriviridae, genus Arterivirus (7). It has a predilection to grow in porcine alveolar macrophages, both in vivo and in vitro. PRRSV is a 50–70 nm enveloped positive-strand RNA virus with a genome of approximately 15 kb long and encodes eight open reading frames (ORF). Three major structural proteins have been identified: a nucleocapsid protein (N; ORF 7) of 14–15 kDa, a membrane protein (M; ORF 6) of 18–19 kDa, and an envelope glycoprotein (E; ORF 5) of 24–25 kDa. Three other less abundant structural glycoproteins are encoded by ORFs 4, 3, and 2 (38). Monoclonal antibodies have been prepared against these major and minor structural proteins (15, 19, 32, 35, 43, 46, 50). The European strains of the virus are antigenically closely related to each other, but distinct from American strains of the virus (4, 47). The American strains in themselves are also antigenically closely related. The presence of PRRS has been reported from various countries in Asia and a few countries in South America. Australia, New Zealand, Sweden and Switzerland are reported to be free from PRRSV infection.

Detailed clinical manifestations and lesions resulting from PRRSV infection of pigs of different age groups have been reviewed (3, 17, 37). Briefly, PRRSV-infected neonatal pigs show dyspnoea (thumping), but also a variety of other signs such as conjunctivitis, fever, rough hair coat, anorexia, diarrhoea, shaking, cutaneous erythema, eyelid oedema and mortality, which may be high. In weaned and grower pigs, fever, pneumonia, failure to thrive and an increase in mortality from concurrent bacterial infections are observed. Subclinical infections are more common in finishing pigs, boars and unbred replacement gilts and sows. Transient fever and inappetence may be common. In PRRSV-infected boars and boars that have been vaccinated with live attenuated vaccine, PRRSV can be shed in semen, and changes in sperm morphology and function have been described. Mortality associated with PRRSV infection of adult swine has been reported. More recently a virulent form of PRRS has been described: sow abortion and mortality syndrome (SAMS). The reproductive disease is fairly well understood. There is increasing evidence that infections earlier in gestation may cause reproductive failure or repeat breeding problems. Limited evidence is available for the existence of various isolates with different reproductive virulence; some countries report the existence of strains that cause transplacental infection without detrimental effect to the fetuses.

The importance of the respiratory infection is less well understood. It has been difficult to constantly reproduce significant respiratory disease with the virus alone and the increase in susceptibility to bacterial infection attributed to PRRSV infection has also been difficult to reproduce experimentally in pigs. Some studies have reported differences in severity of clinical signs and in gross and microscopic lesions following experimental inoculation of pigs with different PRRSV isolates (22, 23). Such a predisposition by PRRSV to exacerbate other diseases is currently a topic of research by various groups, and although it seems that the virus may indeed cause some secondary infections to be more severe, the mechanisms are not yet fully understood.

Gross and microscopic lesions consistent with PRRSV infection are mostly observed in neonatal and nursery pigs. In older pigs, lesions may be similar but less marked. Gross lesions associated with PRRSV infection of adult swine have been reviewed (3, 17, 37). Briefly, PRRSV-infected neonatal pigs show dyspnoea (thumping), but also a variety of other signs such as conjunctivitis, fever, rough hair coat, anorexia, diarrhoea, shaking, cutaneous erythema, eyelid oedema and mortality, which may be high. In weaned and grower pigs, fever, pneumonia, failure to thrive and an increase in mortality from concurrent bacterial infections are observed. Subclinical infections are more common in finishing pigs, boars and unbred replacement gilts and sows. Transient fever and inappetence may be common. In PRRSV-infected boars and boars that have been vaccinated with live attenuated vaccine, PRRSV can be shed in semen, and changes in sperm morphology and function have been described. Mortality associated with PRRSV infection of adult swine has been reported. More recently a virulent form of PRRS has been described: sow abortion and mortality syndrome (SAMS). The reproductive disease is fairly well understood. There is increasing evidence that infections earlier in gestation may cause reproductive failure or repeat breeding problems. Limited evidence is available for the existence of various isolates with different reproductive virulence; some countries report the existence of strains that cause transplacental infection without detrimental effect to the fetuses.

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Gross and microscopic lesions consistent with PRRSV infection are mostly observed in neonatal and nursery pigs. In older pigs, lesions may be similar but less marked. Gross lesions associated with PRRSV vary. Lung lesions vary from none to diffuse consolidation, and are commonly complicated by concurrent bacterial infections. Affected lymph nodes, most commonly in young pigs, can be markedly enlarged. Microscopic lesions, rather nonspecific, most commonly involve lung and lymphoid tissue. Lung lesions are characterised by multifocal interstitial pneumonia showing alveolar septal infiltration by mononuclear cells, type 2 pneumocyte hypertrophy and hyperplasia, and marked accumulation of inflammatory and necrotic alveolar exudate. Lymph nodes demonstrate follicular hyperplasia, foci of follicular necrosis and debris within follicles. Vascular, heart and brain lesions have also been described. Inconsistently observed fetal lesions are characterised by vasculitis, myocarditis and encephalitis. It is important to note that PRRSV infection is but one infectious agent that has been associated with interstitial pneumonia in pigs. Post-weaning multisystemic wasting syndrome, associated with porcine circovirus type 2 infection, is now commonly related to interstitial pneumonia and lymphadenitis in pigs.

In general, antibodies seem to have limited protective value. Infected pigs can remain viraemic for 4–6 weeks after infection, and can transmit the virus to other pigs. It is not known whether maternal antibodies can protect against early infection, but viraemia in pigs born from seropositive females can be detected from week 4 onwards. However, some level of protection is seen in piglets with lower, some antibody, and a rise in neutralising antibody titres often corresponds to a decline in virus titres in the blood. Furthermore, females that are infected for a second time do not show recurring reproductive failure. In short, the relationship between antibody titres
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and protection is not very well understood. Cell-mediated immunity has not been widely studied, but is thought to play a protective role. PRRSV infection, however, appears to result in a weak adaptive cellular immune response. An intriguing aspect of PPRSV infection is the prolonged duration of viraemia and subsequent transmission of the virus to contact animals in comparison with other viral infections. Virus is eventually cleared from the circulation, but a persistent infection is maintained in lymphoid tissues. However, it is generally agreed that lifelong persistence of the infection does not seem to occur.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Virological diagnosis of PRRS is difficult. This is mainly because the cell of choice for virus isolation is the porcine alveolar macrophage, which needs to be harvested from pigs (preferably specific pathogen free [SPF]) under 6–8 weeks of age (48, 51). Not all laboratories have a ready supply of such pigs available, and continuous cell lines cannot fully replace the alveolar macrophages because these cell lines are generally less susceptible to the virus. In addition, different batches of macrophages are not always equally susceptible to the virus; the reason for this is not yet known, but it necessitates the testing of each batch before use.

Certain monkey kidney cell lines (e.g. MA-104) can be a good replacement for macrophages (2, 26), but such cell lines do not support the growth of all isolates, particularly European strains. This chapter therefore only describes virus isolation with alveolar macrophages. Immunohistochemistry and immunofluorescence to detect PRRSV antigen in tissues have been reported. These tests are more rapid than virus isolation and do not necessitate cell culture infrastructure. In addition, immunohistochemistry (21, 28) performed on formalin-fixed tissues enables the visualisation of antigen together with histological lesions and permits retrospective analysis on archival specimens. In situ hybridisation capable of detecting and differentiating North American and European PRRSV genotypes in formalin-fixed tissues has also been reported (29, 41). Reverse-transcription polymerase chain reaction (RT-PCR) and nested PCR are highly sensitive tests for detecting viral RNA (12, 27, 30, 33, 45) and are now more commonly used on different tissues including serum. These tests are also useful when virus isolation is problematic, such as when testing semen (12) and when testing tissues partially degraded by autolysis or by heat during transport of specimens for virus isolation. A multiplex PCR assay has been designed to differentiate North American and European PRRSV isolates (20). Restriction fragment length polymorphism analysis of PCR-amplified products has been developed for the differentiation of field and vaccine PRRSV isolates (49) and recently molecular epidemiological studies of PRRSV strains have been performed using phylogenetic analyses of specific structural gene sequences.

a) Harvesting of alveolar macrophages from lungs

Lungs should preferably be obtained from SPF pigs or from a herd of pigs that is proven to be free from PRRSV infection. Best results are obtained with pigs that are under 8 weeks of age. The macrophages should be harvested from the lung on the same day that the pig is slaughtered. The lungs should be washed three or four times with a total volume of approximately 200 ml sterile phosphate buffered saline (PBS). The harvested wash fluid is then centrifuged for 10 minutes at 1000 g. The resulting pellet of macrophages is resuspended in PBS and centrifuged (washed) twice more. The final pellet is resuspended in 50 ml PBS, and the number of macrophages is counted to determine the cell concentration. The macrophages can then be used fresh, or can be stored in liquid nitrogen according to standard procedures at a final concentration of approximately $4 \times 10^7$ macrophages/1.5 ml. Macrophage batches should not be mixed.

b) Batch testing of alveolar macrophages

Before a batch of macrophages can be used it should be validated. This should be done by titrating a standard PRRSV with known titre in the new macrophages, and by performing an immunoperoxidase monolayer assay (IPMA) with known positive and negative sera on plates seeded with the new macrophages. The cells are suitable for use only if the standard PRRSV grows to its specified titre, (TCID50 or 50% tissue culture infective dose). It is recommended that alveolar macrophages and fetal bovine serum (FBS) to supplement culture medium be pestivirus free.

c) Virus isolation on alveolar macrophages

Alveolar macrophages are seeded in the wells of flat-bottomed tissue-culture grade microtitre plates. After attachment, the macrophages are infected with the sample. Samples can be sera or ascitic fluids, or 10% suspensions of tissues, such as tonsils, lung, lymph nodes, and spleen. In general, the PRRSV gives a cytopathic effect (CPE) in macrophages after 1–2 days of culture, but sometimes viruses are found that give little CPE or give a CPE only after repeat passage. Once a CPE has been observed, the PRRSV needs to be identified by immunostaining with a specific antiserum.
i) **Seeding macrophages in the microtitre plates**

Defrost one vial containing 6 × 10⁷ macrophages/1.5 ml. Wash the cells once with 50 ml PBS and centrifuge the cell suspension for 10 minutes at 300 g (room temperature). Collect the cells in 40 ml RPMI (Rose-Peake Memorial Institute) 1640 medium supplemented with 5% FBS and 10% antibiotic mixture (growth medium). Dispense 100 µl of the cell suspension into each well of a microtitre plate (with one vial of cells, four plates can be seeded at a concentration of 10⁵ cells in each well of the plates).

ii) **Preparation of sample (serum, ascitic fluid, 10% tissue suspension) dilutions in a dummy plate**

Dispense 90 µl of growth medium into each well of a microtitre plate. Add 10 µl samples to the wells of rows A and E (duplicate 1/10 dilution). Shake the plates and transfer 10 µl from rows A and E to rows B and F (1/100 dilution). Shake the plates and transfer 10 µl from rows B and F to rows C and G (1/1000 dilution). Shake the plates and transfer 10 µl from rows C and G to rows D and H (1/10,000 dilution). Shake the plates.

iii) **Incubation of samples**

Transfer 50 µl of the sample dilutions from the dummy plates to the corresponding wells of the plate with macrophages (first passage). Incubate for 2–5 days and observe daily for a CPE. At day 2, seed macrophages in new microtitre plates (see above). Transfer 25 µl of the supernatants from the plates of the first passage to the corresponding wells of the freshly seeded plates (second passage). Incubate for 2–5 days and observe daily for a CPE.

iv) **Reading and interpreting the results**

Wells in which macrophages show CPE in the first passage only are considered to be false positive because of the toxicity of the sample. Wells in which macrophages show CPE in both passages or in the second passage only are considered to be suspect positive. All wells with macrophage monolayers that do not show CPE need to be identified as PRRSV negative by immunostaining with a PRRSV-positive antiserum. CPE-positive samples need to be identified as PRRSV positive by culturing CPE-positive supernatant samples, or the original sample dilutions, for both 24 and 48 hours in macrophages, followed by immunostaining with a PRRSV-positive antiserum.

v) **Immunostaining with a PRRSV-positive antiserum**

Infect macrophages with 50 µl of supernatant or tissue sample as described in Section B.2.a., and grow the infected cells for 24 and 48 hours. Prepare an appropriate dilution of a PRRSV-positive serum in dilution buffer, and immunostain the macrophages as described in Section B.2.a. or B.2.b.

2. **Serological tests**

A variety of assays for the detection of serum antibodies to PRRSV have been described. Serological diagnosis is, in general, easy to perform, with good specificity and sensitivity, especially on a herd basis. Sera of individual pigs sometimes cause difficulties because of nonspecific reactions, but this problem may be solved by resampling the pig after 2–3 weeks. Serology is generally performed with a binding assay, such as the IPMA, immunofluorescence assay, or the enzyme-linked immunosorbent assay (ELISA) – of which many varieties are described (1, 8, 9, 16, 24, 36, 39, 40, 48, 52). These tests are often performed with viral antigen of one antigenic type, which means that antibodies directed against the other, heterologous, antigenic type may be detected with less sensitivity. A blocking ELISA has been used extensively in Denmark (39) and has been described as a double ELISA set-up using both European and American virus as antigen and thus it can distinguish between serological reaction to the European and the American types (40). The first live attenuated vaccine for PRRS based on the American type virus has been observed to spread to nonvaccinated animals (6, 42), and subsequent development in herds of vaccine-virus-induced PRRS reproductive failures has been reported in Denmark (6, 31). Reaction to American-type vaccine-like PRRSV can be anticipated in countries using or having used this vaccine; European countries may therefore observe reactions and isolation of both antigenic types (6, 31). The identification of European-type strains of PRRSV in the USA and Canada has been reported only recently, but the prevalence of infection by such strains is not well documented.

Antibodies to the virus can be detected by antibody-binding assays as early as 7–14 days after infection, and antibody levels reach maximal titres by 30–50 days. Some pigs may become seronegative within 3–6 months, but others remain seropositive for much longer. Neutralising antibodies develop slowly and do not reach high titres. They can be detected from 3 to 4 weeks after infection and they can persist for 1 year or more. The use of complement to make the serum virus neutralisation test more sensitive has been reported (25). Extensive research into the duration of antibody titres after infection has not yet been done, and the results probably depend on the test used. Maternal antibodies have a half-life of 12–14 days, and maternal antibody titre can, in general, be detected until 4–8 weeks after birth, depending on the antibody titre of the sow at birth and the test used. In an infected environment, pigs born from seropositive females can seroconvert actively from the age of 3–6 weeks.
This chapter describes the IPMA in detail as this test can easily be performed in laboratories where virus isolation procedures using macrophages have been established, and can be used with virus of both antigenic types. This assay can also be adapted to the MARC-145 cell line for both the European and American types (39, 40). An indirect immunofluorescence assay (IFA) using MARC-145 cells can also be performed for PRRSV serology and is included in the present chapter. Commercial ELISAs with good sensitivity and specificity are available and have been compared (18).

a) Detection of antibodies with the immunoperoxidase monolayer assay

Alveolar macrophages are seeded in the wells of microtitre plates. After attachment, the macrophages are infected with PRRSV. The object is to infect approximately 30–50% of the macrophages in a well so as to be able to distinguish nonspecific sera. After an incubation period, the macrophages are fixed and used as a cell substrate for serology. On each plate, 11 sera can be tested in duplicate. Test sera are diluted and incubated on the cell substrate. If antibodies are present in the test serum, they will bind to the antigen in the cytoplasm of the macrophages. In the next incubation step, the bound antibodies will be detected by an anti-species horseradish-peroxidase (HRPO) conjugate. Finally, the cell substrate is incubated with a chromogen/substrate¹ solution. Reading of the test is done with an inverted microscope.

• Seeding macrophages in the microtitre plates
  i) Defrost one vial containing 6 × 10⁷ macrophages/1.5 ml.
  ii) Wash the cells once with 50 ml of PBS and centrifuge the cell suspension for 10 minutes at 300 g (room temperature).
  iii) Collect the cells in 40 ml RPMI 1640 medium supplemented with 5% FBS, 100 IU (International Units) penicillin and 100 µg streptomycin (growth medium).
  iv) Dispense 100 µl of the cell suspension into each well of a microtitre plate (with one vial of cells, four plates can be seeded at a concentration of 10⁵ cells in each well of the plates).
  v) Incubate the plates for 18–24 hours at 37°C in a 5% CO₂ cabinet, under humid conditions. Alternatively, use HEPES buffer (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) in the medium.

• Infection of cells with PRRSV
  i) Add to each well 50 µl of a virus suspension containing 10⁵ TCID₅₀/ml, but leave two wells uninfected to act as controls.
  ii) Incubate the plates for 18–24 hours at 37°C in a 5% CO₂ cabinet.

• Fixation of the cells
  i) Discard the growth medium and rinse the plates once in saline.
  ii) Knock the plates gently on a towel to remove excess liquid and then dry them (without lid) for 45 minutes at 37°C.
  iii) Freeze the plates (without a lid) for 45 minutes at −20°C. (Plates that are not used immediately for testing must be sealed and stored at −20°C.)
  iv) Incubate the cells for 10 minutes at room temperature with cold 4% paraformaldehyde (in PBS). Alternatively the cells could be fixed in ice-cold absolute ethanol for 45 minutes at 5°C or in ice-cold 80% acetone for 45 minutes (5).
  v) Discard the paraformaldehyde and rinse the plates once in saline.

• Preparation of serum dilutions in a dummy plate
  i) Dispense 180 µl of 0.5 M NaCl with 4% horse serum and 0.5% Tween 80, pH 7.2 (dilution buffer), to the wells of rows A and E of the dummy plate(s).

¹ Preparation of chromogen solution
Stock solution of chromogen (3-amino-9-ethyl-carbazole [AEC]): (a) 4 mg AEC; (b) 1 ml N,N-dimethyl-formamide.
Dissolve (a) in (b) and store the AEC stock solution at 4°C in the dark.
Preparation of chromogen/substrate solution (prepare shortly before use)
Prepare 0.05 M sodium acetate buffer, pH 5.0, as follows: Dissolve 4.1 g sodium acetate in 1 litre distilled water. Adjust the pH to 5.00 with 100% acetic acid.
Add 1 ml AEC stock solution to 19 ml of 0.05 M sodium acetate buffer. Add 10 µl 30% H₂O₂ for each 20 ml of chromogen/substrate solution.
Filter the solution through a 5 µm filter.
ii) Dispense 120 µl of dilution buffer to all other wells.

iii) Add 20 µl of the test serum or control sera to the wells of rows A and E (= 1/10 dilution), and shake.

iv) Dilute the sera four-fold by transferring 40 µl from rows A and E to rows B and F, and so on to provide further dilutions of 1/40, 1/160 and 1/640.

• Incubation of sera in the plate with fixed macrophages
  i) Transfer 50 µl from each of the wells of the dummy plate(s) to the corresponding wells of the plate with the fixed macrophages. Seal the plate(s) and incubate for 1 hour at 37°C.
  ii) Discard the serum dilutions and rinse the plate(s) three times in 0.15 M NaCl + 0.5% Tween 80.

• Incubation with conjugate
  i) Dilute the rabbit-anti-swine HRPO conjugate to a predetermined dilution in 0.15 M NaCl + 0.5% Tween 80. Add 50 µl of the conjugate dilution to all wells of the plate(s). Seal the plate(s) and incubate for 1 hour at 37°C. Rinse the plates three times.

• Staining procedure
  i) Dispense 50 µl of the filtered chromogen/substrate (AEC) solution to all wells of the plate(s) (see footnote 1).
  ii) Incubate the AEC for at least 30 minutes at room temperature.
  iii) Replace the AEC with 50 µl of 0.05 M sodium acetate, pH 5.0 (see footnote 1).

• Reading and interpreting the results
If antibodies are present in the test serum, the cytoplasm of approximately 30–50% of the cells in a well are stained deeply red by the chromogen. A negative test serum is recognised by cytoplasm that remains unstained. A serum that reacts nonspecifically might stain all cells in a well (compared with a positive control serum). The titre of a serum is expressed as the reciprocal of the highest dilution that stains 50% or more of the wells. A serum with a titre of <10 is considered to be negative. A serum with a titre of 10 or 40 is considered to be a weak positive. Often nonspecific staining is detected in these dilutions. A serum with a titre of ≥160 is considered to be positive.

b) Detection of antibodies with the indirect immunofluorescence assay
Although there is no single standard accepted immuno-fluorescence assay in use at this time, several protocols have been developed and are used by different laboratories in North America. The IFA can be performed in microtitre plates or eight-chamber slides using the MARC-145 cell line and a MARC-145 cell-line-adapted PRRSV isolate. To prevent cross-reactivity with pestivirus, it is recommended that cells and FBS, to supplement culture medium, be pestivirus free. After an incubation period, PRRSV-infected cells are fixed and used as a cell substrate for serology. Serum samples are tested at a single screening dilution of 1/20 and samples are reported as being negative or positive at this dilution. Each porcine serum to be tested is added to wells or chambers containing PRRSV-infected cells. Antibodies to PRRSV, if present in the serum, will bind to antigens in the cytoplasm of infected cells. Following this step, an anti-porcine-IgG conjugated to fluorescein is added, which will bind to the porcine antibodies that have bound to PRRSV antigens in the infected cells. The results are read using a fluorescence microscope. Microtitre plates may also be prepared for serum titration purposes (see Section b1 below).

• Seeding and infection of MARC-145 cells in microtitre plates
  i) Add 50 µl of cell culture medium (e.g. Minimal Essential Medium [MEM] containing 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU penicillin and 100 µg streptomycin) without FBS to each well of columns 2, 4, 6, 8, 10 and 12 of a 96-well plate using a multichannel pipettor.
  ii) Trypsinise confluent MARC-145 cells (grown in culture flasks) to be used for seeding 96-well microtitre plates and resuspend cells in cell culture medium containing 8% FBS at a concentration of 100,000–125,000 cells/ml. The MARC-145 cells are trypsinised from culture flasks for IFA once a week using trypsin/EDTA (ethylene diamine tetra-acetic acid) and are seeded in culture flasks at a concentration of 250,000 cells/ml. After 4 days in culture flasks, new cell culture medium containing 2% FBS is added for 3 additional days.
  iii) Using a multichannel pipettor, add 150 µl of the cell suspension to each well of the 96-well plate.
  iv) Dilute PRRSV preparation in MEM without FBS to 10^{2.2} TCID_{50}/50 µl and distribute 50 µl in each well of columns 1, 3, 5, 7, 9 and 11.
v) Incubate the plates for approximately 48–72 hours at 37°C in a humidified 5% CO₂ cabinet to obtain a monolayer with approximately 40–50% of the cells infected as determined by indirect immuno-fluorescence. Alternatively, microtitre plates may first be seeded with MARC-145 cell suspensions (e.g. concentration of 100,000 cells/ml in medium supplemented with 5–10% FBS) and incubated for up to 72 hours until they are confluent. Then volumes of 50 µl of PRRSV preparations (e.g. 10⁵ TCID₅₀/ml) are added per well and the plates are incubated for an additional 48–72 hours prior to fixation. The use of organic buffers such as HEPES in medium has been suggested to stabilise the pH when CO₂ incubators are not available.

- **Seeding and infection of MARC-145 cells in eight-chamber glass slides**
  i) Add 500 µl of a MARC-145 cell suspension (e.g. in MEM supplemented with 10% FBS) at a concentration of 100,000 cells/ml to each chamber of eight-chamber glass slides.
  ii) Incubate the cells for approximately 48–72 hours at 37°C in a humidified 5% CO₂ cabinet until they are confluent.
  iii) Add to each chamber 50 µl of PRRSV suspension containing 10⁵ TCID₅₀/ml and further incubate cells for approximately 18 hours at 37°C in a humidified 5% CO₂ cabinet. At this time 15–20 infected cells per field of view may be observed by indirect immunofluorescence.

- **Fixation of the cells**
  i) Discard the medium, rinse once with PBS and discard the PBS. For chamber slides, remove the plastic chamber walls, leaving the gasket intact.
  ii) Add volumes of 150 µl cold (4°C) acetone (80% in water) to each well of the 96-well plate. Incubate the plates at 4°C for 30 minutes. For chamber slides, acetone (80–100%) at room temperature is used to fix the cells for 10–15 minutes at room temperature. Some manufactured brands of acetone will degrade the chamber slide gasket leaving a film on the slide. It is recommended to check the acetone before using for routine fixation.
  iii) Discard the acetone and dry the plates and slides at room temperature.
  iv) The plates can then be placed in a plastic bag, sealed and stored at –70°C until use. Chamber slides can be kept similarly in slide cases.

- **Preparation of serum dilutions**
  i) Dilute serum samples to a 1/20 dilution in PBS (0.01 M; pH 7.2) in separate 96-well plates (e.g. add 190 µl of PBS using a multichannel pipettor followed by 10 µl of the sera to be tested).
  ii) Include as controls reference PRRSV antibody positive and negative sera of known titre.

- **Incubation of sera with fixed MARC-145 cells**
  i) Stored plates are removed from the –70°C freezer and when the plates reach room temperature rehydrate the cells with 150 µl PBS for a few minutes. Discard the PBS by inverting the plates and blotting dry on paper towels. Cells of eight-chamber slides are not rehydrated.
  ii) Add volumes of 50 µl of each diluted serum to one well containing the fixed noninfected cells and to one well containing the fixed infected cells. Add similar volumes for each serum to a single chamber.
  iii) Add volumes of 50 µl of the negative control serum and positive control serum dilutions in the same manner.
  iv) Incubate the plates with their lids on at 37°C for 30 minutes in a humid atmosphere. Slides should be incubated similarly in boxes or slide trays with a cover.
  v) Remove the serum samples and blot the plates dry on paper towels. A total of six washes using 200 µl of PBS are performed. The PBS is added to each well, followed by inversion of the plates to remove the PBS. After removing serum samples, slides are rinsed in PBS followed by a 10-minute wash.

- **Incubation with conjugate**
  i) Add volumes of 50 µl of appropriately diluted (in freshly prepared PBS) rabbit or goat anti-swine IgG (heavy and light chains) conjugated with FITC (fluorescein isothiocyanate) to each well using a multichannel pipettor. Similar volumes are added to individual chambers.
  ii) Incubate plates or slides with their lids on at 37°C for 30 minutes in a humid atmosphere.
iii) Remove the conjugate from the plates and blot the plates dry on paper towels. A total of four washes using PBS are performed as described above. Discard the conjugate from the slides, rinse in PBS, wash for 10 minutes in PBS and rinse in distilled water. Tap the slides on an absorbent pad to remove excessive water.

iv) The plates and the slides are read using a fluorescence microscope.

- Reading and interpreting the results

The presence of a green cytoplasmic fluorescence in infected cells combined with the absence of such a signal in noninfected cells is indicative of the presence of antibodies to PRRSV in the serum at the dilution tested. The degree of intensity of fluorescence may vary according to the amount of PRRSV-specific antibody present in the serum tested.

Absence of specific green fluorescence in both infected and noninfected cells is interpreted as absence of antibody to PRRSV in that serum at the dilution tested. The test should be repeated if the fluorescence is not seen with the use of the positive control sera on infected cells or if fluorescence is seen using the negative control serum on infected cells. No fluorescence should be seen on noninfected cells with any of the control sera. Any test serum giving suspicious results should be retested at a 1/20 dilution and if results are still unclear, a new serum sample from the same animal is requested for further testing.

b1) Evaluation by IFA of sera for antibody titres

Microtitre plates and IFA may also be used for serum titration purposes. Up to 16 sera may be titred per 96-well microtitre plate.

i) Seed 96-well microtitre plates with MARC-145 cells and incubate at 37°C in a humidified 5% CO₂ cabinet until they are confluent.

ii) Inoculate all wells with the PRRSV preparation except the wells of columns 1, 6 and 11, and incubate the plates at 37°C in a humidified 5% CO₂ cabinet for 48–72 hours.

iii) Discard culture medium and rinse the monolayers once with PBS (0.01 M, pH 7.2). Fix the monolayers with cold acetone (80% aqueous solution) for 10 minutes at ambient temperature. Discard the acetone, air-dry the plates and keep the plates with lids at –20°C for short-term storage or –70°C for long-term storage, until use.

iv) Serially dilute sera including a PRRSV-positive control serum using a four-fold dilution in PBS, beginning at 1/16 or 1/20. Dilute a negative control serum at 1/16 or 1/20 dilution. Dispense 50 µl of each dilution (1/16, 1/64, 1/256, 1/1024 or 1/20, 1/80, 1/320, 1/1280) in wells containing viral antigen of columns 2, 3, 4, 5 or 7, 8, 9, 10. For each serum, also dispense 50 µl of dilution 1/16 or 1/20 in control wells of columns 1 and 6. Similarly dispense dilutions of positive and negative control sera in wells of columns 11 and 12.

v) Incubate the plates at 37°C for 30 minutes in a humid chamber. Discard the sera and rinse the plates three times using PBS.

vi) Add 50 µl of appropriately diluted anti-swine IgG conjugated with FITC and incubate plates at 37°C for 30 minutes in a humid chamber. Discard conjugate, rinse plates several times and tap the plates on absorbent material to remove excessive liquid.

- Reading and interpreting the results

Following examination with a fluorescence microscope, the titre of a serum is recorded as the reciprocal of the highest serum dilution in which typical cytoplasmic fluorescence is observed. For paired serum samples, a four-fold increase in titre with a 2-week interval is indicative of active infection in an individual animal. No specific fluorescence should be observed with test sera or positive and negative control sera on noninfected control cells. No fluorescence should be seen on infected cells with negative control serum. Specific fluorescence should be observed on infected cells with positive control serum at appropriate dilutions. The IFA end-point may vary among laboratories. Test results may also vary depending on the PRRSV isolate used in the test because of antigenic diversity.

c) Detection of antibodies with the enzyme-linked immunosorbent assay

Several laboratories have developed ELISAs (indirect or blocking) for serological testing (1, 8, 9, 16, 24, 39, 40). A double-blocking ELISA format that can distinguish between serological reactions to the European and the American antigenic type has been described (40). ELISA kits are available commercially to determine the serological status of swine towards PRRSV. These kits use as antigens either the European or the American PRRSV types separately or as combined antigens. Their main advantage is the rapid handling of a large number of samples. Commercial ELISAs using recombinant proteins of both PRRSV types as antigens have also been developed and are available.
C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

In the USA, several modified-live virus (MLV) vaccines are approved for the control of the reproductive and/or respiratory forms of PRRS. Also, in the USA and Europe, a killed virus vaccine is licensed as an aid in the reduction of abortions and weak piglets caused by the reproductive form of PRRS. All PRRS vaccines currently licensed in the USA contain the American antigenic type. In Europe, two MLV vaccines are licensed and available commercially. Antigenic types (American or European) used should be appropriate to the region of isolation (44).

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.

Although vaccination of pigs does not prevent PRRSV infection, it may be helpful in herds experiencing problems with PRRS or herds at high risk of PRRSV infection. MLV vaccines are not intended to be used in naive herds, pregnant sows or gilts or boars of breeding age. MLV vaccines are intended to be used in sows and gilts 3–6 weeks prior to breeding and in piglets from 3 weeks of age or older as an aid in the reduction of diseases caused by PRRS. Vaccine virus can persist in boars and be disseminated through semen (11, 12). MLV vaccine virus may be shed and transmitted to nonvaccinated contact pigs (42).

1. Seed management

a) Characteristics of the seed

The isolate of PRRSV used for vaccine production must be accompanied by a history describing its origin and passage history. The isolate must be safe in swine at the intended age of vaccination and provide protection against challenge. Isolates for a MLV vaccine must be shown not to revert to virulence after passage in host animals.

b) Method of culture

The PRRSV is propagated in a continuous African green monkey kidney cell line, such as MA-104 or Vero. Viral propagation should not exceed five passages from the master seed virus (MSV) unless further passages prove to provide protection in swine.

c) Validation as a vaccine

The MSV should be free from bacteria, fungi and mycoplasma. The MSV must be tested for and free from extraneous viruses, including transmissible gastroenteritis virus, porcine adenovirus, porcine haemagglutinating encephalitis virus, porcine parvovirus, bovine viral diarrhoea virus, reovirus, and rabies virus by the fluorescent antibody technique. The MSV must be free from extraneous virus by CPE and haemadsorption on the Vero cell line and an embryonic swine cell type.

Attenuated PRRSV isolates are known to cause viraemia and will transmit to susceptible animals. The MSV should be shown to be nonvirulent in piglets and pregnant animals by five serial passages of the MSV through susceptible swine using the most natural route of infection.

In an immunogenicity trial, the MSV at the highest passage level intended for production must protect susceptible swine against a virulent, heterologous challenge strain. For the respiratory form, 3-week-old piglets are vaccinated with the highest passage level of MSV. The piglets are challenged with a virulent isolate of PRRSV 2–16 weeks later to determine protection from respiratory clinical signs of PRRS. To determine protection from the losses caused by the reproductive form of PRRS, vaccinated animals are challenged at approximately 85 days’ gestation. A significant number of the vaccinates must be protected from the clinical signs of reproductive disease, including fetal mummification, stillborn piglets and/or weak piglets, when compared with the controls. Field trial studies should be conducted to determine the safety of the vaccine. Nonvaccinated sentinel pigs should be included at each site for monitoring the shed of the attenuated virus.

2. Method of manufacture

The African green monkey kidney cell line is seeded into suitable vessels. MEM supplemented with FBS is used as the medium for production; the FBS must be free from pestivirus or antibodies to pestivirus and free from bovine spongiform encephalopathy risk. Incubation is at 37°C.
Cell cultures are inoculated directly with PRRS working virus stock, which is generally from 1 to 4 passages from the MSV. Inoculated cultures are incubated for 1–8 days before harvesting the culture medium. During incubation, the cultures are observed daily for CPE and bacterial contamination.

Killed virus vaccines are chemically inactivated with either formalin or binary ethylenimine and mixed with a suitable adjuvant. MLV vaccines are generally mixed with a stabiliser before bottling and lyophilisation. If formalin is used as an inactivant, the final product should be tested for residual formaldehyde concentration and should not exceed 0.5 g/litre.

3. In-process control

Production lots of PRRSV must be titrated in tissue culture for standardisation of the product. Low-titred lots may be concentrated or blended with higher-titred lots to achieve the correct titre.

4. Batch control

Final container samples are tested for purity, safety and potency. MLV vials are also tested for the maximum allowable moisture content.

a) Purity

Samples are examined for bacterial, fungal and pestivirus contamination. To test for bacteria, ten vessels, each containing 120 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The ten vessels are incubated at 30–35°C for 14 days and observed for bacterial growth. To test for fungi, ten vessels, each containing 40 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The vessels are incubated at 20–25°C for 14 days and observed for fungal growth.

b) Safety

Safety tests can be conducted in a combination of guinea-pigs, mice or pigs.

c) Potency

Final container samples of an MLV vaccine are titrated (log_{10}) in microtitre plates for determination of the titre.

- Test procedure
  i) Prepare tenfold dilutions from 10^{-1} through 10^{-5} by using 0.2 ml of rehydrated test vaccine and 1.8 ml of MEM. An internal positive control PRRSV should be titrated in the appropriate range.
  ii) Inoculate 0.1 ml/well from each dilution into five wells of a 96-well plate containing African green monkey kidney monolayers.
  iii) Incubate the plate at 37°C in a CO₂ atmosphere for 5–7 days.
  iv) Read the plates microscopically for CPE. The internal positive control PRRSV should give a titre within 0.3 log_{10} TCID_{50} from its predetermined mean.
  v) Determine the TCID_{50}/dose by the Spearman–Kärber method. The release titre must be at least 1.2 logs higher than the titre used in the immunogenicity trial. The 1.2 logs include 0.5 logs for stability throughout the shelf life of the product and 0.7 logs for potency test variability.

Killed virus vaccines may use host animal or laboratory animal vaccination/serology tests or vaccination/challenge tests to determine potency of the final product. Parallel-line assays using ELISA antigen-quantifying techniques to compare a standard with the final product are acceptable in determining the relative potency of a product. The standard should be shown to be protective in the host animal.

d) Duration of immunity

Duration of immunity studies are conducted before the vaccine receives final approval. For the respiratory form of PRRS, duration should be shown up to the market age in pigs. Duration of immunity for the reproductive form should be shown through weaning of the piglets.

e) Stability

All vaccines are initially given a shelf life of 24 months before expiry. Real-time stability studies are then conducted to confirm the appropriateness of the expiry date.
f) **Preservatives**

Antibiotics are added during production, generally gentamicin sulphate or neomycin not to exceed 30 µg/ml.

g) **Precautions (hazards)**

Vaccination is only recommended for pigs in PRRSV-positive herds. MLV vaccines intended as an aid in the reduction of disease associated with the respiratory and/or reproductive form of PRRS are not recommended to be used in pregnant sows or gilts or boars of breeding age. Vaccination of breeding boars with a MLV vaccine is not recommended due to the shedding of the vaccine virus in semen (11, 12). The first MLV vaccine for PRRS based on the American-type virus has been observed to spread to nonvaccinated animals (6, 42) and subsequently cause vaccine-virus-induced PRRS reproductive failures in the affected herds (6, 31).

5. **Tests on the final product**

a) **Safety**

See Section C.4.b.

b) **Potency**

See Section C.4.c.

**REFERENCES**


* *

**NB:** There is an OIE Reference Laboratory for Porcine reproductive and respiratory syndrome (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 2.6.6.
PORCINE CYSTICERCOSIS

See Chapter 2.10.1. Cysticercosis

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* *
SECTION 2.7.

AVIAN DISEASES IN LIST B

CHAPTER 2.7.1.

INFECTIOUS BURSAL DISEASE
(Gumboro disease)

SUMMARY

Infectious bursal disease (IBD) is caused by a virus that is a member of the genus Avibirnavirus of the family Birnaviridae. Although turkeys, ducks, guinea fowl and ostriches may be infected, clinical disease occurs solely in chickens. Only young birds are clinically affected. Severe acute disease of 3–6-week-old birds is associated with high mortality, but a less acute or subclinical disease is common in 0–3-week-old birds. This can cause secondary problems due to the effect of the virus on the bursa of Fabricius. IBD virus (IBDV) causes lymphoid depletion of the bursa, and if this occurs in the first 2 weeks of life, significant depression of the humoral antibody response may result. Two serotypes of IBDV are recognised. These are designated serotypes 1 and 2. Both serotypes can be differentiated by cross-neutralisation assays. Clinical disease has been associated with and vaccines prepared against serotype 1 only. Serological variants of IBD serotype 1 occur and these may require special vaccines for maximum protection. Very virulent strains of classical serotype 1 are now common and are causing serious disease in many countries.

Clinical disease due to infection with the IBDV, also known as Gumboro disease, can usually be diagnosed by a combination of characteristic signs and post-mortem lesions. Laboratory confirmation of disease, or detection of subclinical infection, can be carried out by demonstration of a humoral immune response in unvaccinated chickens or by detecting the presence of viral antigen or viral genome in tissues. In the absence of such tests, histological examination of bursae may be helpful.

Identification of the agent: Isolation of IBDV is not usually carried out as a routine diagnostic procedure. Specific antibody-negative chickens may be used for this purpose, as may cell cultures or embryonating eggs from specific antibody-negative sources. However, some difficulty may be experienced if using the latter two systems as the virus does not readily adapt to them. If successful, the identity of the virus can be confirmed by the virus neutralisation (VN) test.

The agar gel immunodiffusion (AGID) test can be used to detect viral antigen in the bursa of Fabricius. A portion of the bursa is removed, homogenised, and used as antigen in a test against known positive antiserum. This is particularly useful in the early stages of the infection, before the development of an antibody response. An immunofluorescence test using IBDV-specific chicken antiserum can also be used to detect antigen in bursal tissue. Antigen-capture enzyme-linked immunosorbent assays (ELISAs) based on plates coated with IBDV-specific antibodies have also been described for the demonstration of IBDV antigens in bursal homogenates. The reverse-transcription polymerase chain reaction (RT-PCR) with specific primers may be used to detect viral genomic RNA in the bursa of Fabricius.

Strain characterisation: IBDV strains can be further identified by testing their pathogenicity in specific antibody-negative chickens, by investigating their antigenic reactivity in cross VN assays or using monoclonal antibodies, by determining the nucleotide sequence of RT-PCR amplification
products derived from IBDV genome, or by studying the number and size of the restriction fragments obtained following digestion of such RT-PCR products with restriction endonucleases. Several protocols have been described for each of these different approaches. Tests should be performed by specialised laboratories and should include a panel of reference strains as controls. Although the molecular basis for antigenic variation is now better understood, no validated virulence marker has been described yet.

Serological tests: An AGID, VN or ELISA may be carried out on serum samples. The infection usually spreads rapidly within a flock of birds. Because of this, only a small percentage of the flock needs to be tested to detect the presence of antibodies. If positive reactions are found in unvaccinated birds then the whole flock must be regarded as infected.

Requirements for vaccines and diagnostic biologicals: Both live attenuated and inactivated (killed) vaccines are available to control the disease. A live recombinant vaccine expressing the VP2 antigen of IBDV has also been licensed recently. It is important that live vaccines be stable, with no tendency to revert to virulence on passage. To be effective, the inactivated vaccines need to have a high antigen content.

Live vaccines: Attenuated strains of IBD viruses are used. These are referred to as either mild, intermediate, or ‘intermediate plus’ (‘hot’) vaccines. The mild vaccines cause limited bursal damage, while the intermediate and intermediate plus vaccines cause some lymphocytic depletion in the bursa of Fabricius. Usually none of the vaccine types causes immunosuppression when used in birds over 14 days old that have been hatched from IBD immune parents.

Mild vaccines are rarely used in broilers, but are used widely to prime broiler parents prior to inoculation with inactivated vaccine. Intermediate and ‘hot’ vaccines are more capable of overcoming low levels of maternally derived antibodies (MDA). Live vaccines may be administered by intramuscular injection, spray or in drinking water. In the absence of MDA, mild vaccines are given at 1-day old. When MDA are present at 1 day of age, vaccination should be delayed until MDA in most of the flock has waned. The best schedule can be determined by serological testing of the birds to detect the time at which MDA has fallen to a low level. More recently, vaccines have been developed that can be administered in ovo at 18 days of incubation.

Killed vaccines: These are used to stimulate high and uniform levels of antibody in parent chickens so that the progeny will have high and uniform levels of MDA. The killed vaccines are manufactured in oil emulsion adjuvant and given by injection. They must be used in birds already sensitised by primary exposure, either to live vaccine or to field virus. This can be checked serologically. High levels of MDA can be obtained in breeder birds by giving, for example, live vaccine at approximately 8 weeks of age, followed by inactivated vaccine at approximately 18 weeks of age.

**A. INTRODUCTION**

Infectious bursal disease (IBD) is caused by a virus that is a member of the genus *Avibirnavirus* of the family *Birnaviridae*. Although turkeys, ducks, guinea fowl and ostriches may be infected, clinical disease occurs solely in chickens. Only young birds are clinically affected. Severe acute disease of 3–6-week-old birds is associated with high mortality, but a less acute or subclinical disease is common in 0–3-week-old birds. This can cause secondary problems due to the effect of the virus on the bursa of Fabricius. IBD virus (IBDV) causes lymphoid depletion of the bursa, and if this occurs in the first 2 weeks of life, significant depression of the humoral antibody response may result. Two distinct serotypes of infectious bursal disease virus (IBDV) are known to exist. Serotype 1 virus causes clinical disease in chickens younger than 10 weeks. Older chickens usually show no clinical signs. Antibodies are sometimes found in other avian species, but no signs of infection are seen. Serotype 2 antibodies are very widespread in turkeys and are sometimes found in chickens and ducks. There are no reports of clinical disease caused by infection with Serotype 2 virus (19).
B. DIAGNOSTIC TECHNIQUES

Isolation and identification of the agent provide the most certain diagnosis of IBD, but are not usually attempted for routine diagnostic purposes as the virus may prove difficult to isolate (22). In practice, laboratory diagnosis of IBD depends on detection of specific antibodies to the virus, or on detection of the virus in tissues, using immunological or molecular methods.

1. Identification of the agent

Clinical IBD has clearly characteristic signs and post-mortem lesions. A flock will show very high morbidity with severe depression in most birds lasting for 5–7 days. Mortality rises sharply for 2 days then declines rapidly over the next 2–3 days. Usually between 5% and 10% of birds die, but mortality can reach 30–40%. The main clinical signs are watery diarrhoea, ruffled feathers, reluctance to move, anorexia, trembling and prostration. Post-mortem lesions include dehydration of the muscles with numerous ecchymotic haemorrhages, enlargement and orange discoloration of the kidneys, with urates in the tubules. The bursae of Fabricius shows the main diagnostic lesions. In birds that die at the peak of the disease outbreak, the bursa is enlarged and turgid with a pale yellow discoloration. Intrafollicular haemorrhages may be present and, in some cases, the bursa may be completely haemorrhagic giving the appearance of a black cherry. Peribursal straw-coloured oedema will be present in many bursa. Confirmation of clinical disease or detection of subclinical disease is best done by using immunological methods as the IBDV is difficult to isolate. For virus isolation, the methods described below should be followed. Differentiation between serotypes 1 and 2 or between serotype 1 subtypes or pathotypes should be undertaken by a specialised laboratory (e.g. the OIE Reference Laboratories for infectious bursal disease [see Table given in Part 3 of this Terrestrial Manual]).

a) Sample preparation

Remove the bursae of Fabricius aseptically from approximately five affected chickens in the early stages of the disease. Chop the bursae using two scalpels, add a small amount of peptone broth containing penicillin and streptomycin (1000 µg/ml each), and homogenise in a tissue blender. Centrifuge the homogenate at 3000 g for 10 minutes. Harvest the supernatant fluid for use in the investigations described below. Filtration through a 0.22 µ filter may prove necessary to further control bacterial contamination, although this may cause a reduction in virus titre.

b) Identification by the agar gel immunodiffusion test

A protocol for the AGID test is described in Section B.2.a. For detection of antigen in the bursa of Fabricius by AGID, the bursae should be removed aseptically from about ten chickens at the acute stage of infection. The bursae are minced using two scalpels in scissor movement, then small pieces are placed in the wells of the AGID plate against known positive serum. Freeze–thaw cycles of the minced tissue may improve the release of IBDV antigens from the infected bursal tissue, and the freeze–thaw exudate may be used to fill the wells.

c) Identification by immunofluorescence

Sections of bursa are prepared using a microtome cryostat, dried at room temperature and then fixed in cold acetone. Fluorescent-labelled IBDV-specific antisera are applied to the sections, which are then incubated at 37°C for 1 hour in a humid atmosphere. At the end of the incubation period, they are washed for 30 minutes using phosphate buffered saline (PBS), pH 7.2, then rinsed in distilled water. The sections are mounted using buffered glycerol, pH 7.6, and examined by UV microscopy for IBDV-specific fluorescence (24).

d) Identification by antigen-capture enzyme-linked immunosorbent assay (AC-ELISA)

Different protocols have been described for the detection of serotype 1 IBDV using an antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) (11, 18, 33). Briefly, ELISA plates are coated with IBDV-specific antibodies. Depending on the chosen AC-ELISA protocol, the capture antibody may be a mouse anti-IBDV monoclonal antibody (MAb), or a mix of such MAbs, or a chicken post-infectious anti-IBDV polyclonal serum. It has been suggested that AC-ELISAs using polyclonal antibodies may have a higher sensitivity. Samples of bursal homogenates (see above) diluted 1/10 to 1/25 (w/v) in a suitable dilution buffer are incubated in the coated wells. Unbound antigens are discarded at the end of the incubation period by washing with a suitable washing buffer (e.g. PBS, pH 7.2 ± 0.2% Tween 20). The captured antigens are then revealed, as in an indirect ELISA, with a detection antibody (which must have been developed from a different animal species than the capture antibody), followed by an enzyme conjugate that binds to the detection antibody only, followed by the enzyme substrate. Finally, optical densities, which parallel the amount of captured IBDV antigens, are read with an ELISA reader.
The AC-ELISA is based on the use of samples possibly containing live virus and should be performed only in suitable containment facilities such as a class II safety cabinet. All liquid (washing buffers) and solid wastes should be considered to be contaminated by IBDV and decontaminated accordingly before disposal.

Critical steps in the implementation or assessment of AC-ELISA are i) the need to perform extensive washings between each step of the reaction to keep background reactions low, ii) the requirement for known positive and negative samples to be included in each assay as controls, and iii) the need for both the capture and detection antibodies to positively react with all serotype 1 IBDV strains (i.e. neither capture nor detection should critically depend on IBDV antigenic variation that occurs among serotype 1 strains).

e) Identification by molecular techniques

Molecular virological techniques have been developed that allow IBDV to be identified more quickly than by virus isolation (7, 15, 40). The most frequently used molecular method is the detection of IBDV genome by the reverse-transcription polymerase chain reaction (RT-PCR) (21, 40). This method can detect the genome of IBDV, which is unable to grow in cell culture, because it is not necessary to grow the virus before amplification.

RT-PCR is performed in three steps: extraction of nucleic acids from the studied sample, reverse transcription (RT) of IBDV RNA into cDNA, and amplification of the resulting cDNA by PCR. The two latter steps require that the user selects oligonucleotidic primers that are short sequences complementary to the virus-specific nucleotidic sequence. Different areas of the genome will be amplified depending on the location from which the primers have been selected. The example below allows the amplification of the middle third of the gene encoding the outer capsid protein (8, 9).

- Extraction of nucleic acids

Single-stranded RNA is extremely susceptible to degradation by RNases. IBDV double-stranded RNA (dsRNA) genome resists degradation by RNases. However, infected cells also contain IBDV-derived positive-sense single-stranded RNA species that can be used as a template at the RT step and may contribute to improving the sensitivity of the assay. It is thus important that RNA extraction be performed using gloves and RNase-free reagents and labware.

IBDV RNA can be extracted from infected tissues using some kits available from commercial suppliers of molecular biology reagents. Alternatively, IBDV RNA can be extracted by adding 1% sodium dodecyl sulphate and 1 mg/ml proteinase K to 700 µl of virus suspension (e.g. bursal homogenate). Incubate for 60 minutes at 37°C. Nucleic acids are obtained using a standard protocol for phenol/chloroform extraction (caution; phenol is toxic and should be handled and disposed accordingly). Nucleic acids are harvested from the final aqueous phase by ethanol precipitation and may contribute to improving the sensitivity of the assay. It is thus important that RNA extraction be performed using gloves and RNase-free reagents and labware.

- Reverse transcription

A variety of reverse transcriptases are commercially available. Follow the supplier’s instructions to prepare the RT reaction mix. Use the 'lower' PCR primer (complementary to the positive strand of IBDV genome, see below) for reverse transcription, as this allows the synthesis of cDNA both from the positive strand of IBDV dsRNA genome and from IBDV-derived positive-sense single-stranded RNAs previously contained in infected cells.

The IBDV RNA matrix must be denatured before transfer to the RT reaction mix. Add 20% (by volume) molecular biology grade dimethylsulphoxide to the unfrozen solution of IBDV RNA. Heat for 3 minutes at 92°C and chill on ice; an alternative method is to heat for 5 minutes and immediately incubate the mixture in liquid nitrogen. Transfer the relevant volume of denaturated matrix to the reaction mix. Incubate according to the instructions of the enzyme supplier.

The cDNA solution obtained after the RT step should be kept frozen at a temperature below –20°C until use.

- Polymerase chain reaction

A variety of DNA polymerases suitable for PCR are commercially available. Follow the manufacturer’s instructions to prepare the PCR reaction mix. The U3/L3 pair of PCR primers shown below has been found useful for amplifying the middle third of the VP2 gene in serotype 1 IBDV strains (8, 9).

Nucleotide sequence of the U3 and L3 IBDV-specific PCR primers:

Upper U3:  5’-TGT-AAA-AGG-AGG-GCC-AGT-GCA-TGC-GGT-ATG-TGA-GGC-TTG-GTG-AC-3’

Lower L3:  5’-CAG-GAA-ACA-GCT-ATG-ACC-GAA-TTC-GAT-CCT-GTT-GCC-ACC-CTT-TC-3’

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The U3 and L3 primers are 44-nucleotide-long hybrid primers. They include an IBDV-specific 3’ extremity (in italics in the sequence shown above) corresponding to nucleotide positions 657 to 676 and 1193 to 1212 of IBDV segment A, respectively (numbering as in segment A of strain P2, Acc No X84034). The IBDV-specific extremity is coupled to a non-IBDV 5’ extremity (bold type in the sequence above) corresponding to the M13 and RM13 universal primers in the U3 and L3 primers, respectively. The M13 and RM13 universal primers are commonly used in DNA sequencing reactions, so that purified PCR products resulting from amplification with the U3 and L3 primers can be easily sequenced. Finally, restriction sites for the SphI and EcoRI restriction endonucleases are included (underlined in the above sequence) in the U3 and L3 primers, respectively, so that the PCR products resulting from amplification with the U3/L3 pair can be cloned using these sites if required. The U3/L3 pair generates a 604 base pair (bp) product, 516 bp of which are specific of the amplified IBDV sequence.

Perform an initial denaturation step as recommended by the DNA polymerase supplier, followed by 35 cycles, each including one denaturation, one annealing and one elongation step. In such cycles, denaturation at 95°C for 30 seconds and annealing at 64°C for 45 seconds may be used with the U3/L3 pair (the annealing temperature should be adapted if other primers are used). The parameters for the elongation step should be set according to the supplier’s recommendations.

Revelation may be performed by electrophoresis with the PCR products and DNA molecular weight markers in a 1% agarose gel stained with ethidium bromide (caution: ethidium bromide is toxic and carcinogenic. It should be handled and disposed accordingly).

Three PCR reactions should be performed for each cDNA sample (pure, 10- and 100-fold diluted cDNA) to avoid false-negative results due to PCR inhibition in mixes containing high amounts of the cDNA preparation.

Each PCR should include negative and positive control reactions. Protocols that include an internal control to test for the presence of PCR inhibitors have been developed (32).

Delaying the PCR for several weeks after the RT step may cause false negative PCR results.

f) Isolation of virus in cell culture

Inoculate 0.5 ml of sample into each of four freshly confluent chicken embryo fibroblast (CEF) cultures (from a specific pathogen free [SPF] source) in 25 cm² flasks. Adsorb at 37°C for 30–60 minutes, wash twice with Earle’s balanced salt solution and add maintenance medium to each flask. Incubate the cultures at 37°C, observing daily for evidence of cytopathic effect (CPE). This is characterised by small round refractive cells. If no CPE is observed after 6 days, discard the medium, then freeze and thaw the cultures and inoculate the resulting lysate into fresh cultures. This procedure may need to be repeated at least three times. If CPE is observed, the virus should be tested against IBDV antiserum in a tissue culture virus neutralisation (VN) test (see below). The more pathogenic IBDV strains usually cannot be adapted to grow in CEF unless the virus has first been submitted to extensive serial passage in embryos (see below).

g) Isolation of virus in embryos

Inoculate 0.2 ml of sample into the yolk sac of five 6–8-day-old specific antibody negative (SAN) chicken embryos and on to the chorioallantoic membrane of five 9–11-day-old SAN chicken embryos. SAN embryos are derived from flocks shown to be serologically negative to IBDV. Candle daily and discard dead embryos up to 48 hours post-inoculation. Embryos that die after this time are examined for lesions. Serotype 1 IBD produces dwarfing of the embryo, subcutaneous oedema, congestion and subcutaneous or intracranial haemorrhages. The liver is usually swollen, with patchy congestion producing a mottled effect. In later deaths, the liver may be swollen and greenish, with areas of necrosis. The spleen is enlarged and the kidneys are swollen and congested, with a mottled effect.

Serotype 1 IBDV usually causes death in at least some of the embryos on primary isolation.

Serotype 2 IBDV does not induce subcutaneous oedema or haemorrhages in the infected embryos, but embryos are of a smaller size with a pale yellowish discolouration.

For the preparation of embryo-propagated stock virus or for subsequent passaging, embryos with lesions or embryos suspected to be infected, respectively, are harvested aseptically. Their head and limbs are discarded and the main body is minced as described in Section B.1.a. for the preparation a virus suspension.
h) Isolation of virus in chickens

This method has been used in the past but is no longer recommended due to animal welfare concerns. Five susceptible and five IBD-immune chickens (3–7 weeks of age) are inoculated by the eyedrop route with 0.05 ml of sample. Kill the chickens 72–80 hours after inoculation, and examine their bursae of Fabricius. The bursae of chickens infected with virulent serotype 1 IBDV appear yellowish (sometimes haemorrhagic) and turgid, with prominent striations. Peribursal oedema is sometimes present, and plugs of caseous material are occasionally found. The plicae are petechiated.

The presence of lesions in the bursae of susceptible chickens along with the absence of lesions in immune chickens is diagnostic of IBD. The bursae from both groups may be used as antigen in an agar gel immunodiffusion (AGID) test against known positive IBD antiserum (see Section B.1.b).

The extent of bursal damage may vary considerably with the pathogenicity of the studied IBDV strain. However, as the samples submitted for virus isolation may vary in virus content, the extent of bursal damage observed in susceptible chickens at the isolation stage gives a limited indication on strain pathogenicity.

The bursae of chickens infected with serotype 2 IBDV do not exhibit any gross lesions.

i) Strain differentiation

IBDV strains can be further identified by testing their pathogenicity in SAN chickens, by investigating their antigenic reactivity in cross VN tests or using MAbs, by determining the nucleotide sequence of RT-PCR amplification products derived from IBDV genome, or by studying the number and size of the restriction fragments obtained following digestion of such RT-PCR products with restriction endonucleases. Several protocols have been described for each of these approaches. Tests should be performed by specialised laboratories and should include a panel of reference strains as controls. Although the molecular basis for antigenic variation is now better understood, no validated virulence marker has been described yet.

- **Pathogenicity testing**

Studies to compare the pathogenicity of IBDV strains must be carried out in secure biocontainment facilities to avoid the dissemination of the studied virus (see Appendix I.1.6.1. of Chapter I.1.6. International transfer and laboratory containment of animal pathogens). SAN birds with a known microbial status (ideally SPF chickens) must be used to avoid interference by contaminating agents.

The main variables when comparing the results of pathogenicity trials are the breed, age and immune status of the challenged chickens, the dose and route of inoculation of the challenge virus, and the possible presence of contaminating agents in the inoculum. Light layer breeds have been reported to be more susceptible than heavy broilers (39). Differences in susceptibility may also occur between different SPF chicken lines. The highest susceptibility to acute IBD occurs in chickens between 3 and 6 weeks of age (22). (The influence of the immune status is described in Section C.) A high dose of challenge virus such as that recommended in Section C.1.c. is necessary so that all inoculated chickens become infected at once without requiring bird-to-bird transmission of the inoculated virus. Finally, the presence in the inoculum of contaminating agents, such as adenovirus or chicken infectious anaemia virus, may modify the severity of IBD and signs observed after challenge (29).

The terms ‘variant’, ‘classical’ and ‘very virulent’ have been used to qualify IBDV strains that exhibit a different pathogenicity. Based on the signs and lesions observed in two lines of White Leghorn SPF chickens during acute experimental IBD following a 10^5 50% egg infective dose (EID_{50}) challenge, North American ‘Variant’ IBDVs induce little if any clinical signs and no mortality but marked bursal lesions, ‘Classical’ IBDVs induce approximately 10–50% mortality with typical signs and lesions whereas ‘very virulent’ IBDVs induce approximately 50–100% mortality with typical signs and lesions (Eterradossi et al., personal observation).

- **Antigenicity testing**

Antigenic relatedness among IBDV strains may be assayed in cross VN tests, which correlate best with cross protection. Such tests have to be performed in SAN embryonated eggs when the studied viruses do not grow in CEF (e.g. very virulent IBDV [vvIBDV]). Differences in cross VN results among serotype 1 IBDV strains have led to the definition of serotype 1 ‘subtypes’, some of which include the antigenically ‘variant’ North American IBDV isolates (14).

Another approach to the study of genetic relatedness is the use of mouse MAbs that bind to IBDV neutralising epitopes. Several panels of MAbs exist world-wide (11, 12, 34). Some of the MAbs have been included in commercially available kits, but no unified MAb panel as yet been proposed. All neutralising epitopes of IBDV characterised to date have been mapped into a major immunogenic domain in the middle
third (amino acid positions 200 to 340) of the VP2 outer capsid protein (9, 30, 37). This region is termed 'VP2 variable domain' because most amino acid changes observed among IBDV strains are clustered in it. Within vVP2, four amino acid stretches are of critical importance to antigenicity and are referred to as vVP2 hydrophilic peaks. These are amino acid positions 210 to 225 (major peak A), 249 to 252 (minor peak 1), 281 to 292 (minor peak 2) and 313 to 324 (major peak B) (2, 38). Both North American 'variants' and 'very virulent' IBDV exhibit in these areas amino acid changes that correlate with epitope variation (8, 37). To date, no antigenic marker has been shown to correlate strictly with IBDV pathogenicity.

- Molecular identification

Most efforts at molecular identification have focused on the characterisation of the larger segment of IBDV (segment A) and especially of the vVP2 encoding region. Several protocols have been published on characterisation using restriction endonucleases of RT-PCR products. These approaches are known as RT-PCR/RE or RT-PCR-RFLP (restriction fragment length polymorphism) (16, 21, 42). The usefulness of the information they provide depends on the identification of enzymes that cut in restriction sites that are phenotypically relevant. Some sites involved in antigenicity have already been identified (see above), however, restriction sites reliably related to virulence still need to be defined and validated. Nucleotide sequencing of RT-PCR products, although more expensive than restriction analysis, provides an approach to assessing more precisely the genetic relatedness among IBDV strains. Markers have been demonstrated experimentally, using a reverse genetics approach, for cell culture-adapted strains, which exhibit amino acid pairs 279 N–284 T (20) or 253 H–284 T (25). In most very virulent viruses, four typical amino acids are present (222 A, 256 I, 294 I and 299 S) (3, 8, 21). However, it is not yet known whether these amino acids play a role in virulence or if they are merely an indication of the clonal origin of most vvIBDV isolates.

2. Serological tests

a) Agar gel immunodiffusion test

The AGID test is the most useful of the serological tests for the detection of specific antibodies in serum, or for detecting viral antigen or antibodies in bursal tissue.

Blood samples should be taken early in the course of the disease, and repeat samples should be taken 3 weeks later. Because the virus spreads rapidly, only a small proportion of the flock needs to be sampled. Usually 20 blood samples are enough. For detection of antigen in the bursa of Fabricius, the bursae should be removed aseptically from about ten chickens at the acute stage of infection. The bursae are minced using two scalpels in a scissor movement, then small pieces are placed in the wells of the AGID plate against known positive serum. Freeze–thaw cycles of the minced tissue may improve the release of IBDV antigens from the infected bursal tissue.

- Preparation of positive control antigen

Inoculate 3–5-week-old susceptible chickens, by eyedrop, with a clarified 10% (w/v) bursal homogenate known to contain viable IBDV\(^1\). Kill the birds 3 days post-inoculation, and harvest the bursae aseptically. Discard haemorrhagic bursae and pool the remainder, weigh and add an equivalent volume of cold distilled water and an equivalent volume of undiluted methylene chloride. Thoroughly homogenise the mixture in a tissue blender and centrifuge at 2000 \(g\) for 30 minutes. Harvest the supernatant fluid and dispense into aliquots for storage at \(-40^\circ\)\(C\). The antigen contains live virus and should be handled only in suitable containment facilities such as a class II safety cabinet.

- Preparation of positive control antiserum

Inoculate 4–5-week-old susceptible chickens, by eyedrop, with 0.05 ml of a clarified 10% (w/v) bursal homogenate known to contain viable IBDV\(^1\). Exsanguinate 28 days post-inoculation. Pool and store serum in aliquots at \(-20^\circ\)\(C\).

- Preparation of agar

Dissolve sodium chloride (80 g) and phenol (5 g) in distilled water (1 litre) (caution: phenol is toxic and should be handled and disposed accordingly). Add agar (12.5 g) and steam until the agar has dissolved. While the mixture is still very hot, filter it through a pad of cellulose wadding covered with a few layers of muslin. Dispense the medium in 20 ml volumes into glass bottles and store at 4\(^\circ\)\(C\) until required for use.

\(^1\) A suitable strain of IBDV (serotype 1, classical pathotype) is the strain 52/70, obtainable from one of the OIE Reference Laboratories (see Table given in part 3 of this Terrestrial Manual).
Chapter 2.7.1. — Infectious bursal disease (Gumboro disease)

• Test procedure
  i) Prepare plates from 24 hours to 7 days before use. Dissolve the agar by placing in a steamer or boiling water bath. Take care to prevent water entering the bottles.
  ii) Pour the contents of one bottle into each of the required number of 9 cm plastic Petri dishes laid on a level surface. (Some laboratories prefer to pour the gel on 25 × 75 mm glass slides, 3 mm deep.)

![Fig. 1. Protocol for tests for antibody.](image)

![Fig. 2. Protocol for tests for antigen.](image)

Notes:
1. The linear pattern of wells is preferred although a hexagonal pattern may be used. Each test serum or bursa should be placed adjacent to a positive control antibody (AB) or antigen (AG), respectively.
2. Wells, 3 mm deep, 6 mm in diameter, and 3 mm apart (or wells of any other size previously shown to be effective), are used.

  iii) Cover the plates and allow the agar to set, and then store the plates at 4°C. Poured plates may be stored for up to 7 days at 4°C. (If the plates are to be used the same day that they are poured, dry them by placing them opened but inverted at 37°C for from 30 minutes to 1 hour.)
  iv) Cut three vertical rows of wells 6 mm in diameter and 3 mm apart, using a template and tubular cutter.
  v) Remove the agar from the wells by aspiration or remove using a pen and nib, taking care not to damage the walls of the wells.
  vi) Using a pipette, dispense 50 µl of the test sera into the wells as shown in Figure 1.
  Or, for the detection of IBDV antigens in bursae:
  Dispense small pieces of finely minced test bursae by means of curved fine-pointed forceps into the wells, as shown in Figure 2, to just fill the wells. Alternatively, the freeze–thaw exudate of minced tissues can be used to fill the wells.
  vii) Dispense 50 µl of the positive and negative control reagents into the relevant wells.
  viii) Incubate the plates at between 22°C and 37°C for up to 48 hours in a humid chamber to avoid drying the agar.
  ix) Examine the plates against a dark background with an oblique light source after 24 and 48 hours.

• Quantitative agar gel immunodiffusion tests
  The AGID test can also be used to measure antibody levels by using dilutions of serum in the test wells and taking the titre as the highest dilution to produce a precipitin line (4). This can be very useful for measuring maternal or vaccinal antibodies and for deciding on the best time for vaccination; however, this AGID quantitative determination has now been largely been replaced by the ELISA.

b) Virus neutralisation tests
  VN tests are carried out in cell culture. The test is more laborious and expensive than the AGID test, but is more sensitive for detecting antibody. This sensitivity is not required for routine diagnostic purposes, but may be useful for evaluating vaccine responses or for differentiating between IBDV 1 and 2 serotypes.
First, 0.05 ml of virus diluted in tissue culture medium to contain 100 TCID_{50} (50% tissue culture infective doses) per 0.05 ml is placed in each well of a tissue-culture grade microtitre plate (Spearman–Kärber [1] or the Reed & Muench [27]). The test sera are heat-inactivated at 56°C for 30 minutes. Serial doubling dilutions of the sera are made in the diluted virus. After 30 minutes at room temperature, 0.2 ml of SPF chicken embryo fibroblast cell suspension, with a cell density allowing confluent layers to be obtained after 24 hours of incubation, is dispensed into each well. Plates are sealed and incubated at 37°C for 4–5 days, after which the monolayers are observed microscopically for typical CPE. The end-point (serum titre) is expressed as the reciprocal of the highest serum dilution that did not show CPE. To reduce test-to-test and operator-to-operator variation, a standard reference antiserum may be included with each batch of tests.

c) Enzyme-linked immunosorbent assay
ELISAs are in use for the detection of antibodies to IBD. Coating the plates requires a purified, or at least semipurified, preparation of virus, necessitating special skills and techniques. Methods for preparation of reagents and application of the assay were described by Marquardt et al. in 1980 (23). Commercial kits are available.

The test sera are diluted according to the established protocol or kit instructions and each is dispensed into the requisite number of wells. After incubation under the appropriate conditions, the sera are discarded from the plates, and the wells are washed thoroughly. Anti-chicken immunoglobulins conjugated to an enzyme are dispensed into the wells, and the plates are again incubated as appropriate. The plates are emptied and rewashed before substrate containing a chromogen that gives a colour change in the presence of the enzyme used is added to the plate. After a final incubation step, the substrate/chromogen reaction is stopped by addition of a suitable stopping solution and the colour reactions are quantified by measuring the optical density of each well. The Sample to Positive (S/P) ratio for each test sample is calculated.

d) Interpretation of results
The AGID test is surprisingly sensitive, though not as sensitive as the VN test; the latter will often give a titre when the AGID test is negative. Positive reactions indicate infection in unvaccinated birds without maternal antibodies. As a guide, a positive AGID reaction in a vaccinated bird or young bird with maternal antibody indicates a protective level of antibody. ELISA gives more rapid results than VN or AGID and is less costly in terms of labour, although the reagents are more expensive. VN and AGID titres correlate well, but as VN is more sensitive, AGID titres are proportionally lower. Correlation between ELISA and VN and between ELISA and AGID is more variable depending on the source of the ELISA reagents. When testing for the decay of maternally derived antibodies (MDA), it is not uncommon to find residual VN antibodies at an age when ELISA results are already negative. A formula has been devised that allows ELISA titres to be used to calculate the optimal age for vaccination (17), which will vary depending on the vaccine used. Nonspecific positive reactions may occur with most ELISAs because they are usually designed for monitoring vaccine responses, in which case sensitivity is regarded as more important than specificity. This should be taken into account when the ELISA is used for diagnosis. In commercial chicken flocks, the possibility that a serotype 1 ELISA antigen also detects antibodies induced by a natural infection with serotype 2 IBDV cannot be ruled out, however this possible cross reactivity has not yet been demonstrated to interfere with serological monitoring programmes of IBD based on the ELISA.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Two types of vaccine are mostly available for the control of IBD. These are live attenuated vaccines, or inactivated oil-emulsion adjuvanted vaccines (36). A live recombinant vaccine expressing IBDV antigens has also been licensed recently (6).

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.

To date, IBD vaccines have been made with serotype 1 IBDV only, although a serotype 2 virus has been detected in poultry. The serotype 2 virus has not been associated with disease, but its presence will stimulate antibodies. Serotype 2 antibodies do not confer protection against serotype 1 infection, neither do they interfere with the response to type 1 vaccine. There have been numerous descriptions of antigenic variants of serotype 1 virus (28). Cross-protection studies have shown that inactivated vaccines prepared from ‘classical’ serotype 1 virus require a high antigenic content to provide good protection against some of these variants. IBD vaccines that contain both classical and variant IBD serotype 1 viruses are now licensed. vvIBDV strains with limited
antigenic changes as compared with ‘classical’ serotype 1 viruses have emerged since 1986. Active immunisation with a ‘classical’ serotype 1 virus or vaccine provides a good protection against the vvIBDV (10), however the latter viruses are less susceptible to neutralisation by maternally derived antibodies than ‘classical’ pathogenic viruses (39).

- **Live vaccines: methods of use**

Live IBD vaccines are produced from fully or partially attenuated strains of virus, known as ‘mild’, ‘intermediate’, or ‘intermediate plus’ (‘hot’), respectively.

Mild or intermediate vaccines are used in parent chickens to produce a primary response prior to vaccination near to point-of-lay using inactivated vaccine. They are susceptible to the effect of MDA so should be administered only after all MDA has waned. Application is by means of intramuscular injection, spray or in the drinking water, usually at 8 weeks of age (31).

Intermediate or intermediate plus vaccines are used to protect broiler chickens and commercial layer replacements. Some of these vaccines are also used in young parent chickens if there is a high risk of natural infection with virulent IBD. Although intermediate vaccines are susceptible to the presence of MDA, they are sometimes administered at 1-day old, as a coarse spray, to protect any chickens in the flock that may have no or only minimal levels of MDA. This also establishes a reservoir of vaccine virus within the flock that allows lateral transmission to other chickens when their MDA decays. Second and third applications are usually administered, especially when there is a high risk of exposure to virulent forms of the disease or when the vaccinated chicks exhibit uneven MDA levels. The timing of additional applications will depend on the antibody titres of the parent birds at the time the eggs were laid. As a guide, the second dose is usually given at 10–14 days of age when about 10% of the flock is susceptible to IBD, and the third dose 7–10 days later. The route of administration is by means of spray or in the drinking water. Intramuscular injection is used rarely. If the vaccine is given in the drinking water, clean water with a neutral pH must be used that is free from smell or taste of chlorine or metals. Skimmed milk powder may be added at a rate of 2 g per litre. Care must be taken to ensure that all birds receive their dose of vaccine. To this end, all water should be removed (cut off) for 2–3 hours before the medicated water is made available and care must be taken that no residual water remains in the water adduction pipes or in the drinkers. It is possible to divide the medicated water into two parts, giving the second part 30 minutes after the first.

Recently, technology has been developed to deliver live vaccine into eggs during the incubation period. Live vaccine virus is blended with IBD antibody and the complex is injected **in ovo** at 18 days of incubation. The eggs go on to hatch and the vaccine virus is released when the chicks are about 7 days of age. In this way, the problem of maternally derived IBD antibody is overcome and the chicks are effectively immunised (13).

A live recombinant vaccine expressing IBDV VP2 antigen has been licensed recently in Europe. There is limited information available on the use of this vaccine.

Live IBD vaccines are generally regarded as compatible with other avian vaccines. However, it is possible that IBD vaccines that cause bursal damage could interfere with the response to other vaccines. Only healthy birds should be vaccinated. The vials of lyophilised vaccine should be kept at temperatures between 2°C and 8°C up to the time of use.

- **Inactivated vaccines: method of use**

Inactivated IBD vaccines are used to produce high, long-lasting and uniform levels of antibodies in breeding hens that have previously been primed by live vaccine or by natural exposure to field virus during rearing (5). The usual programme is to administer the live vaccine at about 8 weeks of age. This is followed by the inactivated vaccine at 16–20 weeks of age. The inactivated vaccine is manufactured as a water-in-oil emulsion, and has to be injected into each bird. The preferred routes are intramuscular into the leg muscle, avoiding proximity to joints, tendons or major blood vessels or the subcutaneous route. A multidose syringe may be used. All equipment should be cleaned and sterilised between flocks, and vaccination teams should exercise strict hygiene when going from one flock to another. Vaccine should be stored at between 4°C and 8°C. It should not be frozen or exposed to bright light or high temperature.

Only healthy birds, known to be sensitised by previous exposure to IBDV, should be vaccinated. Used in this way the vaccine should produce such a good antibody response that chickens hatched from those parents will have passive protection against IBD for up to about 30 days of age (41). This covers the period of greatest susceptibility to the disease and prevents bursal damage at the time when this could cause immunosuppression. It has been shown that bursal damage occurring after about 15 days of age has little effect on immunocompetence as by that time the immunocompetent cells have migrated into the peripheral lymphoid tissues. However, if there is a threat of exposure to infection with very virulent IBDV, live vaccines should be
applied as described above. The precise level and duration of immunity conferred by inactivated IBD vaccines will depend mainly on the concentration of antigen present per dose. The manufacturing objective should be to obtain a high antigen concentration and hence a highly potent vaccine.

1. Seed management

a) Characteristics of the seed

• Live vaccine

The seed virus must be shown to be free from extraneous viruses, bacteria, mycoplasma and fungi, particularly avian pathogens. This includes freedom from contamination with other strains of IBDV. For vaccine strains that claim to be attenuated and nonimmunosuppressive, the seed virus must be shown to be stable, with no tendency to revert to virulence. This can be confirmed by carrying out at least five consecutive chicken-to-chicken passages at 3–4-day intervals using bursal suspension as inoculum in SPF chickens of the minimum age recommended for vaccination. It must be shown that the virus was transmitted. A histological comparison is then made to show that there is no difference between bursae from birds inoculated with the initial and the final passage material. Bursal scoring (26) and imaging techniques have been developed.

Test for immunosuppression: An important characteristic is that the virus should not produce such damage to the bursa of Fabricius that it causes immunosuppression in susceptible birds. (Live vaccines of the 'intermediate' or 'intermediate plus' type may be licensed even though they may be capable of causing immunosuppression). The vaccine is administered by injection or eyedrop, one field dose per bird, to each of 20 SPF chickens, at 1-day old. A further group of birds of the same age and source are housed separately as controls. At 2 weeks of age, each bird in both groups is given one field dose of live Newcastle disease vaccine by eyedrop. Alternatively, the IBDV vaccine may be administered at the minimum age recommended for vaccination, and the Newcastle disease vaccine at the time when bursal lesions induced by the IBDV vaccine are maximal. The haemagglutination inhibition (HI) response of each bird to Newcastle disease vaccine is measured 2 weeks after the administration of the Newcastle Disease vaccine, and the protection is measured against challenge with $10^{5.0}$ to $10^{6.5}$ ELD$_{50}$ (50% embryo lethal doses) Herts 33/56 strain (or similar) of Newcastle disease virus. The IBD vaccine fails the test if the HI response and protection afforded by Newcastle disease vaccine is significantly less (<0.01) in the group given IBD vaccine than in the control group. In countries where Newcastle disease virus is exotic, an alternative is to use sheep erythrocytes or Brucella abortus-killed antigen as the test antigen, measuring the response using the haemagglutination or serum agglutination test, respectively. However, another live vaccine is a preferable test system because it also evaluates cell-mediated immunity.

• Killed vaccine

For killed vaccines, the most important characteristics are high yield and good antigenicity. Both virulent and attenuated strains have been used. The seed virus must be shown to be free from extraneous viruses, bacteria, mycoplasma and fungi, particularly avian pathogens (35).

b) Method of culture

Seed virus may be propagated in various culture systems, such as SPF chicken embryo fibroblasts, or chicken embryos. In some cases, propagation in the bursa may be used. The bulk is distributed in aliquots and freeze-dried in sealed containers.

c) Validation as a vaccine

Data on efficacy should be obtained before bulk manufacture of vaccine begins. The vaccine should be administered to birds in the way in which it will be used in the field. Live vaccine can be given to young birds and the response measured serologically and by resistance to experimental challenge. In the case of killed vaccines, a test must be carried out in older birds that go on to lay, using the recommended vaccination schedule, so that their progeny can be challenged to determine resistance due to MDA at the beginning and end of lay.

• Live vaccine

Efficacy test: Administer one field dose of the minimum recommended titre to each of 20 SPF chickens of the minimum age of vaccination. Inoculate separate groups for each of the recommended routes of application. Leave 20 chickens from the same hatch as uninoculated controls. After 14 days, challenge each of the chickens by eyedrop with approximately 100 CID$_{50}$ (50% chicken infective dose) of a virulent strain of IBDV as recommended by one of the OIE Reference Laboratories for IBD (see Table given in Part 3 of this Terrestrial Manual). Observe the chickens daily for 10 days. Register the number of birds that die or exhibit IBD signs. The vaccine fails the test unless at least 90% of the vaccinated chickens survive
without showing either clinical signs or severe lesions in the bursae of Fabricius at the end of the observation period. If more than half the controls do not show IBD signs, or one or more control chicken does not exhibit severe lesions of the bursa of Fabricius, the test is invalid. Lesions are considered to be severe if at least 90% of follicles show greater than 75% depletion of lymphocytes. Providing results are satisfactory, this test need be carried out on only one batch of all those batches prepared from the same seed lot.

- **Killed vaccine**

  **Efficacy test:** At least 20 unprimed SPF birds are given one dose of vaccine at the recommended age (near to point-of-lay) by one of the recommended routes, and the antibody response is measured between 4 and 6 weeks after vaccination by serum neutralisation with reference to a standard antiserum.\(^3\)

  Eggs are collected for hatching 5–7 weeks after vaccination, and 25 progeny chickens are then challenged at 3 weeks of age by eyedrop with approximately 100 \(\text{CID}_{50}\) of a recognised virulent strain of IBDV. Ten control chickens of the same breed but from unvaccinated parents are also challenged. Protection is assessed 3–4 days after challenge by removing the bursa of Fabricius from each bird; each bursa is then subjected to histological examination or tested for the presence of IBD antigen by the agar gel precipitin test. Not more than three of the chickens from vaccinated parents should show evidence of IBD infection, whereas all those from unvaccinated parents should be affected.

  These procedures should be repeated towards the end of the period of lay when the vaccinated birds are at least 60 weeks of age, but, on this occasion challenge of the progeny should be undertaken when they are 15 days old.

  The efficacy test should be repeated on primed birds vaccinated by the recommended schedule. The final dose of killed vaccine is given at the earliest recommended age. Chickens hatched from fertile eggs collected at the beginning and the end of lay are tested for protection against challenge as described above.

  These tests need to be performed once only using a typical batch of vaccine.

2. **Method of manufacture**

   The vaccine must be manufactured in suitable clean and secure accommodation, well separated from diagnostic facilities or commercial poultry.

   Production of the vaccine should be on a seed-lot system using a suitable strain of virus of known origin and passage history. SPF eggs must be used for all materials employed in propagation and testing of the vaccine. Live vaccines are made by growth in eggs or cell cultures. Inactivated IBD vaccines may be made using virulent virus grown in the bursae of young birds, or using attenuated, laboratory-adapted strains of IBDV grown in cell culture or embryonated eggs. A high virus concentration is required. These vaccines are made as water-in-oil emulsions. A typical formulation is to use 80% mineral oil to 20% suspension of bursal material in water, with suitable emulsifying agents.

3. **In-process control**

   **Antigen content:** Having grown the virus to high concentration, its titre should be assayed by use of cell cultures, embryos or chickens as appropriate to the strain of virus being used. The antigen content required to produce satisfactory batches of vaccine should be based on determinations made on test vaccine that has been shown to be effective in laboratory and field trials.

   **Inactivation of killed vaccines:** This is often done with either beta-propiolactone or formalin. The inactivating agent and the inactivation procedure must be shown under the conditions of vaccine manufacture to inactivate the vaccine virus and any potential contaminants, e.g. bacteria, that may arise from the starting materials.

   Prior to inactivation, care should be taken to ensure a homogeneous suspension free from particles that may not be penetrated by the inactivating agent. A test for inactivation of the vaccine should be carried out on each batch of both the bulk harvest after inactivation and the final product. The test selected should be appropriate to the vaccine virus being used and should consist of at least two passages in susceptible cell cultures, embryos or chickens, with ten replicates per passage. No evidence of the presence of any live virus or microorganism should be observed.

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\(^3\) See footnote 2
Sterility of killed vaccines: Oil used in the vaccine must be sterilised by heating at 160°C for 1 hour, or by filtration, and the procedure must be shown to be effective. Tests appropriate to oil-emulsion vaccines are carried out on each batch of final vaccine as described, for example, in the European Pharmacopoeia.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter I.1.5.

b) Safety

- Live vaccine safety test
  
  Ten field doses of vaccine are administered by eyedrop to each of 15 SPF chickens of the minimum age recommended for vaccination and not older than 2 weeks. The chickens are observed for 21 days. If more than two chickens die due to causes not related to the vaccine, the test must be repeated. The vaccine fails the test if any chickens die or show signs of disease attributable to the vaccine. This test is performed on each batch of final vaccine.

- Killed vaccine safety test

  Ten SPF birds, 14–28 days of age, are inoculated by the recommended routes with twice the field dose. The birds are observed for 3 weeks. No abnormal local or systemic reaction should develop. The test is performed on each batch of final vaccine.

c) Potency

- Live vaccine potency test

  A potency test (virus titration) in eggs or cell cultures must be carried out on each serial (batch) of vaccine produced. In addition, the method described in Section C.1.c. ‘Live vaccine (efficacy test)’ must be used on one batch representative of all the batches prepared from the same seed lot.

- Killed vaccine potency test

  Ten SPF chickens, approximately 4 weeks of age, are each vaccinated with one dose of vaccine given by the recommended route. An additional ten control birds of the same source and age are housed together with the vaccinates. The antibody response of each bird is determined 4–6 weeks after vaccination in a VN test with reference to a standard antiserum. The mean antibody level of the vaccinated birds should not be significantly less than the level recorded in the test for protection. No antibody should be detected in the control birds. This test must be carried out on each batch of final vaccine.

d) Stability

Evidence should be provided on three batches of vaccine to show that the vaccine passes the batch potency test at 3 months beyond the requested shelf life.

e) Preservatives

A preservative is normally required for vaccine in multidose containers. The concentration of the preservative in the final vaccine and its persistence throughout shelf life should be checked. A suitable preservative already established for such purposes should be used.

f) Precautions (hazards)

Oil-emulsion vaccines cause serious injury to the vaccinator if accidentally injected into the hand or other tissues. In the event of such an accident the person should go at once to a hospital, taking the vaccine package with them. Each vaccine bottle and package should be clearly marked with a warning of the serious consequences of accidental self-injury. Such wounds should be treated by the casualty doctor as a ‘grease gun injury’.

5. Tests on the final product

a) Safety

See Section C.4.b.
Chapter 2.7.1. — Infectious bursal disease (Gumboro disease)

b) Potency

See Section C.4.c.

REFERENCES


Chapter 2.7.1. — Infectious bursal disease (Gumboro disease)


**RECENT REVIEWS**


* * *

**NB:** There are OIE Reference Laboratories for Infectious bursal disease (Gumboro 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 2.72.

MAREK’S DISEASE

SUMMARY

Marek’s disease (MD) is a lymphomatous and neuropathic disease of domestic fowl caused by herpesvirus.

Diagnosis is made on clinical signs and gross or microscopic lesions. Chickens may become persistently infected with MD virus (MDV) without developing clinical disease. Infection by MDV is detected by virus isolation and the demonstration of viral antigen or antibodies.

MD is prevented by vaccination with monovalent or multivalent live virus vaccines of various types. The vaccine is injected in ovo or at hatch.

In chickens, MD occurs at 3–4 weeks of age or older and is most common between 12 and 30 weeks of age. Paralysis of the legs and wings, with enlargement of peripheral nerves, is pathognomonic, but nerve involvement is sometimes not seen, especially in adult birds. Depending on the strain of MDV, lymphomatosis can occur, especially in the ovary, liver, spleen, kidneys, lungs, heart, proventriculus and skin. As opposed to the uniform cell population that comprises the tumours caused by lymphoid leukosis, the nerve infiltration and lymphomas caused by MDV consist of lymphoid cells of various types. Differentiation of MD from lymphoid leukosis is important. Lesions caused by reticuloendotheliosis virus can also be confused with those of MD.

Identification of the agent: Under field conditions, most chickens become infected with MDV during the first few weeks of life and then carry the infection throughout their lives, often without developing overt disease. The infection is usually detected by inoculating live buffy coat cells on to monolayer cultures of chicken kidney cells or duck embryo fibroblasts, in which characteristic viral plaques develop within a few days. Two serotypes of MDV are recognised – 1 and 2 – and a third serotype is represented by the related herpesvirus of turkeys (HVT). Serotype 1 includes the virulent strains and serotype 2 the naturally avirulent strains. MD viral antigen can be detected in the feather tips of infected birds using a radial precipitin test.

Serological tests: Antibodies to MDV develop within 1–2 weeks of infection and are commonly recognised by the agar gel immunodiffusion test, the indirect fluorescent antibody test, and sometimes by other serological tests such as enzyme-linked immunosorbent assay.

Requirements for vaccines and diagnostic biologicals: MD is prevented by vaccinating chickens in ovo or at 1 day of age. Live viral vaccines are used. HVT, in either a cell-free (lyophilised) form, or a cell-associated (‘wet’) form, is most commonly used. Attenuated variants of serotype 1 strains of MDV are also used as vaccines, and serotype 2 strains may also be used, particularly in bivalent vaccines, together with HVT (serotype 3). Serotype 1 and 2 vaccines are only available in the cell--associated form. Bivalent vaccines consisting of serotypes 1 and 3 or trivalent vaccines consisting of serotypes 1, 2, and 3 are also used. The bivalent and trivalent vaccines have been introduced to combat the very virulent strains of MDV that are not well controlled by the usual monovalent vaccines.

Vaccination greatly reduces clinical disease, but not persistent infection by MDV. The vaccine viruses are also carried throughout the life of the fowl.

A. INTRODUCTION

Marek’s disease (MD) (21, 24, 32) is a disease of domestic fowl (chickens) caused by a herpesvirus. It occurs at 3–4 weeks of age or older and is most common between 12 and 30 weeks of age. In the classical form of the
disease, characterised by mainly neural involvement, mortality rarely exceeds 10–15% and can occur over a few weeks or many months. In the acute form, in which there is usually lymphoma formation in the viscera, a disease incidence of 10–30% in the flock is not uncommon and outbreaks involving up to 70% can occur. Mortality may increase rapidly over a few weeks and then cease, or can continue at a steady or slowly falling rate for several months. Currently, the acute form of the disease with extensive visceral lymphomas is most prevalent. In its classical form, the most common clinical sign of MD is partial or complete paralysis of the legs and wings. In the acute form, birds are often severely depressed and some may die without showing preceding signs.

In the classical form, the characteristic finding is enlargement of one or more peripheral nerves. Those most commonly affected and easily seen at post-mortem are the brachial and sciatic plexuses, coeliac plexus, abdominal vagus and intercostal nerves. Affected nerves are often two or three times their normal thickness, the normal cross-striated and glistening appearance is absent, and the nerve may appear greyish or yellowish, and sometimes oedematous. Lymphomas are sometimes present in the classical form of MD, most frequently as small, soft, grey tumours in the ovary, and sometimes also in the lungs, kidneys, heart, liver and other tissues. ‘Grey eye’ caused by an iridocyclitis that renders the bird unable to accommodate the iris in response to light and causes a distorted pupil is common in older (16–18 week) birds, and may be the only presenting sign.

In the acute form, the typical finding is widespread, diffuse lymphomatous involvement of the liver, gonads, spleen, kidneys, lungs, proventriculus and heart. Sometimes lymphomas also arise in the skin around the feather follicles and in the skeletal muscles. Affected birds usually have enlarged peripheral nerves, as in the classical form. In younger birds, liver enlargement is usually moderate in extent, but in adult birds the liver may be greatly enlarged and the gross appearance identical to that seen in lymphoid leukosis, from which the disease must be differentiated. Nerve lesions are often absent in adult birds with MD.

In both the classical and acute forms of MD, the disease starts as a proliferation of lymphoid cells, which is progressive in some cases and regressive in others. The peripheral nerves may be affected by proliferative, inflammatory or minor infiltrative changes, which are termed type A, B, and C lesions, respectively. The A-type lesions consist of infiltration by proliferating lymphoblasts, large, medium and small lymphocytes, and macrophages, and appear to be neoplastic in nature. The B-type lesion is characterised by interneuritic oedema, infiltration by mainly small lymphocytes and plasma cells, and Schwann cell proliferation, and appears to be inflammatory. The C-type lesion consists of a light scattering of mainly small lymphocytes, and is often seen in birds that show no gross lesions or clinical signs. It is thought to be a regressive, inflammatory lesion. Demyelination frequently occurs in nerves affected by the A- and B-type lesions, and is responsible for the clinical paralysis.

Lymphomas in the visceral organs and other tissues are similar cytologically to the lymphoproliferations in the A-type lesions in nerves. Usually the lymphoid cells are of mixed types, often with a preponderance of small and medium lymphocytes, but sometimes, particularly in acute MD in adult birds, large lymphocytes and lymphoblasts may predominate.

The heterogeneous population of lymphoid cells in MD lymphomas, as seen in haematoxylin-and-eosin-stained sections, or in impression smears of lymphomas stained by May–Grünwald–Giemsa, is an important feature in differentiating the disease from lymphoid leukosis, in which the lymphomatous infiltrations are composed of uniform lymphoblasts. Another important difference is that, in lymphoid leukosis, gross lymphomas occur in the bursa of Fabricius, and the tumour has an intrafollicular origin and pattern of proliferation. In MD, although the bursa is sometimes involved in the lymphoproliferation, the tumour is less apparent, diffuse and interfollicular in location. Peripheral nerve lesions are not a feature of lymphoid leukosis as they are in MD. The greatest difficulty comes in distinguishing between lymphoid leukosis and forms of MD sometimes seen in adult birds in which the tumour is lymphoblastic with marked liver enlargement and absence of nerve lesions. Here it may be necessary to resort to specialised techniques, such as detection by immunofluorescence of activated T cell antigens present on the surface of MD tumour cells (MD tumour-associated surface antigen or MATSA), or of B-cell antigens or IgM on the tumour cells of lymphoid leukosis. However, a diagnosis can usually be made on gross lesions and histopathology if several affected birds are examined at post-mortem.

Nerve lesions and lymphomatous proliferations induced by certain strains of reticuloendotheliosis virus are similar, both grossly and microscopically, to those present in MD. Although reticuloendotheliosis virus is not common in chicken flocks, it should be borne in mind as a possible cause of lymphoid tumours; its recognition depends on virological and serological tests on the flock. Reticuloendotheliosis virus can also cause neoplastic disease in turkeys, ducks, quail and other species. Another retrovirus also causes lymphoproliferative disease in turkeys. Although chicken flocks may be seropositive for reticuloendotheliosis virus, neoplastic disease is rare. The main features in the differential diagnosis of MD, lymphoid leukosis and reticuloendotheliosis are shown in Table 1.

There are no recognised health risks to humans working with MD virus (MDV) or the related herpesvirus of turkeys (HVT).
B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Infection by MDV in a flock may be detected by isolating the virus from the tissues of infected chickens. Commonly used sources are buffy coat cells from heparinised blood samples, or suspensions of lymphoma cells or spleen cells. When these samples are collected in the field, it is suggested that they be transported to the laboratory under chilled conditions. As MDV is highly cell associated, it is essential that these cell suspensions contain viable cells. The cell suspensions are inoculated into monolayer cultures of chicken kidney cells or duck embryo fibroblasts (chicken embryo fibroblasts are less sensitive for primary virus isolation). Serotype 2 and 3 viruses (see Section C.1.a.) are more easily isolated in chicken embryo fibroblasts than in chicken kidney cells. Usually a 0.2 ml suspension containing from $10^6$ to $10^7$ live cells is inoculated into duplicate monolayers grown in plastic cell culture dishes (60 mm in diameter). Inoculated and uninoculated control cultures are incubated at 38.5°C in a humid incubator containing 5% CO₂. Alternatively, closed culture vessels may be used. Culture medium is replaced at 2-day intervals. Areas of cytopathic effects, termed plaques, appear within 3–5 days and can be enumerated at about 7–10 days.

Table 1. Features useful in differentiating Marek’s disease, lymphoid leukosis and reticuloendotheliosis

<table>
<thead>
<tr>
<th>Feature</th>
<th>Marek’s disease</th>
<th>Lymphoid leukosis</th>
<th>Reticuloendotheliosis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Any age. Usually 6 weeks or older</td>
<td>Not under 16 weeks</td>
<td>Not under 16 weeks</td>
</tr>
<tr>
<td>Signs</td>
<td>Frequently paralysis</td>
<td>Nonspecific</td>
<td>Nonspecific</td>
</tr>
<tr>
<td>Incidence</td>
<td>Frequently above 5% in unvaccinated flocks</td>
<td>Rarely above 5%</td>
<td>Rare</td>
</tr>
<tr>
<td>Macrophscopic lesions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neural involvement</td>
<td>Frequent</td>
<td>Absent</td>
<td>Infrequent</td>
</tr>
<tr>
<td>Bursa of Fabricius</td>
<td>Diffuse enlargement or atrophy</td>
<td>Nodular tumours</td>
<td>Nodular tumours</td>
</tr>
<tr>
<td>Tumours in skin, muscle and proventriculus, ‘grey eye’</td>
<td>May be present</td>
<td>Usually absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Microscopic lesions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neural involvement</td>
<td>Yes</td>
<td>No</td>
<td>Infrequent</td>
</tr>
<tr>
<td>Liver tumours</td>
<td>Often perivascular</td>
<td>Focal or diffuse</td>
<td>Focal</td>
</tr>
<tr>
<td>Spleen</td>
<td>Diffuse</td>
<td>Often focal</td>
<td>Focal or diffuse</td>
</tr>
<tr>
<td>Bursa of Fabricius</td>
<td>Interfollicular tumour and/or atrophy of follicles</td>
<td>Intrafollicular tumour</td>
<td>Intrafollicular tumour</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Lymphoid proliferation in skin and feather follicles</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Cytology of tumours</td>
<td>Pleomorphic lymphoid cells, including lymphoblasts, small, medium and large lymphocytes and reticulum cells. Rarely can be only lymphoblasts</td>
<td>Lymphoblasts</td>
<td>Lymphoblasts</td>
</tr>
<tr>
<td>Category of neoplastic lymphoid cell</td>
<td>T cell</td>
<td>B cell</td>
<td>B cell</td>
</tr>
</tbody>
</table>

*Reticuloendotheliosis virus may cause several different syndromes. The bursal lymphoma syndrome is most likely to occur in the field and is described here.
Another, less commonly used source of MDV for diagnostic purposes is feather tips, from which cell-free MDV can be extracted. Tips about 5 mm long, or minced tracts of skin containing feather tips, are suspended in an SPGA/EDTA (sucrose, phosphate, glutamate and albumin/ethylene diamine tetra-acetic acid) buffer for extraction and titration of cell-free MDV. The buffer is made as follows: 0.2180 M sucrose (7.462 g); 0.0038 M monopotassium phosphate (0.052 g); 0.0072 M dipotassium phosphate (0.125 g); 0.0049 M L-monosodium glutamate (0.083 g); 1.0% bovine albumin powder (1.000 g); 0.2% EDTA (0.200 g); and distilled water (100 ml). The buffer is sterilised by filtration and should be at approximately pH 6.5.

This suspension is ultrasonicated and then filtered through a 0.45 µm membrane filter for inoculation on to 24-hour-old drained chicken kidney cell monolayers. After absorption for 40 minutes, the medium is added, and cultures are incubated as above for 7–10 days.

Using these methods, MDV of serotypes 1 and 2 may be isolated, together with the HVT (serotype 3), if it is present as a result of vaccination. With experience, plaques caused by the different virus serotypes can be differentiated fairly accurately on the basis of time of appearance, rate of development, and plaque morphology. HVT plaques appear earlier and are larger than serotype 1 plaques, whereas serotype 2 plaques appear later and are smaller than serotype 1 plaques.

MDV and HVT plaques may be identified as such using specific fluorescent antibodies raised in chickens. Monoclonal antibodies may be used to differentiate serotypes.

2. Serological tests

The presence of antibodies to MDV in nonvaccinated chickens from about 4 weeks of age is an indication of infection. Before that age, such antibodies may represent maternal transmission of antibody via the yolk and are not evidence of active infection.

Viruses, antigens and antisera are usually available from OIE Reference Laboratories for Marek’s Disease (see Table in Part 3 of this Terrestrial Manual), but international standard reagents have not yet been produced.

a) Agar gel immunodiffusion

There is no prescribed test for trade, but the agar gel immunodiffusion (AGID) test is employed most commonly to detect antibody. The test is conducted using glass slides coated with 1% agar in phosphate buffered saline containing 8% sodium chloride. Adjacent wells are filled with antigen or serum and these are incubated in a humid atmosphere at 37°C for 24 hours for diffusion to take place; positive sera show reactions of identity with known positive serum and antigen. The antigen used in this test is either disrupted MDV-infected tissue culture cells or an extract of feather tips, or skin containing feather tracts obtained from MDV-infected chickens. The cell culture antigen is prepared by propagating MDV in chicken kidney cells or chicken embryo fibroblast cells. When cytopathic effect is confluent, the cells are detached from the culture vessel and suspended in culture medium or phosphate buffered saline without tryptose phosphate broth (presence of tryptose phosphate broth may produce nonspecific precipitin lines) at a concentration of about 1 × 10^7 cells/ml. This suspension is then freeze–thawed three times and used as antigen.

- Test procedure
  i) Make a 1% solution of Difco Bactoagar in 8% sodium chloride by standing the mixture in a boiling water bath.
  ii) Pipette 4 ml of the agar solution on to a 7.5 cm × 2.5 cm microscope slide and allow to set.
  iii) Cut holes in the agar using a template and No. 1 cork borer. The diameter of wells should be 5 mm, and the wells should be 2 mm apart. Remove agar plugs with a swab stick or pen nib.
  iv) Pipette test sera into the top and bottom rows of wells, and standard positive serum and antigen alternately along the centre row.
  v) Incubate the slide for 24 hours at 37°C in a humid container and read the results over a lamp in a darkened room.

A variation of the AGID test may be used to detect MDV antigen in feather tips as an indication of infection by MDV. Glass slides are prepared with a coating of 0.7% agarose (e.g. A37) in 8% sodium chloride,
containing MDV antiserum. Tips of small feathers are taken from the birds to be examined and are inserted vertically into the agar, and the slides are maintained as described above. The development of radial zones of precipitation around the feather tips denotes the presence in the feather of MDV antigen and hence of infection in the bird.

b) Other tests

Other tests for MDV antibody include the direct and indirect fluorescent antibody test. These demonstrate the ability of a test serum to stain MDV plaques in cell cultures (15, 28). These tests are group specific and more sensitive than the AGID test. A virus neutralisation test for the ability of a serum to neutralise the plaque-forming property of cell-free MDV can also be employed (5). However, this test is more suitable for research purposes than for routine diagnostic use. An enzyme-linked immunosorbent assay (ELISA) for detecting MDV antibodies is available (7, 12). To prepare antigen for the ELISA, wells of a 96-well microtitre plate are coated with MDV-infected chicken embryo fibroblast cells. The details of the procedure have been published (7, 24).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Commercial biological products used in the control of MD are the cell-associated or cell-free (lyophilised) live virus or HVT, respectively (see below). Although genetically engineered recombinant vaccines have been developed (19, 22), they are currently not in commercial use. Marek’s disease vaccines are injected in ovo at the 17th or 18th day of embryonation (25) or subcutaneously at hatch. The requirements for producing vaccines are outlined below, and in Chapter I.1.7. Principles of veterinary vaccine production, but other sources should be consulted for further information on the procedures (8–10, 17, 18, 20, 29, 31). Protocols are given in the British Pharmacopoeia Monograph 589, and the US Code of Federal Regulations, Volume 9, part 113 (30). The guidelines in this Terrestrial Manual are intended to be general in nature and maybe supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

Viruses of the MDV group are classified under three serotypes – 1, 2, and 3 – on the basis of their antigenic relatedness.

**Serotype 1:** This includes all the pathogenic strains of the virus, ranging from strains that are very virulent plus (e.g. 648A), very virulent (e.g. Md/5, Md/11, Ala-8, RB-1B), virulent (e.g. HPRS-16, JM GA), mildly virulent (e.g. HPRS-B14, Conn A) and finally to weakly virulent (e.g. CU-2, CVI-988). These strains may be attenuated by passage in tissue culture, with loss of pathogenic properties but retention of immunogenicity, to provide strains that have been used as vaccines. Those that have been used commercially include attenuated HPRS-16 and CVI-988 (Rispens) strains. Attenuated variants of the very virulent stains have been used in experimental vaccines to protect against the variant form of acute MD caused by the very virulent stains, and the R2/23 vaccine strain derived from Md/5 is licensed in the United States of America. Serotype 1 vaccines are prepared in a cell-associated ('wet') form that must be stored in liquid nitrogen.

**Serotype 2:** This includes naturally avirulent strains of MDV (e.g. SB-1, HPRS-24, 301B/1, HN-1), and several of these have been shown to provide protection against virulent strains. The SB-1 and 301B/1 strains have been developed commercially and used, particularly with HVT, in bivalent vaccines for protection against the very virulent strains. Serotype 2 vaccines exist only in the cell-associated form.

**Serotype 3:** This contains the strains of naturally avirulent HVT (e.g. FC126, PB1), which are widely used as a monovalent vaccine, and also in combination with serotype 1 and 2 strains in bivalent or trivalent vaccines against the very virulent strains of MDV. HVT may be prepared in a cell-free form as a freeze-dried (lyophilised) vaccine or in a cell-associated (‘wet’) form.

b) Method of culture

The substrates used for commercial vaccine production are primary chicken embryo fibroblasts (CEF) derived from specific pathogen free (SPF) flocks or duck embryo fibroblasts. CEF from SPF flocks are preferred to duck cells because more is known about chicken-embryo-transmitted pathogens and methods for their detection.

c) Validation as a vaccine

Methods for testing SPF flocks for freedom from infection are available (18, 29). SPF chicken flocks should be free from avian adenoviruses, including egg-drop syndrome 76 virus, avian encephalomyelitis virus, avian leukosis virus (subgroups A, B and J), avian nephritis virus, avian reoviruses, avian rotaviruses,
Chapter 2.7.2. – Marek’s disease

chicken anaemia virus, fowl pox virus, infectious bronchitis virus, infectious bursal disease virus, infectious laryngotracheitis virus, influenza type A virus, MDV, Mycoplasma gallisepticum, Mycoplasma synoviae, Newcastle disease virus, reticuloendotheliosis virus, Salmonella spp., and turkey rhinotracheitis virus.

SPF duck flocks should be free from avian adenoviruses, avian reoviruses, Chlamydia, duck virus enteritis, duck virus hepatitis types I and II, influenza type A virus, Newcastle disease virus, Pasteurella (now Riemerella) anatipestifer, reticuloendotheliosis virus, and Salmonella infections.

Freedom from other infections may also be required as they become recognised.

The master seed virus should be shown to be nonpathogenic for chickens by inoculating ten times the field dose into 1-day-old SPF chickens of a strain susceptible to MD, to ensure that it does not cause gross lesions or significant microscopic lesions of MD by 120 days of age. It should be noted that some vaccine strains of MDV and HVT may produce minor and transient microscopic nerve lesions under these circumstances.

No increase in virulence should occur during six serial passages of the vaccine strain in 1-day-old SPF MD-susceptible chickens. Ten times the field dose of vaccine is inoculated initially and then passaged by inoculation of heparinised blood at 5–7-day intervals, and tests for viraemia are run to check that virus is transferred at each passage. The birds receiving the final passage are kept for 120 days and should be free from MD lesions. However, some strains such as Rispens, may cause some mild MD lesions. The important observation is that the virulence should not change. This is a difficult test because the genetic resistance of the chickens fundamentally affects the apparent virulence of the virus, so does the type of inoculum. After successful completion of laboratory safety tests, the safety of the strain should be confirmed in extensive field trials.

Seed virus must be free from the agents listed for SPF flocks and from other contaminants that may be acquired in the laboratory. A vaccine strain derived from turkeys must also be free from lymphoproliferative disease virus and haemorrhagic enteritis virus.

The ability of the master seed virus – and derived virus at the limit of the passage range used to produce vaccinal virus (usually not more than five tissue culture passages) – to protect against MD must be determined. Standardised protection tests are published. They involve vaccination of MD-susceptible SPF chickens at 1 day of age and challenge with sufficient virulent MDV 8 days later to cause at least a 70% incidence of MD in unvaccinated chickens. Two types of tests are used. In the protection index test, a single field dose (1000 PFU) (plaque-forming units) of vaccine is given and the incidence of MD in vaccinated birds is compared with that in unvaccinated birds. Protective indices should be greater than 80, i.e. vaccinated birds should show at least 80% reduction in the incidence of gross MD, compared with unvaccinated controls.

A PD_{50} (50% protective dose) test is also used, involving the inoculation of five four-fold serial dilutions of vaccine virus selected to provide protection above and below the 50% level, followed by challenge 8 days later to determine the PD_{50} value. The assays are conducted using a standard reference vaccine for comparison. The PD_{50} may be as low as 4 PFU, but higher values can be obtained depending on the vaccine strain, whether cell-free or cell-associated and the presence or absence of maternal antibodies in the test chickens. On the basis of the PD_{50} test, it has been suggested that the minimum vaccine field dose should be the greater of two values: 10^3 PFU or 100 PD_{50}.

Extensive field trials of a new vaccine strain in the presence of field challenge should be conducted, using different breeds of birds of varying MDV maternal antibody status, to ensure efficacy and persistence of immunity. Experience suggests that vaccinal immunity, once acquired, is lifelong.

2. Method of manufacture

Substrate cells are seeded into flat-bottomed vessels for stationary incubation, or into cylindrical vessels for rolled incubation. Media commonly used are Eagle’s minimal essential medium, or 199 medium, buffered with sodium bicarbonate and supplemented with 5% calf serum. Incubation is at 38–39°C for 48 hours.

For cell-associated vaccine, cultures are infected with production HVT or MDV seed-virus stock, in cell-associated form, which is usually two passages beyond the master seed stock. Cultures are incubated for 48 hours then the infected cells are harvested by treating the washed cell sheet with an EDTA/trypsin solution to allow the cells to begin to detach. The flasks are then returned to the incubator (38.5°C) to allow complete detachment. The cells are subjected to low-speed centrifugation, and then resuspended in the freezing mixture consisting of cell growth medium containing 7.5–15% dimethyl sulphoxide, and held at 4°C or dispensed immediately into the final vaccine containers, usually glass ampoules, which are flame sealed and frozen in liquid nitrogen.
Cell-free lyophilised vaccine may be prepared from HVT, but not from MDV strains. For the production of this form of vaccine, HVT-infected cultures are incubated for 72 hours, infected cells are detached from the vessel as described above, or scraped from the walls of the vessel. The cells are suspended in a small volume of growth medium, centrifuged, and resuspended in a buffered stabiliser solution containing 8% sucrose, but free from protein to prevent frothing. The cell suspension is sonicated to release virus, the cell debris is removed, the suspension is diluted with a complete stabiliser – such as SPGA – filled into the final containers, and lyophilised.

The dilution rate for both cell-associated and cell-free vaccines is based on previous experience, as is the number of doses required per container, because the virus content of the harvested material cannot be assayed prior to filling the final containers. The virus content of the finished product can subsequently be added to the label.

3. In-process control

For optimal results in preparing cell-associated vaccine, a slow rate of freezing (1–5°C per minute) and rapid thawing are essential. The infectivity titre of the infected cells, and hence the number of doses per ampoule, are determined after filling the ampoules. Similarly for cell-free vaccine, the virus content of the final suspension, and hence the number of doses per container, is determined after filling.

4. Batch control

a) Identity

Using monospecific neutralising serum, checks should be carried out to show that the product is of the same specificity as the seed virus. This is best done using monoclonal antibodies.

b) Safety and sterility

Extensive testing is required of the materials used to produce the vaccine, and of the final product. Substrate cells should come from an SPF flock, in particular, free from vertically transmitted agents. Substances of animal origin used in the preparation of vaccines such as serum, trypsin, and bovine serum albumin, must be free from extraneous agents.

Batches of the final vaccine produced should be tested for freedom from contaminating bacteria, fungi, mycoplasma and the viruses listed for SPF flocks; tests for purity of the diluent should also be conducted. Suitable tests for the detection of extraneous agents at all stages of vaccine production are recommended by several official bodies (18, 20, 29) and in Chapter I.1.5.

Ten doses of vaccine or a quantity of diluent equivalent to two doses of vaccine should be inoculated into separate groups of ten 1-day-old SPF chickens. No adverse reactions should occur during a 21-day observation period.

c) Potency

The standard dose of each type of vaccine is 1000 PFU per chicken or egg. Virus content assays are conducted on batches of vaccine to ensure that the correct dose per bird will be achieved.

d) Duration of immunity

A test for duration of immunity is carried out on the seed virus only. Such immunity is apparently lifelong.

e) Stability

Tests for stability are carried out on six representative batches of vaccine to show that titre is maintained during the stated shelf life of the vaccine. These tests should be conducted under the conditions of storage of the vaccine. The lyophilised product should have a shelf life of 12 months when stored at 2–8°C. Manufacturers may double the virus content of the vaccine to compensate for some loss of titre during storage. Appropriate diluting fluids are provided for use with cell-associated and freeze-dried vaccines. The stability of reconstituted vaccine over a 2-hour period should be tested.

f) Preservatives

Preservatives are not included in the vaccine or diluent.

g) Precautions (hazards)

With cell-associated vaccine, care is necessary to avoid injury from ampoules that may explode when they are removed from liquid nitrogen. Eye protection must be worn. During use, reconstituted vaccine must be kept cool and cell-associated vaccine should be agitated to keep cells in suspension.
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 Marek’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 2.7.3. AVIAN MYCOPLASMOSIS (Mycoplasma gallisepticum)**

**SUMMARY**

**Definition of the disease:** Avian mycoplasmosis is caused by several pathogenic mycoplasmas of which Mycoplasma gallisepticum (MG) is the most important and the only one that causes an OIE notifiable disease.

**Description of the disease:** MG is a cause of chronic respiratory disease, especially in the presence of management stresses and/or other respiratory pathogens. Disease is characterised by coryza, conjunctivitis, sneezing, and by sinusitis, particularly in turkeys and game birds. It can result in loss of production and downgrading of meat-type birds and loss of egg production. MG strains vary in infectivity and virulence, and infections may sometimes be inapparent.

**Identification of the agent:** MG can be identified by immunological methods after isolation in mycoplasma media or by detection of its DNA in field samples or cultures.

Samples for isolation can be swabs of organs or tissues, exudates, diluted tissue homogenates, aspirates from the infraorbital sinuses or joint cavities, or material from yolk or embryos. Clinical signs and lesions will influence the sample selection. Broth and agar are used for isolation, but it is normally necessary to obtain mycoplasma colonies on agar before attempting identification. Basic biochemical tests can be helpful in preliminary classification of isolates but final identification is by immunological tests, the most satisfactory being fluorescent antibody and immunoperoxidase tests.

DNA detection methods, based on the polymerase chain reaction, are used in specialised laboratories and commercial kits are available.

**Serological tests:** Several serological tests are used to detect MG antibodies, but due to variations in specificity and sensitivity, they are more satisfactory for flock screening than for testing individuals.

The most commonly used are the rapid serum agglutination (RSA) test, the enzyme-linked immunosorbent assay (ELISA) and the haemagglutination inhibition (HI) test. In the RSA test, sera are mixed with commercially produced stained MG antigen and those that react within 2 minutes are heated at 56°C for 30 minutes and retested. Sera that still react, especially when diluted, are considered to be positive. ELISA or HI can be used as confirmatory tests. Several commercial MG antibody ELISA kits are available.

**Requirements for vaccines and diagnostic biologicals:** Although the preferred method of control is maintenance of MG-free flocks, both live and inactivated vaccines are used in chickens. Vaccination should be considered only on multi-age sites where infection is inevitable. The usual use is to prevent egg-production losses in commercial layers, although vaccines may also be used to reduce egg transmission in breeding stock or to aid MG eradication on multi-age sites. It is important to vaccinate before field challenge occurs.

Available live vaccines are produced from the F strain of MG, and, more recently, strains ts-11 and 6/85, which are apathogenic strains with improved safety characteristics. Administration of the F strain by the intranasal or eyedrop route is preferred, but aerosol or drinking water administration may be used. The eyedrop method is recommended for ts-11, and a fine spray for 6/85. Pullets are generally vaccinated between 12 and 16 weeks of age. One dose is sufficient and vaccinated birds remain permanent carriers. Long-term use of the F strain on multi-age sites results in displacement
of field strains. The ts-11 strain has been successfully used to eradicate F strain in multi-age commercial layers.

Bacterins consist of a concentrated suspension of MG organisms in an oil emulsion. They are administered parenterally to pullets at 12–16 weeks of age, usually subcutaneously in the neck. Two doses are desirable. Bacterins are effective in preventing egg-production losses and respiratory disease, but they do not prevent infection with wild-type MG.

A. INTRODUCTION

Mycoplasma gallisepticum (MG) belongs to the class Mollicutes, order Mycoplasmatales, family Mycoplasmataceae. It should be noted, however that M. synoviae (MS), M. meleagridis and M. iowae can also cause disease in poultry, but MG is considered to be the most important of the pathogenic mycoplasmas and the OIE has designated the disease caused by MG as notifiable. MG occurs world-wide and is particularly important in chickens and turkeys as a cause of respiratory disease and decreased production (3, 15). It can also cause upper respiratory disease in game birds. More recently MG has been recognised in North America in house finches as a cause of conjunctivitis (16). In poultry the infection is spread vertically through infected eggs and horizontally by close contact; the MG nucleic acid has been identified in environmental samples (18). Other methods of spread are less well documented.

The clinical signs in infected poultry can vary from asymptomatic to obvious respiratory signs including coryza, conjunctivitis, coughing and sneezing. Nasal exudate, tracheal rales and breathing through the partially open beak may occur. Unilateral or bilateral sinusitis may also be a feature, particularly in turkeys and game birds and the infraorbital sinuses may become so swollen that the eyelids are closed. Conjunctivitis, with frothy ocular exudate is also a common feature in turkeys and game birds, and sometimes in chickens. In turkeys there is often soiling of the wing feathers as the result of attempts to remove exudate from the eyes. Infected finches may reveal ocular and nasal discharge and swollen eyelids in addition to the conjunctivitis.

Under certain circumstances, MG may be associated with acute respiratory disease in chickens and turkeys, especially in young birds, with the turkey being more susceptible. The severity of the disease is greatly affected by the degree of secondary infection with viruses such as Newcastle disease and infectious bronchitis, and/or bacteria such as Escherichia coli. In turkeys there is synergism with avian pneumovirus infection. A more chronic form of the disease may occur and can cause reduced egg production in breeders and layers.

Lesions of the respiratory tract take the form initially of excess mucous exudate followed by catarrhal and caseous exudate, which may form amorphous masses in the air sacs. In turkeys and game birds the swollen infraorbital sinuses contain mucoid to caseous exudate.

MG disease in chickens may superficially resemble respiratory disease caused by other pathogens such as mild strains of Newcastle disease (Chapter 2.1.15.) and avian infectious bronchitis (Chapter 2.7.6.). These may be present in mixed infection with MG. Infections with Haemophilus paragallinarum, Pasteurella multocida, and MS should also be ruled out. MG in turkeys may be confused with avian pneumovirus infections and the presence of sinusitis may also suggest infection with Pasteurella multocida, Chlamydia (Chapter 2.7.4.) or MS.

B. DIAGNOSTIC TECHNIQUES

The presence of MG can be confirmed by isolating the organism in a cell-free medium or by detecting its DNA directly in infected tissues or swab samples. When results are equivocal, chicken embryos or chickens may be inoculated with suspect material. Serological tests are also widely used for diagnosis.

1. Identification of the agent

   • Culture

   Samples are taken from live birds, fresh carcasses or the carcasses of birds that have been frozen when fresh. Samples may also be collected from dead-in-shell embryos or chickens or poults that have broken the shell but failed to hatch. From live birds, swabs may be taken from the choanal cleft, oropharynx, oesophagus, trachea, cloaca and phallus. In the case of dead birds, samples may be taken from the nasal cavity, infraorbital sinus, trachea, or air sacs. Exudates can be aspirated from the infraorbital sinuses and joint cavities.

   Samples can also be taken from embryonated eggs, e.g. from the inner surface of the vitelline membrane, and from the oropharynx and air sacs.
All samples should be examined as soon as possible after collection. If transportation is necessary, small pieces of tissue should be placed in mycoplasma broth, or swabs should be vigorously agitated in 1–2 ml of mycoplasma broth and then discarded. Alternatively, the swabs can be dipped in mycoplasma broth before the specimens are taken (23) and then replaced in the swab holders for transportation. An ice pack or some other means of chilling should be included. Serial dilutions of specimens may be of value because the presence of specific antibodies or antibiotics or inhibitory substances in tissues may inhibit mycoplasma growth unless they are diluted out.

Several suitable culture media have been formulated (7) and medium suitable for isolation of avian mycoplasmas can be purchased from Mycoplasma Experience, Reigate, Surrey, United Kingdom. Mycoplasma media generally contain a protein digest and a meat-infusion base supplemented with serum or a serum fraction, yeast factors, glucose and bacterial inhibitors. It is important that each new batch of medium be tested with recently isolated MG cultures of low in-vitro passage because some components, especially the yeast extract and the serum may vary in their ability to support growth.

The medium developed by Frey et al. is widely used in the United States of America (USA) and other countries for isolation of MG (and M. synoviae) (2, 8). For the cultivation of MG, nicotinamide adenine dinucleotide may be omitted from the medium.

For the growth of MG the following broth and agar media are also satisfactory:

- Part A: Pleuropneumonia-like organism (PPLO) broth base without crystal violet (Difco) (14.7 g); distilled or deionised water (700 ml).

- Part B: Pig serum (heated at 56°C for 1 hour) (150 ml); 25% (w/v) fresh yeast extract (100 ml); 10% (w/v) glucose solution (10 ml); 5% (w/v) thallous acetate (10 ml); 200,000 International Units (IU)/ml penicillin G (5 ml); and 0.1% (w/v) phenol red solution (20 ml). The pH is adjusted to 7.8. The pig serum may be replaced by horse serum, but it is important to ascertain that it supports the growth of MG.

Part A is autoclaved at 121°C, at 1 atmosphere for 15 minutes and, after cooling, is added to Part B, which has previously been sterilised by filtration. For the corresponding solid medium, 10 g of purified agar, known to support the growth of MG, is added to part A above. The mixture is autoclaved as before and kept in a water bath at 56°C. The constituents of part B, omitting the phenol red, are mixed separately and then incubated at 56°C. Parts A and B are mixed carefully to avoid the production of air bubbles, and are dispensed into 50 mm dishes using 7–9 ml/dish. Excess surface moisture can be removed by a short incubation at 37°C. Plates are stored in an airtight container at approximately 4°C for up to 2 weeks.

Fresh yeast extract is available commercially, although it is preferable to prepare it ‘in-house’ by taking active dry baker’s yeast (250 g) and suspending it in distilled water (1 litre). This is heated to boiling point, cooled and then centrifuged for 20 minutes at 3000 g. The supernatant fluid is decanted and adjusted to pH 8.0 with 0.1 M NaOH. This is clarified by centrifugation or by filtration, and then sterilised by filtration. The extract is stored at –20°C. Reagent grade glucose (10 g) is dissolved in distilled or deionised water (100 ml) and adjusted to pH 7.8–8.0 with 0.1 M NaOH. It is sterilised by filtration and stored at 4°C. Reagent grade thallous acetate is dissolved (5 g) in distilled or deionised water (100 ml), filter-sterilised and stored at –20°C. Penicillin solution (10^6 IU benzyl penicillin in 5 ml distilled water) is stored at 4°C, and has a shelf life of 1 week. For isolation from heavily contaminated samples, penicillin concentration can be increased to 2000 units/ml or ampicillin, 0.5–1.0 mg/ml, maybe used instead. Phenol red (0.1 g) is ground in 0.1 M NaOH (2.8 ml), and then made up to 100 ml in sterile distilled water and autoclaved at 115°C at 1 atmosphere for 30 minutes. It is stored at 4°C. (Note: Thallous acetate is highly toxic and care should be taken, especially when preparing the stock solution.)

Specimens are inoculated on to mycoplasma agar and into broth. Solid medium may help detection of slow-growing mycoplasma colonies, which can be overgrown by saprophytes in broth. It may be necessary to make serial dilutions up to 10^-3 for successful isolation. Inoculated plates are incubated at 37°C in sealed containers. Increased humidity and CO₂ tension in the atmosphere have been reported to enhance growth; these conditions may be obtained by the inclusion of damp paper or cotton wool, and by flushing the container with 5–10% CO₂ in nitrogen, by placing a lighted candle in the container, or by using a CO₂ incubator or suitable gas.generating system.

The caps of liquid medium containers should be tightly sealed before incubation at 37°C to avoid spurious changes in pH. For the first few days, the plates are examined daily for colonies with a stereoscopic microscope; after that they are examined less frequently. Cultures from field material should not be discarded as negative for at least 20 days.
Broth medium should be examined daily for acidity, indicated by a change from red to orange or yellow in the indicator. Any observable growth is subcultured on to solid medium immediately. Even if no colour change occurs, subculture on to solid medium should be made after 7–10 days or earlier as the presence of an arginine-hydrolysing (alkali-producing) mycoplasma species may mask the acid colour change produced by MG.

Mycoplasma colonies on solid medium can usually be recognised, although they may not have the typical 'fried egg' appearance. Bacterial colonies may appear on the first passage, but they are often more pigmented and fail to pass on mycoplasma media.

Biochemical reactions (e.g. fermentation of glucose and failure to hydrolyse arginine) can assist in identification, but they are not specific for MG and necessitate purification of the culture by cloning.

Immunological and DNA detection methods can be used to identify mycoplasma isolates. They include the indirect fluorescent antibody (IFA) and immunoperoxidase (IP) tests, both of which are simple, sensitive, specific and rapid to perform; growth inhibition (GI); and metabolism inhibition (MI). Purified (cloned) cultures are required for the GI and MI tests, but not for the IFA or IP test. IFA and IP can detect the presence of more than one species of mycoplasma, as the colonies specific for the antiserum will react while the others will not. However, *M. imitans*, a mycoplasma species that is serologically related to MG and that presents the same biochemical properties has been isolated from ducks, geese and sometimes from other nondomestic bird species in some countries. It may be distinguished from MG by use of a PCR-RFLP (polymerase chain reaction/restriction fragment length polymorphism), as described by Kempf (10). Alternatively, colonies of the isolate can be examined by immunofluorescence using serial dilutions of antiserum to MG and *M. imitans* in parallel. The homologous antiserum should have a considerably higher titre.

DNA detection methods for identifying MG directly in tissues or for identifying laboratory isolates are discussed below and are usually based on the PCR.

In certain circumstances where results of the above methods are not conclusive, inoculation of chick embryos or bioassays in live chicks may be appropriate. However these techniques are time-consuming and costly and tend to have been replaced by PCR technology, although they remain a useful research tool.

The specimens required for inoculation of chicken embryos are the same as those used for the inoculation of artificial media. They are prepared in broth from which thallous acetate is omitted, incubated for 30–60 minutes at 37°C, and then a 0.05–0.1 ml aliquot is inoculated into the yolk sac of several 6–8-day-old embryos or bioassays in live chicks may be appropriate. However these techniques are time-consuming and costly and tend to have been replaced by PCR technology, although they remain a useful research tool.

Samples such as nasal turbinates, infraorbital sinus, trachea, lungs, air sacs, oviduct, or joint fluid are homogenised in from 5 to 10 volumes of broth, and immediately inoculated into at least four test birds. Each bird is injected intranasally, supra-conjunctivally, intratracheally, into the infraorbital sinus and the air sacs. The chickens are kept in isolation. Two additional control birds are inoculated by the same routes with broth only and kept separately in isolation. An alternative procedure that has been found to be useful is to place mycoplasma-free turkeys in contact with birds from a suspicious flock in an isolation facility as ‘in-contact’ sentinels.

At intervals after inoculation, the sera of all birds are tested for MG antibodies by the rapid serum agglutination (RSA) test. Any turkeys showing respiratory signs are cultured for MG from the air sacs and lungs. After the final bleed at 35 days post-inoculation, all birds are killed and examined for gross and microscopic lesions. Tissues, especially those showing gross lesions, are cultured for mycoplasma. The original samples are considered to be positive for MG if the organism or its DNA is detected in the chickens inoculated with the homogenate or in the 'in-contact' turkeys, or if they develop an RSA titre of 1/4 or more. Titres less than this are interpreted as being doubtful. An enzyme-linked immunosorbent assay (ELISA) or a haemagglutination inhibition (HI) test should be performed. The organism or its DNA should not be detected in the control birds, nor should they develop any RSA titres. There should be no RSA reaction to *M. synoviae* in either group of birds.

- **Immunological methods**
  
  Immunofluorescence and IP procedures for diagnosis are generally applied to suspect laboratory isolates rather than directly to infected exudates or tissues. This is because the organisms are too small to
recognise conclusively under the light microscope and because the corresponding negative and positive control exudate/tissue is unlikely to be readily available.

a) Indirect fluorescent antibody test

The recommended technique for the IFA test (19) requires an agar culture of the unknown isolate, consisting of numerous small discrete colonies, a known MG culture as a positive control, and a culture of a further mycoplasma species, such as M. synoviae or M. gallinarum as a negative control. Also required are polyclonal rabbit anti-MG serum, a normal rabbit serum and an anti-rabbit immunoglobulin fluorochrome-conjugated serum. Sera may be prepared in species other than rabbits, but monoclonal antibodies (MAbs) should not be used because MG demonstrates variable expression of its surface epitopes and an MAB may fail to recognise the organism. Suitable working dilutions in sterile phosphate buffered saline (PBS; 0.01 M, pH 7.2) of the anti-MG serum and the conjugate are first determined by cross-titration, and are selected for use at two-to-four-fold dilutions less than the actual end-points. These are applied to the colonies of mycoplasmas to be identified that have been previously grown on agar plates as indicated below.

- Test procedure
  i) From colony-bearing agar plates cut blocks of about 1.0 × 0.5 cm and place them on to microscope slides with the colonies uppermost.
  ii) To make subsequent orientation possible, cut off the lower right hand corner of the block. A block with the unknown isolate, a block with the known MG culture, and a block with a different but known mycoplasma culture are placed on one slide. A block of the unknown isolate is placed on another slide.
  iii) Add a drop of suitably diluted MG antiserum to each block of the first slide and add normal rabbit serum to the single block on the second slide.
  iv) Incubate all blocks for 30 minutes at room temperature in a humid atmosphere.
  v) Place each block in a labelled tube containing PBS, pH 7.2 and wash for 10 minutes in a rotary mixer, then similarly rewash, and finally return the blocks to the original microscope slides.
  vi) Blot excess moisture from the sides of the blocks. Add one drop of the diluted conjugate to each block, and incubate and wash as before.
  vii) Return the blocks to their original slides, and examine the colonies by incident light using fluorescence microscopy.

Interpretation of the results is subjective and requires some expertise; comparisons with the controls are essential, and they must give the correct reactions.

Some laboratories use fluorescein-conjugated antiserum in a direct immunofluorescence test (direct IMF). A technique that is widely used for direct IMF is one in which the reagents are applied successively within stainless steel rings placed on the original mycoplasma agar plate. Although this is quick and easy to perform, the results obtained are less specific than using the indirect method, which is therefore preferred.

b) Indirect immunoperoxidase test

This involves a similar principle to the IFA test except that the binding of specific antibodies to colonies in situ is detected by adding an anti-rabbit immunoglobulin that has been conjugated to the enzyme peroxidase. A positive reaction is then developed by adding an appropriate substrate which, on oxidation, produces coloured colonies. An immunobinding procedure can also be used in which the test colonies are blotted on to nitrocellulose (12) and then reacted in a similar manner. As with IFA, polyclonal sera should be used for serotyping isolates by IP. The advantage of the IP test over immunofluorescence is that the IP test does not require an expensive fluorescence microscope.

c) Growth inhibition test

In the GI test, the growth of mycoplasmas is inhibited by specific antiserum, enabling species to be identified. It is relatively insensitive and sera must be high-titred, monospecific and prepared in mammalian hosts as poultry sera do not inhibit mycoplasma growth efficiently. The organism under test must be in pure culture (cloned) and several dilutions should be tested; a concentration of 10⁴ colony-forming units (CFU/ml) is optimal. The rate of growth of the organism may influence growth inhibition, and it is helpful to retard growth initially by incubating at 27°C for 24 hours, followed by incubation at 37°C thereafter. Details of the test and its interpretation are published elsewhere (5).
Nucleic acid recognition methods

An alternative to conventional culture and identification is the use of specific DNA detection methods. MG may be detected by hybridisation with DNA probes, but now it is much more common to use the PCR to amplify specific portions of DNA in the test material. One commercial MG DNA test kit uses a PCR directly on material extracted from swabs. If the amplified product is present it is detected using a nonradio labelled probe. Two versions are available; one that detects MG field strains and one that identifies the vaccine F strain. Several ‘in-house’ PCR-based tests have also been published for MG including a multiplex PCR, which is designed to detect all four avian mycoplasma pathogens (21), but which has not been validated with clinical samples. Several methods are cited by Kempf (10) and, in addition, a manual published by Lauerman (13) contains a validated PCR assay for MG and other avian mycoplasmas. This method is presented below.

a) DNA isolation

DNA is extracted from swab samples (three–five may be pooled) suspended in 1 ml of PCR-grade PBS in a 1.5 ml snap-cap Eppendorf tube. The suspension is centrifuged for 30 minutes at 14,000 g at 4°C. The supernatant is carefully removed with a Pasteur pipette and the pellet is suspended in 25 µl PCR-grade water. The tube and the contents are boiled for 10 minutes and then placed on ice for 10 minutes before centrifugation at 14,000 g for 5 minutes. The DNA is in the supernatant.

b) Primers

The MG primers consist of the following sequences.

MG-14F: 5’-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3’

MG-13R: 5’-GCT-TCC-TTG-CGG-TTA-GCA-AC-3’

c) Polymerase chain reaction

The reaction mixture should be prepared in a separate clean area using a set of dedicated pipettes. For one 50 µl PCR reaction the mixture is as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O Ultra-pure</td>
<td>35.75 µl</td>
</tr>
<tr>
<td>10 × PCR Buffer</td>
<td>5.00 µl</td>
</tr>
<tr>
<td>dNTP(l0 mM)</td>
<td>1.00 µl</td>
</tr>
<tr>
<td>F Primer (20 pmole/µl)</td>
<td>0.50 µl</td>
</tr>
<tr>
<td>R Primer (20 pmole/µl)</td>
<td>0.50 µl</td>
</tr>
<tr>
<td>Taq (5 U/µl)</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>MgCl₂ (50 mM)</td>
<td>2.00 µl</td>
</tr>
</tbody>
</table>

A 45 µl volume of the reaction mixture is dispensed into each PCR tube. The reaction mixture should be overlaid with a few drops of light weight mineral oil unless the thermocycler is equipped with a heated lid. The tubes are then taken to another clean area where the appropriate DNA sample (5 µl) is added to each tube. Positive and negative controls should be used in each run.

The tubes are then placed in a thermal cycler for the following cycles: 40 cycles: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, 1 cycle (final extension): 72°C for 5 minutes and soak at 4°C.

d) Electrophoresis

PCR products are detected by conventional agarose gel electrophoresis, incorporating appropriate size markers, followed by examination under UV light. The PCR product for MG is 185 bp. Visualisation of the PCR products should be carried out in a separate laboratory area, well separated from all other steps in the PCR procedure.

At present PCR tests tend to be carried out by specialist laboratories and should probably be regarded as useful adjuncts to the present diagnostic methods until their validity has been firmly established. Great care needs to be taken to avoid contamination of samples with MG DNA from nearby culture laboratories or from positive amplificates from previous PCR runs. However the commercial kit referred to above is now licensed by the United States Department of Agriculture (USDA) as a diagnostic method and approved for use in the National Poultry Improvement Plan (NPIP).

Molecular methods are also available for differentiation of MG strains (10), but their use tends to be restricted at present to specialist laboratories.
Chapter 2.7.3. – Avian mycoplasmosis (Mycoplasma gallisepticum)

2. Serological tests

The serological tests in common use may lack specificity and/or sensitivity; their use is advised for monitoring flocks rather than for testing individual birds. Diagnosticians wishing to use such tests are advised to establish the test sensitivity and specificity (Chapter I.1.3) under their own laboratory conditions. It should also be noted that these tests have not been validated for use with sera from game birds.

The most commonly used tests are RSA, ELISA and HI although several others have been described such as radioimmunoassay, microimmunofluorescence and IP assay. The number of sera to be tested within a flock depends on the level of detection and the confidence limits required. Minimal requirements may be laid down for international trade and the frequency of testing may also be stipulated as, for example, in the European Communities Council Directive 90/539/EEC. Minimal requirements and approved tests are also set out for members of the NPIP of the USA.

Poultry companies using ELISA technology for screening large numbers of sera for virus antibodies may find this type of assay convenient also for mycoplasma testing. The ELISA technology will not be described in detail here because several MG kits are available commercially. Instead, the details of the HI test are provided as the reagents needed for this test are not widely available commercially.

a) Rapid serum agglutination test

Sera are collected from a sample of the flock and, if not tested immediately, are stored at 4°C and not frozen. The test should be carried out at room temperature (20–25°C) within 72 hours of serum collection and the reagents should also be at room temperature. Prior centrifugation will reduce nonspecific reactions. The RSA antigens are available commercially, but they may vary in specificity and sensitivity from different manufacturers and from batch to batch. They must be stored according to the manufacturer’s instructions. Suitable RSA-stained antigens may also be prepared ‘in-house’ using culture methods as described in Section B.1.; these are then stained with crystal violet dye. Quality control standards for mycoplasma antigens for serological tests are described below.

- Test procedure
  i) Drop one volume (approximately 0.02 ml) of serum on to a clean white tile or glass plate followed by one volume of stained MG antigen. Do not allow the serum to dry out before addition of the antigen. It is important to shake the antigen bottle vigorously and frequently during use to keep the correct amount of antigen in suspension.
  ii) Use a stirring rod to spread the mixture over a circular area of approximately 1.5 cm diameter. Rock the tile or plate for 2 minutes. Agglutination is indicated by flocculation of the antigen within 2 minutes.
  iii) Include known positive and negative controls in the test.
  iv) Retest serial dilutions of any sera that agglutinate after heating at 56°C for 30 minutes. If they still react strongly, they are considered to be positive, especially if they do so on dilution (1/4 or more).

In the USA, MG positive reference antisera can be obtained from the USDA National Veterinary Services Laboratories (NVSL), and in Europe from AFFSA Ploufragan1, France. Sera produced in chickens or in turkeys and with a range of titres can be purchased. Sets of antisera can be purchased also from the University of Georgia Department’s of Avian Medicine, subject to availability. They are produced by contact infection with chickens infected with the virulent MG R strain.

There are no international standards for interpreting these tests, but a high proportion of positive sera in a flock (10% or more) indicates MG infection, especially if confirmed by HI test or ELISA. For further confirmation, the flock should be restested within a month. Inconclusive results make it necessary to attempt to isolate the organism or to demonstrate the presence of its DNA. Doubtful results should be investigated by performing tests with *M. synoviae* antigen as infection with this organism sometimes causes cross-reactions.

Tests can be conducted on yolk as well as sera although the yolk must first be diluted or extracted.

b) Haemagglutination inhibition test

MG is capable of haemagglutinating avian red blood cells (RBCs), and specific antibodies in sera cause inhibition. A strain should be selected that grows well and haemagglutinates reliably. The HI test requires a satisfactory haemagglutinating MG antigen, washed fresh chicken or turkey RBCs, as appropriate, and the

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1 Agence française de sécurité sanitaire des aliments (AFFSA) Ploufragan, Mycoplasmology Bacteriology Unit, 22440 Ploufragan, France.
test sera. The antigen can be either a fresh broth culture or a concentrated washed suspension of MG in PBS. It may be difficult to sustain a supply of high-titred broth culture antigen; however, the use of concentrated antigen (usually containing 25–50% glycerol and stored at –70°C), increases the likelihood of nonspecific reactions. In the USA, MG haemagglutination (HA) antigen can be purchased from the NVSL.

The HI test follows well known procedures (1). The HA titre of the antigen is first determined in doubling dilutions, the HA unit being defined as the least amount of antigen giving complete HA in the test system employed. The HI test should be performed using 4 HA units by the following method or a method having equivalent sensitivity as determined by tests with known positive sera.

All HA titrations and HI tests are best performed in multiwell plastic plates with V-shaped wells and using constant volumes of 50 µl. A positive and negative control serum are incorporated into each test. One row of eight wells is required for each serum under test.

- **Test procedure**
  
i) Add 50 µl of PBS to the first well in each row.
  
ii) Add 8 HA units of antigen in 50 µl volumes to the second well in each row and add 50 µl of 4 HA units of antigen to each of wells 3 to 8.
  
iii) Add 50 µl of a previously-prepared 1/5 dilution of the serum under test to the first well, mix, and transfer 50 µl to the second well, and so on, and discard 50 µl from the last well. The first well is the serum control well.
  
iv) Six wells are required for the antigen control. Add 50 µl of PBS to wells 2 to 6, inclusive, and add 50 µl of the 8 HA unit antigen to wells 1 and 2. Mix the contents of well 2 and transfer 50 µl to well 3, mix and repeat up to well 6, and discard 50 µl.
  
v) Two wells are required for the RBC control. Add 50 µl of PBS to each of these.
  
vi) Add 50 µl of a 0.5% suspension of RBCs (chicken cells for chicken serum and turkey for turkey serum) to all wells.
  
vii) Shake the plate lightly to ensure thorough mixing of the well contents, and read after standing for approximately 50 minutes at room temperature or when the antigen titration is reading 4 HA units. For reading, the plate should be tilted and only those wells in which the RBCs 'stream' at the same time as those in the RBC control wells should be considered to be inhibited. The serum control should show a clear button of RBCs and the positive and negative controls should react as expected. The HI titre is the highest serum dilution exhibiting complete inhibition of HA.

Sera giving nonspecific HA must be adsorbed to remove all nonspecific haemagglutinins so that a clear button is obtained in the control well without HA antigen. The adsorption is carried out by incubating 1 ml of the serum dilution with 6–8 drops of packed washed chicken or turkey RBCs. The cells are removed after incubation at 37°C for 10 minutes, and the supernatant is tested for haemagglutinating activity.

There is no official definition of positive and negative results for international trade but the NPIP of the USA states that titres of 1/80 or above are considered to be positive and titres of 1/40 are strongly suspicious.

c) **Enzyme-linked immunosorbident assay**

Several commercial MG antibody ELISA kits are marketed. The sensitivity is determined to some extent by the manufacturer's recommendations for the cut-off levels for positive and suspicious reactions. Sensitivity may sometimes be 'damped down', to avoid the well-known cross-reaction between MG and *Mycoplasma synoviae*. One ELISA uses an MAb that recognises an epitope on a 56 kDa polypeptide of MG (6). In this system, ELISA plates are coated with whole cell MG antigen and the sera under test are added as in the conventional indirect ELISA, but the reaction is assessed by the extent of blocking that occurs when the conjugated MAb is added. One advantage is that the system can be used for sera from any avian species without adaptation.

- **Quality control of *Mycoplasma gallisepticum* and *M. synoviae* antigens**
  
i) *Mycoplasma gallisepticum* antigens

  Antigens are usually prepared from the S6 strain or the A5969 strain. Antigens prepared from other strains may also be used when necessary.

  **MG antigen for the RSA test:** The methods of quality control described below apply solely to suspensions of MG stained with a suitable dye and containing preservative and intended for use in the rapid plate agglutination test with serum. Such antigens are available commercially.
On microscopic examination, the antigen should appear as a homogeneous suspension without floccules or precipitates and the suspending liquid should be free from residual dye. It must be free from contamination with bacteria and fungi. The pH must be between 6.5 and 7. It must be stored at 5±3°C and be warmed to room temperature before use.

The sensitivity and specificity of the antigen is determined with respect to its reaction with known positive sera of high and low titre and known negative sera. A positive reaction is recognised by the formation of coloured floccules and the clearing of the suspending medium. The criteria described above continue to apply until the expiry date declared by the manufacturer.

**MG antigen for the HI test:** The test is preferably performed with live, actively growing cultures. The antigen must be free from contamination with bacteria and fungi.

**MG antigen for the ELISA:** It may be difficult to prepare satisfactory antigen for use in the indirect ELISA without considerable prior experimentation and confirmation of sensitivity and specificity. Use of a reliable commercial kit is probably the best approach for most diagnostic laboratories. Some kits are now USDA-licensed and approved for use in the NPIP in the USA.

1. **Mycoplasma synoviae antigens**

   Antigens prepared from the WVU 1853 strain or other suitable strains should be used.

   **Mycoplasma synoviae antigen for the RSA test:** the specifications apply as for MG antigen for the RSA test.

   **Mycoplasma synoviae antigen for the HI test:** the same specifications apply as for MG antigen for the HI test.

2. **Additional comments**

   Sera giving nonspecific reactions to the RSA test do not usually give a positive reaction in the HI test using live HA antigen. Positive RSA reactions can be confirmed by the HI test with sera taken after the first 2–3 weeks of infection (the time taken for HI antibodies to develop). However, the HI test tends to be strain specific and therefore may lack sensitivity. ELISA may be a useful alternative.

   Samples of serum should not be frozen before use in RSA tests. They should be free from haemolysis and contamination to avoid nonspecific reactions. The use of inactivated vaccines for other diseases may result in nonspecific reactions. Samples should be tested as soon as possible (within 72 hours) because mycoplasma antibodies may deteriorate on storage. Sera may be inactivated in a water bath at 56°C for 30 minutes.

### C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

The preferred method of control is to maintain MG-free flocks. Vaccination should be considered only in situations where field exposure is inevitable, such as on multi-age sites. Potential exposure of neighbouring poultry flocks should also be carefully considered.

Two types of vaccines are available for the control of MG. These are mild to avirulent MG strains used as live vaccines, or inactivated oil-emulsion bacterins. The subject of MG vaccination has been reviewed by Whithear (22). Although there is antigenic variability among MG strains, it is thought that vaccination with a single strain is sufficient.

Guidelines for the production of veterinary vaccines are given in Chapter 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.

- **Live vaccines: methods of use**

   The use of live vaccines is equivalent to ‘controlled exposure’. The objective is to infect the flock with a mild, immunogenic MG strain at an age when little or no significant damage occurs. Such exposure results in resistance to challenge later in life, such as on multi-age commercial sites. Vaccinated birds are resistant to respiratory disease, airsacculitis, and egg production drops caused by MG. Vaccination also results in reduced levels of egg transmission in breeders.

   The F strain of MG has been the most commonly used vaccine strain (4). It is a naturally occurring strain of mild to moderate virulence for chickens, but it is virulent for turkeys. It ordinarily spreads slowly from bird to bird. When administered to healthy chickens via the upper respiratory tract, little or no respiratory reaction is observed. However, when administered by aerosol or in the presence of other respiratory disease agents, such as Newcastle disease or infectious bronchitis virus, respiratory signs and airsacculitis may result. Vaccinated
chickens are permanent carriers, so a single dose is adequate. Use of F strain vaccine in each replacement flock on a multi-age site will eventually result in displacement of the field strain with the vaccine strain. Strains ts-11 and 6/85 are avirulent and spread to unvaccinated birds does not occur or occurs very poorly when birds are in very close contact (14).

Commercial pullets are usually vaccinated between 12 and 16 weeks of age, but vaccination of younger or older birds is permissible. It is essential that vaccination occurs before the flock is naturally infected. Vaccination in cases of probable early field exposure can be carried out in birds as young as 2–4 weeks of age. For the F strain, intranasal or eyedrop administration is preferred. Administration in the drinking water may result in some birds being missed unless the procedure is carried out properly. Aerosol administration should also be done carefully, so that all birds are exposed. A respiratory reaction should be expected at approximately 5–7 days after vaccination if aerosol administration is used. Vaccinated flocks should be tested with the agglutination test approximately 3–4 weeks post-vaccination to be sure that all birds were properly exposed. It is desirable that birds be vaccinated at an age when there is no reaction to other respiratory vaccines. Strain ts-11 should be administered by eyedrop, and 6/85 is given as a fine spray. Vaccination with ts-11 results in a low but distinctive serological response by serum plate agglutination, HI, and ELISA, but vaccination with 6/85 does not ordinarily result in a serological response. No post-vaccination reaction should be observed with 6/85 or ts-11. Flocks vaccinated with F strain or ts-11 are culture positive for the life of the flock, but 6/85 may be difficult to recover later than 4–6 weeks after vaccination.

Commercial live vaccines should be used within 1–2 hours after reconstitution. Lyophilised vaccine should be stored at 4°C. Some manufacturers supply the vaccine frozen. Such vaccine should be stored in liquid nitrogen, dry ice, or at −70°C or colder. Live MG vaccine is not stable for long periods at ordinary freezer temperatures. Storage for more than a few days at −20°C should be avoided.

Strains 6/85 and ts-11 are inherently safer than F strain, although the level of protection may be somewhat less, and may be useful as the primary vaccine strain on a multi-age site or as a ‘second generation vaccine’ on sites previously using F strain vaccine. They may also be preferred in situations where inadvertent exposure of neighbouring poultry flocks is of concern. F strain displaces wild-type MG more efficiently than either ts-11 or 6/85, but ts-11 has been used to eradicate F strain MG from a multi-age commercial egg-production site (20). Multi-age sites where strain 6/85 is consistently used often test MG-negative, suggesting that it has displaced the wild-type strain.

Live vaccines have also been used in some countries in broiler breeder pullets. In Australia, ts-11 live vaccine is being extensively used in broiler breeder pullets as well as in commercial layers. F strain vaccine has been used in breeder breeder pullets raised under multi-age conditions in some Latin American countries for several years; more recently there has been limited use of strains ts-11 and 6/85. Anecdotal evidence and personal experience regarding the performance of the breeders and respiratory disease in their broiler progeny, show that such vaccines have been useful and effective when properly applied. There has been limited use of the 6/85 strain as a vaccine for commercial turkeys in the USA, but no good data on its effectiveness are available. Generally, vaccination of turkeys with live vaccines is not recommended and vaccination of broilers with either live or inactivated vaccines has not been successful.

- **Inactivated vaccines: method of use**

MG bacterins are prepared from a concentrated suspension of whole cells that is emulsified into an oil adjuvant. A high antigen content is essential.

Bacterins are ordinarily used in commercial pullets to provide protection against egg-production drops that occur after MG exposure on multi-age layer sites (9). They may also be used to reduce the level of egg transmission in breeder pullets. Use of bacterins in broilers is limited by the fact that birds vaccinated before 1–2 weeks of age are not protected. Although bacterins may provide protection against respiratory signs, airsacculitis, and egg-production losses, vaccinated flocks are readily infected. The duration of immunity is not known, but most flocks are exposed within 1–2 months after vaccination.

Administration is by the intramuscular or subcutaneous route, usually with a dose of 0.5 ml per bird. There is a risk that a persistent reaction at the site of vaccination will require trimming of carcasses of spent fowl vaccinated by the intramuscular route, so subcutaneous administration in the upper dorsal part of the neck is the most commonly used route. Two doses are preferred, but cost and labour considerations may dictate the use of a single dose, usually between 16 and 18 weeks of age for commercial pullets. A multidose syringe may be used. All equipment should be cleaned and sterilised between flocks, and vaccination crews should exercise proper methods of biosecurity when travelling between flocks. Vaccine should be stored at 2–8°C up to the time of use. It should not be frozen or exposed to strong light.
1. Seed management

a) Characteristics of the seed

- Live vaccine

The vaccine strain should be immunogenic, have the ability to readily colonise the upper respiratory tract, and cause minimal damage to the respiratory system. A strong antibody response does not necessarily correlate with immunity.

The seed culture should be free from all extraneous agents. The culture should be cloned to ensure purity. If desired, restriction endonuclease patterns of the mycoplasmal DNA on agarose gels can be run to be sure of the identity and purity of the strain.

The seed culture should be stable with no tendency to revert to virulence. This can be confirmed with ten back passages in susceptible chickens. Contact chickens can be introduced at weekly intervals. If necessary, tracheal swabs can be taken from infected chickens and can then be inserted into the trachea of contact chickens. Transmission of the organism should be proven. The resulting isolate can then be used to challenge susceptible chickens.

- Killed vaccine

For killed vaccines the most important characteristics are high yield and good antigenicity. It is assumed, but not proven, that virulent strains are desirable. The seed culture should be free from all extraneous organisms.

b) Method of culture

The seed culture may be propagated in a medium similar to that described above (Section B.1.). For live vaccines, the broth culture is lyophilised or frozen at –70°C or colder. For bacterins the culture must be concentrated and resuspended in a small volume of saline or PBS before the emulsion is prepared.

c) Validation as a vaccine

Data on efficacy should be obtained before bulk manufacture of vaccine begins. Chickens should be vaccinated by the same route that will be used in the field. Vaccinated birds should be challenged, and protection should be determined against respiratory signs, nasal discharge, and/or airsacculitis. Ideally, protection against egg-production losses should be evaluated, but such challenge trials are expensive and cumbersome.

Efficacy test: Groups of 20 specific pathogen free (SPF) or at least mycoplasma-free chickens, 2 weeks of age or older, are vaccinated by eyedrop or other route of administration with one field dose of live vaccine, or subcutaneously or intramuscularly with one dose (usually 0.5 ml) of bacterin. A similar group of unvaccinated chickens is maintained separately as controls. All chickens should be challenged with a 24-hour broth culture of a virulent strain of MG, 2–3 weeks post-vaccination. A simple challenge method is inoculation of 0.1 ml of the challenge culture into the posterior thoracic air sac. All birds are necropsied 7–10 days post-challenge, and air sac lesions are scored. Alternative methods are to challenge by inoculating 0.1 ml into the infraorbital sinus and examining the birds for nasal discharge from 7 to 14 days post-challenge or to challenge by aerosol and measure the thickness of the tracheal mucosa on microscopic sections at four to six equidistant predetermined points (22).

2. Method of manufacture

The vaccine must be manufactured in suitable clean and secure accommodation, well separated from diagnostic facilities or commercial poultry. Special care must be taken to avoid MG contamination of other products manufactured in the same facility.

Production of vaccine should be on a seed-lot system, using a suitable MG strain of known origin, passage history, and purity. The growth medium is similar to that given above. The serum used in the growth medium should be inactivated at 56°C for 1 hour to prevent contamination with any mycoplasmal organism that may be present, and filter sterilised. A source of SPF serum is desirable.

Broth medium is inoculated, with a rapidly growing inoculum, at a rate of approximately 5% (v/v). Incubation is at 37°C. Production can be in batches using large flasks or in a fermenter. In batch cultures, harvest is approximately 24 hours after inoculation. Live vaccines are preserved by lyophilisation or by freezing at –70°C, in liquid nitrogen, or on dry ice.
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For bacterin production, the antigen must be concentrated, usually by centrifugation, ultrafiltration, or other suitable method. Bacterins are made as water-in-oil emulsions, typically 80% mineral oil, 20% aqueous, with suitable emulsifying agents.

3. In-process control

Antigen content: At harvest, the titre should be from $10^8$ to $10^9$ CFU/ml. The antigen concentration of bacterins is difficult to standardise but may be based on packed cell volume, which is typically 1% (v/v) packed cells in the final product.

Inactivation of killed vaccines: Inactivation is frequently done with either beta-propiolactone or formaldehyde. The inactivating agent and the inactivation procedure must be shown under the conditions of vaccine manufacture to inactivate the vaccine organism and potential contaminants.

Prior to inactivation, care should be taken to ensure a homogeneous suspension free from particles that may not be penetrated by the inactivating agent. A test for inactivation should be carried out by culture in mycoplasma broth on each batch of both the bulk harvest after inactivation and the final product. No evidence of growth of mycoplasma should be observed.

Sterility of killed vaccines: Oil used in the vaccine must be sterilised by heating at 160°C for 1 hour, or by filtration, and the procedure must be shown to be effective. Tests appropriate to oil-emulsion vaccines are carried out on each batch of final vaccine as described, for example, in the British Pharmacopoeia (Veterinary) 1985.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter I.1.5.

b) Safety

- Live vaccine safety test
  
  The birds vaccinated in the efficacy test given above can be used to evaluate the safety of the vaccine.

- Killed vaccine safety test
  
  Birds vaccinated in the efficacy test described above may be observed for adverse local or systemic effects.

c) Potency

Potency tests for both live and killed vaccine can be conducted by the procedures given above for the efficacy test. The titre of live vaccines should be sufficient to induce infection by the route recommended; $10^9$ CFU/dose is sufficient for eyedrop administration of live F strain vaccine. The recommended dose of ts-11 is $10^{7.7}$ colour changing units (CCU)/dose and for 6/85 a dose of $10^7$–$10^8$ CFU was effective in challenge trials.

d) Duration of immunity (killed vaccine)

Because flocks are generally exposed within 1–2 months after vaccination, duration of immunity is not a primary consideration. After field challenge, resistance is considered to be permanent.

e) Stability

Evidence should be provided on three batches of vaccine to show that the vaccine passes the batch potency test at 3 months beyond the requested shelf life.

f) Preservatives

A preservative is normally required for vaccine in multidose containers. The concentration of the preservative in the final vaccine and its persistency throughout the shelf life should be checked.

A suitable preservative that has already been established for such purposes should be used. Mycoplasmas are susceptible to many antibacterials except for penicillins; such antibiotics should not be included as preservatives.
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5. Tests on the final product

a) Safety
See Section C.4.b.

b) Potency
See Section C.4.c.

REFERENCES


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NB: There are OIE Reference Laboratories for Avian mycoplasmosis (Mycoplasma gallisepticum) (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 2.7.4.

AVIAN CHLAMYDIOSIS

SUMMARY

Avian chlamydiosis (AC) is caused by the bacterium Chlamydophila psittaci. AC occurring in humans and all birds was originally called psittacosis, but later the term ornithosis was introduced to identify the disease contracted from or occurring in domestic and wildfowl, the name of the disease contracted from or occurring in psittacine birds remained psittacosis. These diseases are similar when contracted by humans. The genus Chlamydia was recently divided into two genera, Chlamydia and Chlamydophila. All known avian strains are now in the species Chlamydophila psittaci. Chlamydiosis is still the term used for diseases produced by both genera. The avian strains include at least six serotypes that correlate with the avian species from which they are usually isolated. Chlamydiosis as it occurs naturally in mammalian species and not contracted from avian species, is caused by distinctly different strains of the organism.

Depending on the chlamydial serovar and the avian host, chlamydiae cause pericarditis, conjunctivitis, sinusitis, airsacculitis, pneumonia, lateral nasal adenitis, peritonitis, hepatitis, and splenitis. Generalised infections result in fever, anorexia, lethargy, diarrhoea, and occasionally shock and death. Special laboratory handling is recommended because avian chlamydial strains can cause serious illness and possibly death in humans. The disease in ducks and turkeys is of particular concern as transmission to humans is common during handling and slaughter of the birds. The diagnosis of AC requires the isolation and identification of the organism, the demonstration of chlamydiae in tissues, or the demonstration of a four-fold increase in specific humoral antibody, as well as typical clinical signs. A tentative diagnosis can be made in a flock that includes birds with clinical signs of AC as well as a high incidence of birds with high antibody levels.

Identification of the agent: Isolation of chlamydiae requires the inoculation of embryonated eggs, laboratory animals, or cell cultures, and testing for chlamydiae by cytochemical stains or immunohistochemical methods. The direct inoculation of samples into cell cultures of buffalo green monkey (BGM), African green monkey kidney (Vero), McCoy, or L cells is preferable. Cell cultures are as sensitive for the isolation of most avian strains of chlamydiae as are chicken embryos. To enhance the infectivity of samples, a preferred method is the centrifugation of the inoculum on to monolayers and addition of cell-division inhibitors, such as cycloheximide. The cell cultures are then stained by direct immunofluorescence or by other appropriate stains at appropriate times to demonstrate the presence of inclusions.

Enzyme-linked immunosorbent assays (ELISAs) developed for detecting trachomatis antigen in humans have been used for diagnosing chlamydiae in birds. Many of the earlier tests were developed using monoclonal or polyclonal antisera against lipopolysaccharide epitopes, some of which were shared with other Gram-negative bacteria. Their use when screening individual birds is questionable, as their sensitivity and specificity for use with avian samples has not been proven. Their main value is in confirming chlamydiosis in a bird showing signs of disease. However, these tests have not been approved and licensed for use in testing birds.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and immunohistochemical staining of histological sections are two new techniques showing much promise for the future. Both of these are very rapid and do not require the live agent. The current PCR tests target the MOMP gene or the ribosomal RNA genes (16S–23S), and will amplify all chlamydial strains and allow identification at the level of the chlamydial species. There has been an increase in the use of immunohistochemical staining of histological sections because of the recent development and availability of automated staining equipment. It has the advantage that
most diagnostic laboratories routinely collect materials for haematoxylin and eosin (H&E)-stained sections, and extra sections can easily be cut at the time or retrospectively be cut for immunohistochemical staining.

**Serological tests:** The standard serological test for chlamydial antibodies is the complement fixation (CF) test. The antigen is a group-reactive lipopolysaccharide antigen present in all strains. The occurrence of high CF titres in the majority of individuals in a flock with clinical signs is presumptive evidence of active infection. The demonstration of a four-fold increase in titre in an individual bird is considered to be diagnostic of a current infection.

Other serological tests, such as the ELISA, latex agglutination, elementary body agglutination, micro-immunofluorescence, and the agar gel immunodiffusion tests are available. These tests are of value in specific cases and may replace the CF test; however, comparisons of reliability and reproducibility are not yet available.

**Requirements for vaccines and diagnostic biologicals:** There are no commercial vaccines available for chlamydiosis control in poultry. Antibiotics are the only current means of control. *Chlamydia psittaci* is susceptible to a number of antibiotics. The drug of choice varies from country to country.

### A. INTRODUCTION

Avian chlamydiosis (AC) is caused by the bacterium *Chlamydia psittaci*. The disease in birds was originally called psittacosis, but the term ornithosis was introduced later to differentiate the disease in domestic and wild fowl from the disease in psittacine birds. The two syndromes are currently considered to be the same (5). Their earlier separation was based on the assumption that in humans ornithosis was a milder disease than psittacosis. However, it should be noted that the disease in humans contracted from turkeys and ducks is often as severe as that contracted from psittacine birds.

*Chlamydia psittaci* produces a systemic and occasionally fatal disease in birds. The clinical signs vary greatly in severity and depend on the species and age of the bird and the strain of chlamydia. AC can produce lethargy, hyperthermia, abnormal excretions, nasal and eye discharges, and reduced egg production. Mortality rates will vary greatly. In pet birds the most frequent clinical signs are anorexia and weight loss, diarrhoea, yellowish droppings, sinusitis, conjunctivitis, biliverdinuria, nasal discharge, sneezing, lacrimation and respiratory distress (27). Many birds, especially older psittacine birds, may show no clinical signs; nevertheless, they may often shed the agent for extended periods. Necropsy of affected birds will often reveal spleen and liver enlargement, fibrous airsacculitis, pericarditis and peritonitis (5, 40). Histological lesions are non-pathognomonic unless there are identifiable chlamydialae present.

The severity of disease in turkeys depends on the chlamydial strain and the presence of other diseases. Serovar D strains are usually the most severe and are particularly hazardous for poultry workers. At the peak of disease in a flock infected with serovar D strains, 50–80% of the birds can show clinical signs and mortality is often 10–30% (5). In broiler turkeys, mortality rates as high as 80% have been reported (41). Strains of other serovars, such as serovars B and E, often have morbidity rates of 5–20% and mortality rates of under 5%.

Clinical signs and necropsy lesions in turkeys vary greatly. Turkeys infected with the highly virulent strains show cachexia, anorexia, and elevated temperatures. The birds will excrete yellow-green gelatinous droppings. In laying hens, egg production will drop rapidly and remain low until recovery is complete. In broiler turkeys, a respiratory syndrome having the characteristics of rhinotracheitis has been reported (41). Signs included are conjunctivitis, swelling of the infra-orbital sinuses and sneezing. In turkeys infected with strains of low virulence, the disease signs are milder and usually include anorexia and, in some birds, loose green droppings. Leg problems (arthritis) have also been associated with *Chlamydia psittaci* infection of turkeys. Characteristic lesions on necropsy of birds infected with the virulent strain include enlarged spleen and liver, and a fibrous to fibropurulent exudate on respiratory, peritoneal, and pericardial surfaces. The lesions can include sinusitis, tracheitis, airsacculitis, pneumonia, and enteritis. Pneumonia is usually seen only in birds that die of the infection. The lesions in birds infected with the low virulence strains are similar, but not as extensive or severe.

Chlamydiosis in ducks is important both economically and as a public health hazard in a number of parts of the world. The disease is usually severe with morbidity as high as 80% and mortality ranging from 0 to 40% depending on the age of the ducks and the presence of concurrent infections (5). Clinical signs include head tremors, unsteady gait, conjunctivitis, serous to purulent nasal discharge, depression and death. On necropsy, enlarged spleens, focal necrosis of the liver, fibrinous polyserositis, and pneumonia are common. In recent years, a mild form has been recognised in which disease signs are minimal or absent, and death is associated.
only with stress of handling or with other disease. Human infections have been known to occur following handling or slaughter of both clinically and inapparently infected birds.

Chlamydiosis in ostriches and rheas has been reported in many parts of the world. The only isolates that have been serotyped were serovar E, which has been isolated from pigeons, ducks and humans. Its reservoir is thought to be in wild pigeons or other wild birds. Ratites are usually housed outdoors where they are exposed to wild birds. Chlamydiosis usually occurs in younger birds, but can occur in adults. It is usually very acute with a high mortality; however, studies have not been reported giving the per cent of the infected birds showing clinical signs. Because of the widespread occurrence of the disease in ratites and the potential of transmission to humans, clinically sick birds should be handled with caution.

The family Chlamydiaceae was recently reclassified into two genera and nine species based on sequence analysis of its 16S and 23S rRNA genes (15). The two new genera, Chlamydia and Chlamydophila, correlate with the former species Chlamydia trachomatis and C. psittaci. The genus Chlamydia includes C. trachomatis (human), C. suis (swine), and C. muridarum (mouse, hamster). The genus Chlamydophila includes C. psittaci (avian), C. felis (cats), C. abortus (sheep, goats, cattle), C. caviae (guinea pigs), and the former species C. pecorum (sheep, cattle) and C. pneumonia (human).

The two genera and nine species have merit both molecularly and for classification of host range and clinical disease. The species show a high degree of correlation with host range, disease syndrome, and virulence, and thus provide an understanding of the epidemiology of the various species and serovars affecting livestock and birds. The terms ‘chlamydiosis’ and ‘chlamydia(e)’ are used as generic terms to refer to members of either and both genera. However, the new scientific names are used when referring to a specific chlamydial species.

The avian strains all belong to the species Chlamydophila psittaci. This species includes six known avian serovars, and two mammalian serovars, M56 from muskrats and WC from cattle (15). M56 and WC were each isolated from a single outbreak. The six avian serovars are labelled A through F and each shows host specificity. The hosts that each serovar has been associated with are: A, psittacine birds; B, pigeons; C, ducks and geese; D, turkeys; E, pigeons and ratites; and F is a single isolate from a psittacine bird. What is not known is how many of these birds and mammals are the natural hosts of the serovars.

The strains of avian chlamydiae can infect humans and should be handled carefully under conditions of biocontainment (12, 13). Most infections occur through inhalation of infectious aerosols. Post-mortem examinations of infected birds and handling of cultures should be done in laminar flow hoods or with proper protective equipment. Human infection can result from transient exposures. The incubation period is usually 5–14 days; however, longer incubation periods are known. Human infections vary from inapparent to severe systemic disease with interstitial pneumonia and encephalitis. The disease is rarely fatal in properly treated patients; therefore, awareness of the danger and early diagnosis are important. Infected humans typically develop headache, chills, malaise and myalgia, with or without signs of respiratory involvement. Pulmonary involvement is common; auscultatory findings, however, may appear to be normal or to underestimate the extent of involvement. Diagnosis can be difficult and is usually established through testing paired sera for antibodies to chlamydia by the complement fixation (CF) test. In humans, tetracycline, doxycycline, or azithromycin are usually the drugs of choice unless contraindicated. The length of treatment will vary with the drug, but should be continued for at least 14 days for tetracycline.

### B. DIAGNOSTIC TECHNIQUES

#### 1. Identification of the agent

The preferred method for the identification of AC is the isolation and identification of the organism. Because of the time involved, the need for high quality samples, and the hazard to laboratory personnel, other techniques are often used. These include histochemical staining of smears of exudate and faeces, and impression smears of tissues, immunohistochemical staining of cytological and histological preparations, antigen-capture enzyme-linked immunosorbent assays (ELISA), polymerase chain reaction (PCR) and PCR-RFLP (restriction fragment length polymorphism).

#### a) Collection and treatment of samples

The samples to be collected will depend on the disease signs in evidence. They must be taken aseptically. Contaminant bacteria may interfere with the isolation of the chlamydiae. Specimens from acute cases should include inflammatory or fibrinous exudate in or around organs that display lesions, ocular and nasal exudates, whole blood and tissue samples from kidney, lung, pericardium, spleen, and liver. In cases with diarrhoea, colon contents or excrement should be cultured. In live birds, the preferred samples are pharyngeal and nasal swabs (2). Intestinal excrement, cloacal swabs, conjunctival scrapings, and peritoneal exudate can also be taken.
Proper handling of clinical samples is necessary to prevent loss of infectivity of chlamydiae during shipping and storage. A special medium consisting of sucrose/phosphate/glutamate (SPG) was developed for rickettsiae and has proven to be satisfactory for transport of chlamydial field samples. The medium as recommended for chlamydiae (36) consists of SPG buffer: sucrose (74.6 g/litre); KH$_2$PO$_4$ (0.512 g/litre); K$_2$HPO$_4$ (1.237 g/litre); and L-glutamic acid (0.721 g/litre), which can be sterilised by autoclaving or filtering. Added to this are fetal calf serum (10%), vancomycin and streptomycin (200–500 µg/ml), nystatin and gentamicin (50 µg/ml). The addition of antibiotics reduces the effect of contamination, even when samples are shipped at ambient temperatures. This medium can also be used as a laboratory diluent and for freezing of chlamydiae.

Contaminated samples must be pretreated before being used to inoculate animals or cell cultures. There are three basic methods: treatment with antibiotics (7, 8), treatment with antibiotics together with low-speed centrifugation (4, 5), and treatment with antibiotics with filtration (4, 7, 8, 11). A number of antibiotics that do not inhibit chlamydia can be used. Samples are homogenised in phosphate buffered saline (PBS), pH 7.2, containing a maximum of the following: streptomycin (1 mg/ml), vancomycin (1 mg/ml), and kanamycin (1 mg/ml). Gentamicin (200 µg/ml) can be used. Amphotericin B (50 µg/ml) can be added to control yeast and fungal growth. Other antibiotic solutions are often used. Penicillin, tetracycline and chloramphenicol should be avoided as these inhibit the growth of chlamydiae.

When contamination is light, samples should be homogenised in an antibiotic solution prior to inoculation into chicken embryos or tissue cultures. Samples are often left to stand in the antibiotic solution for 24 hours at 5°C before inoculation. Heavily contaminated samples, such as faecal samples, should be homogenised in antibiotics and then centrifuged at 500 g for 20 minutes. The surface layer and the bottom layer are discarded. The supernatant fluid is collected and recentrifuged. The final supernatant fluid is used for inoculation. Samples should be passed through a filter of 450–800 µm average pore size if contamination persists.

### b) Isolation in cell culture

Cell cultures are the most convenient method for the isolation of C. psittaci. Cell lines are satisfactory, the more common ones being buffalo green monkey (BGM), McCoy, HeLa, African green monkey kidney (Vero), and L cells (39). The cells are grown as monolayers using standard tissue culture media containing 5–10% fetal calf serum and antibiotics that are not inhibitory to chlamydia (as described previously).

When selecting cell culture equipment, it is important to remember that:

- **Chlamydiae can be identified by direct or indirect immunofluorescence or some other appropriate staining technique;**
- **The inoculum is usually centrifuged on to the monolayer to enhance its infectivity;**
- **The sample may need to be blind passaged at 5–6 days to increase sensitivity of isolation;**
- **The sample will need to be examined from two to three times during any one passage; and**
- **Chlamydia can be infectious to humans.**

Small flat-bottomed vials, such as 1 dram (3.7 ml, 15 × 45 mm) shell vials or bottles containing cover-slips that are 12 mm in diameter, will meet these requirements (7, 8, 11). A number of vials, often four to six, are inoculated with each sample to permit fixing and staining at various intervals, and to permit repassaging of apparently negative samples 6 days after inoculation. When testing multiple samples, 96-well multiwell dishes can also be used as they have a labour-saving advantage. However, it should be noted that cross-contamination between samples can be a problem.

Chlamydiae can be isolated from cells that are replicating normally, but the use of nonreplicating cells is preferable as these may provide increased nutrients for the growth of chlamydiae. Suppressed cells can also be observed for longer periods. Host cell division can be suppressed either by irradiation or, more commonly, by cytotoxic chemicals. The latter include 5-iodo-2-deoxyuridine, cyto-cholasin B, cycloheximide, and emetine hydrochloride (32). Cycloheximide is the most commonly used and can be added to the medium at the rate of 0.5–2.0 µg/ml at the time of inoculation of the monolayer. Emetine is removed after treatment and replaced by medium (4, 5, 7, 8). The monolayer is first treated for 5 minutes with emetine (0.5 µg/ml), after which the emetine is removed and replaced with culture medium; the monolayer is then ready for use. The growth of most chlamydial strains will be enhanced by the treatment of the monolayer by one of these drugs; the treatment will have no effect on the growth of other strains.

Attachment of chlamydia to cells is increased by centrifuging the inoculum on to the monolayer at 500–1500 g for 30–90 minutes at 37°C. The inoculum is removed and replaced with tissue culture medium containing a cell-division inhibitor, and then incubated at 37–39°C. Cultures must be examined for chlamydiae at regular intervals using an appropriate staining method. This is usually done on day 2 or 3, as well as on day 5 or 6. Cultures that appear to be negative at the sixth day are harvested and repassaged.
When repassaging chlamydiae, cells and culture media should be passaged without freezing as freeze-thawing should not be used to disrupt cells as this may destroy the chlamydiae.

Before staining the cultures, the medium is first removed, the cultures are washed with PBS and fixed with acetone for 2–10 minutes. The fixation time will depend on the tissue culture vessel used. As acetone will soften most plastics, the use of a mixture of 50% acetone and 50% methyl alcohol may be preferable. A number of staining methods can be employed to demonstrate chlamydial inclusions. The preferred method is direct immunofluorescence (4, 7, 28). A chlamydial fluorescein-conjugated antiserum is applied to the infected cells and incubated in a humid chamber for 30 minutes at 37°C. The cover-slips are then washed three times with PBS, air-dried, mounted, and examined. Chlamydial inclusions fluoresce a green colour. Commercial conjugate preparations using monoclonal antibodies (MAbs) are available and are highly specific. Conjugates may also be prepared from polyclonal sera, but it is important to obtain specific, high-titre antisera. Polyclonal antisera can be prepared in rabbits, guinea-pigs, sheep or goats. Sheep and goats are excellent sources because of the volume and high titres that are readily obtained following infection. Conjugates are then prepared using standard techniques (4, 5, 7).

Chlamydial inclusions can also be demonstrated by indirect fluorescent antibody and immunoperoxidase techniques (4, 6, 28). Direct staining can be done with Gimenez, Giemsa, Ziehl–Neelsen, or Macchiavello’s stains. Except for immunofluorescence, all these stains have the advantage that standard light microscopes can be used.

c) Isolation in eggs

Chicken embryos are still used for the primary isolation of chlamydiae. The standard procedure is to inject up to 0.5 ml of inoculum into the yolk sac of a specific pathogen free 6–7-day-old embryo (4, 5). The eggs are then incubated in a humid atmosphere at 39°C, rather than at 37°C, as multiplication of chlamydia is greatly increased at the higher temperature. Replication of the organism usually causes the death of the embryo within 3–10 days. If no deaths occur, two additional blind passages are usually made before designating any sample as negative. Chlamydial infections will give rise to a typical vascular congestion of the yolk sac membranes. These are harvested and homogenised as a 20% (w/v) suspension in SPG buffer, and can be frozen to preserve the strain, or inoculated into eggs or on to cell cultures.

The organism can be identified by preparing an antigen from an infected yolk sac and testing it by direct staining of smears using appropriate stains or by using the antigen in a serological test. Cell culture monolayers can be inoculated with the yolk sac suspension and examined by direct immunofluorescence 48–72 hours later for the presence of chlamydial inclusions. Typical inclusions are intracytoplasmic, round, or hat-shaped bodies. With some virulent strains, the inclusions rapidly break up and the chlamydial antigen is dispersed throughout the cytoplasm.

d) Differentiating among species/strains

All avian isolates are in the Chlamydophila psittaci group, as discussed earlier (15). The avian strains can be differentiated from other chlamydiae by PCR-RFLP of either the MOMP gene or the 16S–23S rDNA operon (14). A provisional C. psittaci determination can be made using the source of the isolate and serovar-specific MAbs.

The avian strains of C. psittaci contain a number of specific serotypes (1, 3, 6). The syndromes caused by the various strains are quite specific; the natural host range of a particular strain may also be fairly specific. There are at least six serotypes that infect birds. These are labelled A through F. The hosts from which they are mainly isolated are: serotype A, psittacine birds; serotype B, pigeons; serotype C, ducks; serotype D, turkeys; serotype E, pigeons and rats; and one isolate of serotype F from a psittacine bird.

Serovar-specific MAbs to the six serotypes have been developed and are being used by a limited number of laboratories to serotype new isolates (1, 3). PCR-RFLP techniques have been developed that will also differentiate the strains (3, 35, 38). Again the use is mainly experimental with only a limited number of laboratories using either the PCR-RFLP technique or serovar-specific MAbs. Serotyping is relatively easy to perform and laboratories that need it can easily set up the procedures.

e) Histochemical staining

Giemsa, Gimenez, Ziehl–Neelsen and Macchiavello’s stains are commonly used to detect chlamydiae in impression smears of liver and spleen. The following modified Gimenez technique is used by several laboratories (4).

- Modified Gimenez technique or (Pierce-van der Kamp) stain

- Reagents:

  Solution 1: Distilled H$_2$O (450.0 ml) and phenol (5.0 ml) added to basic fuchsins (2.5 g) and 95% ethanol (50.0 ml). Incubate at 37°C for 48 hours. Filter and store in the dark at room temperature.
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Solution 2: Na₂HPO₄ (11.65 g); Na₂HPO₄.H₂O (2.47 g); distilled H₂O, pH 7.5 (to 1.0 litre).
Solution 3: Solution 1 (20.0 ml); and solution 2 (25.0 ml). Let stand for 10 minutes, filter and use.
Solution 4: 0.5% citric acid.
Solution 5: Fast green (0.2 g); distilled H₂O (100.0 ml); and glacial acetic acid (0.2 ml).
Solution 6: Solution 5 (20.0 ml); and distilled H₂O (50.0 ml).

- Procedure for smears is as follows:
  i) Fix in methanol for 5 minutes.
  ii) Stain in Solution 3 for 10 minutes and rinse in tap water.
  iii) Counterstain in Solution 6 for 2 minutes.
  iv) Rinse in tap water and air-dry.

- Procedure for paraffin sections is as follows:
  i) Deparaffinise and hydrate with distilled H₂O.
  ii) Stain in Solution 3 for 10 minutes and rinse in tap water.
  iii) Dip in Solution 4 until no more red runs out of the section. Rinse in tap water.
  iv) Counterstain in Solution 6 for 20 dips.
  v) Dip in two changes of 95% alcohol, for five dips each. Dehydrate, clear, and mount.

Chlamydiae will appear red against a green background.

f) Immunohistochemical staining

Immunohistochemical staining can be used to detect chlamydiae in cytological and histological preparations. The technique is more sensitive than histochemical staining, but some experience is necessary as cross-reactions with some bacteria and fungi require that morphology must be considered.

Most widely used immunohistochemical staining procedures can be adapted to give satisfactory results. The selection of the primary antibody is very important. Both polyclonal and monoclonal antibodies have been used. Because formalin affects chlamydial antigens, it is recommended that polyclonal antibodies be made to purified formalin-inactivated chlamydiae. The chlamydial strain used is not important as the antibodies will be mainly to the group-reactive antigens. MAbs should also be selected for reactions to formalin-fixed chlamydia. A pool of group-reactive MAbs can be used.

g) Enzyme-linked immunosorbent assays

The ELISA is a relatively new technique that has been extensively promoted as kits for use in the diagnosis of human chlamydiosis. These test kits detect the lipopolysaccharide (LPS) antigen (group reactive) and will detect all species of chlamydiae. A number of these kits have been tested for use in detecting chlamydia in birds (42), but none of the kits has been licensed for detection of C. psittaci. One problem with some of these tests is that the chlamydial LPS shares some epitopes with other Gram-negative bacteria, and these epitopes can cross-react, resulting in a high number of false-positive results. This problem has been reduced or eliminated in more recently developed kits by careful selection of the MAbs used. These kits, however, still lack sensitivity because a few hundred organisms are still needed to give a positive reaction. Most diagnosticians believe that a diagnosis of AC can be made when a strong positive ELISA reaction is obtained from birds with signs of psittacosis. Because of the number of false-positive results, a positive in an individual bird without signs of disease is not considered to be significant, but indicates the need for further testing using different methods.

h) Polymerase chain reaction

PCR techniques have been reported for the detection of chlamydiae in animals. Current PCR tests for detection of C. psittaci target the MOMP gene or the 16S–23S rRNA gene (16, 22, 26, 37). The sensitivity and specificity varies on sample preparation and the PCR test. Sensitivity is increased by targeting a relatively short DNA segment, using a nested procedure or using the new rapid-cycle real-time PCR techniques. The nested procedure increases the risk of contamination. The real-time PCR requires a labelled probe and special equipment which increases costs. Targeting the 16S–23S gene also increases sensitivity as multiple copies are usually present in the organism; however, cross-reactions with other bacteria can be a problem. Sequencing of the product will allow comparison with the sequences of reference avian chlamydia isolates and the sequence can be used in phylogenetic analysis for classification.
and epidemiological purposes. DNA sample preparation has improved with the availability of DNA extraction kits that work on most clinical samples.

2. Serological tests

a) Modified direct complement fixation test for Chlamydia

The following is a widely used modified direct CF test for the detection of antibody. The reagents are relatively easy to prepare and standardise. There are other CF tests; each has advantages. The modified direct CF test is performed in 96-well round-bottom multiwell dishes. Incubation steps are usually done by floating the plates in a 37°C water bath. The chlamydial antigen can be prepared from either infected yolk sacs or cell culture preparations. The modified direct CF test differs from the direct CF test in that normal, unheated chicken serum from chickens without chlamydial antibody is added to the complement dilution. The normal serum increases the sensitivity of the CF procedure so that it can be used to test sera from avian species whose antibodies do not normally fix guinea-pig complement.

- Test procedure
  
  i) Dilution of sera

  Figure 1 gives a suggested pattern for performing the test in round-bottom, 96-well multiwell dishes. All sera must be heat-inactivated at 60°C for 30 minutes prior to use. The sera are diluted in Veronal (barbiturate) buffer saline (VBS) as shown in Figure 1. The dilutions are made in the multiwell dish by adding 100 µl of VBS to each well of rows A and E, and then adding 25 µl of the undiluted sera, positive serum, or negative serum to each of three wells. This gives a starting dilution of 1/5. Then, 25 µl of VBS is added to each well in row B through to D and row F through to H. Twofold dilutions are made, using a 25 µl micropipette, from row A through to D and row E through to H. Appropriate volumes are discarded from the starting and finishing rows to give 25 µl per well. Diluters are rinsed twice in distilled H₂O and once in VBS between each serum.

  ii) Addition of antigen

  To each well in columns 1, 4, 7, and 10, add 25 µl of positive chlamydial antigen. In columns 2, 5, 8, and 11, add 25 µl of VBS (anticomplementary control wells), and in columns 3, 6, 9, and 12, add 25 µl of negative antigen (normal yolk sac or cell culture prepared the same as the chlamydial antigen). The chlamydial antigens are stored undiluted at 4°C and diluted to proper concentration in VBS prior to use.

  iii) Addition of complement

  Complement (C') is stored at –70°C and should be thawed and diluted in VBS prior to the addition of the antigen. Fresh chicken serum is added before diluting the C' to give a 5% concentration in the complement. Dilutions of C' are made as in previous tests or from titrations. C' should be allowed to

![Fig. 1. Suggested test pattern for the modified direct complement fixation test when using 96-well dishes.](image-url)
stand in an ice bath to stabilise for 15 minutes. The diluted C' should be stored at 4°C following
stabilisation and should be used within 2 hours: 50 µl of the C' is added to each well immediately
following the addition of the antigens. The plates are incubated uncovered in a 37°C water bath for
2 hours.

iv) Addition of sheep red blood cells

Mix 4% standardised sheep red blood cells (SRBCs) with an equal volume of VBS. To this add an
equal volume of haemolysin diluted in VBS. The final dilution is incubated in a 37°C water bath for
15 minutes to sensitise the SRBCs. To each well add 50 µl of sensitised SRBCs. The plates are then
incubated for 1 hour in a 37°C water bath. The plates can be centrifuged at 400 g for 5 minutes before
reading or they can be refrigerated at 4°C overnight prior to reading.

v) Interpretation of the results

The wells are often scored 1+, 2+, 3+, or 4+ corresponding to reduction of haemolysis of 25, 50, 75, or
100%. A positive reaction is 2+ or higher, which is equivalent to 50% or less lysis of the SRBCs. This
indicates that the C' was fixed by antibody prior to the addition of the SRBCs. Negative wells are
indicated by the complete lysis of the cells: the C' remains unbound and reacts with the SRBCs and
the haemolysin to produce lysis of the SRBCs.

Invalid tests occur when the serum is anticomplementary and a positive reaction occurs in the dilution
with VBS as the antigen. Nonspecific serum reactions give positive reactions in both the positive and
negative wells.

- Reagents

  i) Antigen preparation

  The simplest methods start with the growth of chlamydiae in cell culture. The two methods described
  below produce antigens that can be used in the micro-CF test. The procedures are quite similar: both
  include the growth of chlamydiae in cell culture, the inactivation of the chlamydiae, partial purification
  of the antigen, mechanical disruption, and dilution into the appropriate buffer. The method selected will
  depend on the equipment available.

  The first procedure (17, 19) starts with the chlamydiae and cell culture debris harvested when
cytopathic effects are noted. The culture is inactivated by the addition of phenol to a final
concentration of 1.0%, incubated for 24 hours at 37°C, and concentrated by centrifugation at 10,000
for 1 hour. The sediment is reconstituted to 10% of the original volume using VBS, pH 7.2, containing
1.0% phenol and 1.0% glycerol.

  The sediment is then homogenised in an omnimixer at top speed for three 1-minute periods while
cooled in ice water. The homogenate is centrifuged for 15 minutes at 100 g to remove debris. Some
procedures suggest heating the antigen for 30 minutes in a boiling water bath at this time. The
supernatant is saved and diluted to the desired concentration.

  In the second procedure for the production of antigen for the CF test (9, 10), antigen is prepared from
L cells infected with a psittacine strain. The cell culture medium is discarded, and the cells are heated
for 40 minutes at 56°C. The cells are lysed in distilled water, the chlamydiae are disrupted by
ultrasonication and then made isotonic in VBS. The antigen is tested against a standard sheep
convalescent serum and used at 2 units in the micro-CF test.

  There are a number of procedures for preparing the antigen from infected yolk sacs, some of which
are quite elaborate. However, with the following procedure it is relatively easy to prepare a crude
infected yolk sac antigen that works well in the modified direct CF test. An egg-adapted strain of
chlamydia is used to inoculate 6–7-day-old embryonated chicken eggs via the yolk sac. The yolk sacs
are harvested from embryos that die between 3 and 7 days post-inoculation. The yolk-sac harvest is
diluted 1/3 in PBS, Tris buffer, or cell culture medium, and then autoclaved for 20 minutes. The
suspension is cooled and then homogenised thoroughly. The use of a high-speed tissue homogeniser
for 3–5 minutes is recommended. After homogenisation, phenol is added to make a final concentration
of 0.5% phenol (prepare a 5% phenol stock solution and add 1 ml for every 9 ml of antigen). The
antigen preparation is prepared, held for 3 days, and then used after centrifugation for 20 minutes at
1000 g. The antigen can be stored for long periods of time at 4°C.

  ii) Preparation of sensitised SRBCs

  Defibrinated SRBCs are preserved by mixing in an equal volume of Alsever's solution. These can be
stored at 4°C for up to 4 weeks. Wash 25 ml of the stock SRBCs with 25 ml of VBS. Centrifuge at
400 g for 10 minutes. Aspirate off the VBS and resuspend in 50 ml of VBS. Repeat the wash a total of
three times. Following the final wash, dilute the SRBCs at a ratio of 2.2 ml of packed SRBCs to 98 ml
of VBS. The SRBCs can then be standardised by optical density: mix 1 ml of the diluted, washed
SRBCs with 14 ml distilled H₂O, determine the absorbance using a spectrophotometer, and standardise to 0.25 at a wave length of 550 mm. The reading obtained can be used in the following formula to determine the dilution needed:

\[
\text{Final volume of SRBCs} = \frac{(\text{absorbance reading} \times \text{current volume})}{0.25 \text{ desired absorbance}}
\]

The SRBCs are sensitised by rapidly adding an equal volume of VBS containing the appropriate dilution of haemolysin (dilution determined by titration). Incubate at 37°C for 15 minutes prior to use.

iii) **Veronal buffer saline**

VBS is prepared as a 5 × stock solution and diluted 1/5 with distilled H₂O prior to use. The following formula makes 4 litres. To distilled water add sodium barbital (7.5 g); barbital H₂O (dissolve in boiling H₂O) (11.5 g); MgSO₄·7 H₂O (4.056 g); NaCl (170.0 g); and CaCl₂ (0.078 g): Add distilled H₂O to make to 4 litres.

iv) **Complement titration**

Complement (C') is unstable and will deteriorate if improperly handled. Normally it should be kept frozen at -70°C in aliquots that are used at one time to eliminate refreezing. To obtain the desired working concentration (2 units per test well) first add 5% normal chicken serum for the modification to enhance sensitivity as described earlier. Then estimate a starting point based on previous lots. A good starting point is a dilution of 1/30 after the chicken serum has been added. Set up a series of tubes with various amounts of complement in VBS. The VBS should contain the antigen to be used in the reaction and take into account any anticomplementary properties of the antigen. A common method is to dilute 0.10 ml C’ + 0.90 ml VBS; 0.12 ml complement + 0.88 ml VBS, etc. through 0.25 ml C’ + 0.75 ml VBS. Incubate the tubes for 2 hours in a 37°C water bath. Add 0.5 ml of sensitised SRBCs to each tube. Incubate for 1 additional hour in the 37°C water bath. The highest dilution giving complete haemolysis equals 1 unit. Twice that amount equals 2 units. The following formula can be used to obtain 2 units/0.05 ml:

\[
x = \frac{(di) \times v}{2dh}
\]

where:

\[
x = \text{reciprocal of C’ dilution desired to yield 2 units C'/well}
\]
\[
di = \text{reciprocal of C’ initial dilution used in titration (1/30)}
\]
\[
v = \text{volume of diluted C’ to be added}
\]
\[
dh = \text{twice the volume of C’ giving complete haemolysis in titration}
\]

v) **Titration of haemolysin**

Haemolysin can be obtained from commercial sources. It must be standardised by titration. The following procedure is recommended:

Prepare a 1/100 dilution of the stock haemolysin in VBS. From this, prepare 1/300, 1/400, and 1/500 dilutions in tubes. From each of these dilutions, make 0.5 ml of twofold dilutions in VBS for a block titration.

To determine haemolysin concentration, add the following to 0.5 ml of each dilution: 0.5 ml of C’ at 1/30 dilution, 0.5 ml of unsensitised SRBCs at 0.25 optical density, and 1.5 ml of VBS. Incubate for 1 hour at 37°C, and then centrifuge at 400 g for 5 minutes. One unit of the haemolysin is the dilution that gives complete lysis of the SRBCs. The haemolysin solution is prepared in VBS at the dilution containing 2 units of haemolysin. This is then added to an equal volume of SRBCs at the proper concentration.

vi) **Titration of antigen and positive control serum**

In order to standardise the CF test, it is also necessary to have titres of both the antigen and the positive control serum. If the titre is known for either the positive serum or antigen, the titre of the other component can be determined by performing the CF test using dilutions of the component being titred. If titres of both the positive serum and antigen are unknown, a block titration (chequerboard) can be
used to determine the limiting dilutions of both the antigen and the antibody where haemolysis starts. It is very critical to obtain these titres accurately.

For both the antigen and the positive control serum, 4 units are used. A unit is the highest dilution that will give a positive test. That is, if a dilution of 1/160 gives a positive test, then a 1/40 dilution has 4 units and is used for the test.

Complement-fixing antibodies usually appear within 7–10 days of infection. For a positive diagnosis, a four-fold rise in CF antibody titre is required. A presumptive diagnosis by serological tests on a flock can only be made if typical clinical signs are present and a majority of the birds have antibody titres of >1/64.

b) Other tests

Other serological tests have been developed, but their specificity has not yet been sufficiently evaluated. The ELISA for group-specific chlamydial antibodies is more rapid and sensitive than the CF test; it can be automated. Evaluations of ELISA for the detection of antibodies to both C. trachomatis and C. psittaci (15, 24, 33, 34) indicate that it can be substituted for the CF test in most cases. However, it has yet to be tested extensively, standards for its use have not been established, and conjugates are not commercially available for all species of birds.

Other tests include the agar gel immunodiffusion test (29), the latex agglutination (LA) test, the elementary body agglutination (EBA) test (18, 21) and the micro-immunofluorescence test (MIFT). Immunodiffusion is less sensitive than the CF test. The LA test will detect antibodies to C. psittaci, and is easy and rapid to perform (20). Latex beads are coated with purified chlamydial antigen, mixed thoroughly with the test serum on a glass plate, and rotated for 2 minutes to enhance agglutination. The test is read against a dark background. Sera giving positive reactions should be retested with uncoated beads to eliminate possible nonspecific agglutination. The LA and direct CF tests correlate in 72.5% of tests with paired sera. The LA test has a sensitivity of 39.1% and a specificity of 98.8% relative to the direct CF test (20). The test detects both IgM and IgG, but it is best at detecting IgM. It has been suggested for use in detecting recent or active infections. The EBA test detects only IgM, and it is indicative of a current infection. The MIFT is rapid and easy to perform; however, fluorescence-conjugated anti-species sera are not always available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no commercial vaccines available for chlamydiosis in poultry. Attempts to produce a vaccine have met with limited success, and most have been based on bacterins produced by formalin inactivation of concentrated suspensions of chlamydiae. There is evidence that immunity involves cell-mediated immune responses (30, 31), but vaccine manufacture has not been directed towards reactions of this type.

Antibiotics are the only current means of control. *Chlamydophila psittaci* is susceptible to a number of antibiotics: the drug of choice varies from country to country. Chlorotetracycline, doxycycline, and other tetracyclines are the most commonly used. Fluoroquinolone antibiotics have also proven their value (25). Treatment needs to be maintained for extended periods of time. For pet birds, 45 days is often recommended (23, 40).

REFERENCES


Chapter 2.7.4. — Avian chlamydiosis


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* *
CHAPTER 2.7.5.

FOWL TYPHOID AND PULLORUM DISEASE

SUMMARY

Definition of the disease: Pullorum disease of chickens is caused by Salmonella enterica subspecies enterica serovar Pullorum (Salmonella Pullorum)\(^1\) and, in its acute form, is almost exclusively a septicaemic disease of young chickens. However, the organism may also be associated with disease in turkey pouls. Ovarian transmission is a major route by which the organism can spread. Game birds and 'backyard' poultry flocks may act as reservoirs of infection, and wild birds may act as vectors for the organism and as such are important in the epidemiology of the disease.

Fowl typhoid in chickens and turkeys is caused by S. Gallinarum and is more often observed in the later growing period and in mature stock.

Description of disease: Clinical signs in chicks and pouls comprise anorexia, diarrhoea, dehydration, weakness and death. In mature birds disease is less severe but decreased egg production, poor hatchability and some increased mortality may occur.

Identification of the agent: Samples should not be taken from birds or eggs that have recently been treated with antimicrobial drugs. They can be swabs or aseptically collected samples from infected tissues, or intestinal and cloacal contents. Other materials that may be sampled include eggs, embryos, faecal droppings and hatcher debris, especially fluff, dust and broken eggshells and chick box linings. Samples of tissues such as caecal tonsils and spleen from infected birds are preferable to faecal and environmental samples. Tissue samples should be inoculated into nonselective and selective enrichment broth and into selective agar medium, such as brilliant green agar, as soon as possible after collection. In case of delay, samples should be stored at 4°C. Typical colonies can be identified by serological and biochemical tests.

Serological tests: These are satisfactory for establishing the presence and estimating the prevalence of infection within a flock. The test used in the field is the rapid whole blood plate agglutination test. This test is unreliable in turkeys and ducks, many uninfected turkeys may give positive reactions. In the laboratory a serum agglutination test is used, either as a rapid plate test or as a tube test. These can be applied as macro- or microagglutination tests though the latter may be more likely to give false-positive results with turkey sera. Any positive reactors should be confirmed as being infected by culture at post-mortem examination. Enzyme-linked immunosorbent assays have been reported but no commercial test is available.

The use of vaccines to control S. Enteritidis infections in chickens may cause problems in the interpretation of serological results.

Requirements for vaccines and diagnostic biologicals: Live and inactivated vaccines are available for fowl typhoid in some countries. The most commonly used vaccine is a live vaccine derived from the stable rough strain of S. Gallinarum known as ‘9R’.

A. INTRODUCTION

Fowl typhoid and pullorum disease, caused by Salmonella enterica subspecies enterica serovars Gallinarum and Pullorum, respectively, are widely distributed throughout the world but they have been eradicated from commercial poultry in many developed countries of Western Europe, the United States of America (USA),

\(^1\) See the note in Chapter 2.10.3. Salmonellosis for the principles followed concerning the nomenclature of Salmonella.
Canada, Australia and Japan. *Salmonella* Pullorum remains in wild and game birds however. *Salmonella Gallinarum* and *S. Pullorum* are host adapted to avian species and are considered to pose a minimal zoonotic risk (11).

Clinical signs are typical of a septicaemic condition in poultry and include increased mortality and poor quality in chicks hatched from infected eggs. Older birds show signs of anaemia, depression, laboured breathing and diarrhoea causing adherence of faeces to the vent. The highest mortality occurs in birds of 2–3 weeks of age. In older birds disease may be mild or inapparent. In breeding flocks reduced egg production and hatchability may be the only signs, and trans-ovarian infection resulting in infection of the egg and hatched chicks or poults is one of the most important transmission routes for the disease.

Post-mortem signs of pullorum disease in newly hatched chicks are those of peritonitis with generalised congestion of tissues and inflamed unabsorbed yolk sac. Longer standing infections commonly lead to typhlitis with development of necrotic caecal casts and small necrotic foci in the liver, lungs and other viscera. Adult birds may develop misshapen or shrunken ovaries. In fowl typhoid, as well as generalised signs of septicaemia, the liver is usually enlarged, dark and friable with a distinctive coppery bronze sheen. The bone marrow is also often dark brown. Although clinical signs and post-mortem findings of pullorum disease and fowl typhoid may be highly suggestive of the conditions, they are not sufficiently distinct from other causes of septicaemia to be pathognomonic. It is therefore necessary to confirm disease by isolation of the organisms. Serological tests can be used to establish the presence of the disease in a flock.

**B. DIAGNOSTIC TECHNIQUES**

In its acute form, pullorum disease is almost exclusively a disease of young chickens, and the agent can be recovered from almost all organs, tissues and faeces. In older birds that have become carriers, *S. Pullorum* is most commonly recovered from the ova, and only exceptionally from other organs and tissues, including the alimentary tract. In the acute phase of fowl typhoid the organism is also widely distributed, but in carrier birds, the organism is found most often in the liver and caecae.

- **Culture**

*Salmonella* Pullorum and *S. Gallinarum* belong to the Kauffmann–White scheme serogroup D, along with *S. Enteritidis*, which is thought to be closely related. The organisms are Gram negative nonsporogenic rods 1.0–2.5 µm in length and 0.3–1.5 µm in width. They are considered to be nonmotile under normal conditions but inducement of flagellar proteins and motility has been shown in special media (8).

To recover the organisms, the birds should not have been treated with antimicrobial drugs for approximately 2–3 weeks previously.

Samples may be obtained from live birds, fresh or freshly frozen carcasses, egg materials, fresh faeces, or any contaminated materials from housing, incubators or transport boxes. Swabs may be taken from the cloaca of live birds. Samples from visibly abnormal tissues are preferable to faecal and environmental samples. Aseptic samples can be taken from the spleen, liver, gall-bladder, kidneys, lungs, heart, ova, testes, alimentary tract or joint lesions. The surface is seared with a hot spatula and a sample is obtained by inserting a sterile cotton swab or sterile loop through the heat-sterilised surface. The demonstration of infection in serological reactor birds that are apparently normal may require the culture of large volumes of homogenised tissues as well as direct swabbing. Tissue pools may be made from tissues collected from a number of birds.

When floor litter or faecal material is sampled, it should be remembered that *S. Pullorum* and *S. Gallinarum* are more difficult to isolate from faecal and environmental samples than other *Salmonella*ae. Samples should include floor faeces, moist and dry litter and swabs from open drinkers. These samples should be cultured by direct inoculation of a selective enrichment broth such as selenite cysteine, followed by plating on selective media such as brilliant green agar.

Both *S. Pullorum* and *S. Gallinarum* grow well on nonselective media, but selective and enrichment media have been described that contain substances to inhibit the growth of extraneous organisms. The efficiency of recovery of *Salmonella* varies according to circumstances, and experience in the use of a medium is an important but unquantifiable factor. Some complex media may have an inhibitory effect on these organisms, so that it is advisable to use both nonselective and selective media for isolation from tissues. Both solid media and broths can be employed. As the toxic properties of selective media may vary, it is preferable to monitor these by comparing growth of control cultures on both types of medium. The inhibitory media should grow at least 75% of the colonies of the corresponding non-inhibitory medium (3, 4, 7, 10).
All the media mentioned below are examples of commonly used media, but there are many others found to be equally satisfactory.

Non-inhibitory media include nutrient agar and blood agar, on which colonies are seen to be smooth, translucent, slightly raised, and about 2 mm in diameter. Broths include buffered peptone water and nutrient and meat infusion broths.

- **Selective media include:**
  
  **MacConkey agar:** The agar is inhibitory to non-enteric organisms, it differentiates lactose fermenters (pink colonies) from nonlactose fermenters (colourless colonies). NaCl is omitted to limit the spread of *Proteus* colonies. *Salmonella* colonies are smooth and colourless. *Salmonella Pullorum* produces smaller colonies than other salmonellae. MacConkey is the agar of choice for direct plating from tissues.

  **Xylose lysine deoxycholate agar:** The agar is inhibitory to non-enteric organisms. *Salmonella Pullorum* grows sparsely as small red colourless colonies. *S. Gallinarum* colonies are small, dome-shaped, and may have a central black spot due to H₂S production, but this reaction may be delayed or variable.

  **Brilliant green agar (BGA):** The agar is inhibitory to coliforms and most *Proteus* strains; useful for distinguishing enteric organism colonies. *Salmonellae* form low, convex, pale red, translucent colonies of 1–3 mm in diameter, similar to *Citrobacter*. *Proteus* forms pin-point colonies, *Pseudomonas aeruginosa* appears as small red colonies, and lactose fermenters are green. *Salmonella Pullorum* produces smaller more pale colonies than other salmonellae. BGA is the agar of choice following enrichment.

  **Brilliant green sulphapyridine agar:** The agar is inhibitory to coliforms and *Proteus* strains. The sulphapyridine is added to stabilise selectivity in the presence of egg materials. *Salmonella Pullorum* produces small colonies.

  *Salmonella Pullorum* and *Gallinarum* grow poorly and do not produce typical colonies on newer chromogenic agars such as Rambach agar.

- **Liquid enrichment and selective media include:**
  
  **Selenite F broth:** Inhibitory to coliforms but not *Proteus*, improved by addition of brilliant green. Loss of activity after 24 hours. Selenite cysteine broth is more stable. Although selenite broths are considered to be preferable for isolation of *S. Pullorum* and *S. Gallinarum* from faeces, if there are difficulties with issues of toxicity or shelf life in particular laboratories the other enrichment broths mentioned below may be used.

  **Tetrathionate/brilliant green broth:** Inhibitory to coliforms and *Proteus*, but may also inhibit some strains of *S. Pullorum*/*Gallinarum*.

  **Rappaport–Vassiliadis soya peptone broth:** For selective enrichment following pre-enrichment, use 1 part inoculum to 100 parts medium. *Salmonella Pullorum* and *Gallinarum* are more likely to be overgrown by other organisms during pre-enrichment of faeces or intestinal contents than salmonellae that are not host-adapted.

- **Recovery of salmonellae**

  The methods for recovering *S. Pullorum* and *S. Gallinarum* vary according to the origin of the samples. Although their isolation from cloacal swabs and faeces may be unrewarding, examination of tissues taken at post-mortem is usually more successful. The methods are as follows:

  **Cloacal swabs and fresh faeces from live birds:** Swabs dipped in nutrient broth are suitable, small swabs being used for young chickens. The swabs are streaked on selective media, and placed in enrichment broth. The plates and the broth are incubated at 37°C. At this temperature, some *Proteus* and *Pseudomonas* organisms tend to be inhibited. Higher temperatures may be used with some broths, e.g. 41.5°C for Rappaport–Vassiliadis (RV), but care needs to be exercised because some enrichment media may be too inhibitory at high temperatures and RV broth is more inhibitory than selenite cysteine broth for *S. Pullorum* and *S. Gallinarum*. Subcultures are made on to selective media after 24 and 48 hours.

  **Gall-bladder contents:** Swabs of gall-bladder contents are streaked on to nonselective and selective agars and placed in inhibitory and non-inhibitory broths, followed by incubation at 37°C and subculture on to selective agar after 24 and 48 hours.

  **Organs and tissues:** Swabs or segments of tissues are taken in an aseptic manner from individual tissues and lesions and cultured on to nonselective and selective media, and into similar broths. These are incubated at 37°C
and subcultured on to selective agar after 24 and 48 hours. Intestinal material in selective broths may also be incubated at 40°C; *S. Gallinarum* grows well but there may be some inhibition of *S. Pullorum* at this temperature.

**Carrier birds:** Larger amounts of material are required to identify the carrier birds. The ovary is the tissue of choice for *S. Pullorum*, and the liver and gall-bladder should be tested for *S. Gallinarum*. In practice it is usually best to pool samples from a variety of tissues. Tissues are homogenised in a small volume of broth and directly plated out. Approximately 10 ml of homogenate is also added to 100 ml of nonselective enrichment broth (e.g. buffered peptone water) and selective enrichment broth (e.g. selenite cysteine broth, brilliant green broth), and incubated at 37°C. These broths are subcultured on to nonselective and selective agar after 24 hours.

**Alimentary canal, including the caecal tonsils and intestinal contents:** After grinding or homogenisation in a small volume of broth, 10 ml of the homogenate is incubated in 100 ml of selective enrichment broth at 37°C. In general better isolation is achieved with selenite cysteine broth.

**Eggshells:** The broken eggshells are placed in a tenfold volume of enrichment broth (e.g. selenite cysteine broth). The broth is incubated at 37°C and subcultured on to selective agar after 24 and 48 hours.

**Egg contents:** Aseptically taken contents of fresh eggs are homogenised and mixed with 200 ml of buffered peptone water or nutrient broth, incubated at 37°C, and subcultured on to nonselective and selective agar after 24 and 48 hours. Incubated eggs, whether infertile or containing small embryos, can be similarly treated.

**Embryos:** Homogenised viscera and swabs from the yolk sacs of well developed embryos may be streaked on to nonselective and selective agar, one swab being placed in 10 ml of both nonselective and enrichment broth (e.g. selenite cysteine broth, brilliant green broth). Incubation is carried out at 37°C, and subcultures are made on to nonselective and selective agar after 24 and 48 hours.

**Environmental samples:** These include hatcher fluff, debris and macerated egg/chick waste samples and chick box liners or floor faecal or litter samples; 25 g is mixed with 225 ml of enrichment broth (e.g. selenite cysteine broth, brilliant green broth). Incubation is carried out at 37°C, and subcultures are made on to nonselective and selective agar after 24 and 48 hours.

1. **Identification of the agent**

Typical *S. Gallinarum* colonies on nonselective media are round, transluscent, glistening, domed, smooth, and 1–2 mm in diameter after 24–48 hours’ incubation. *Salmonella Pullorum* colonies are slightly smaller and transluscent. On selective media their appearance varies with the medium, but suspect colonies can be investigated serologically by testing for ‘O’9 somatic antigens, observing for motility and testing biochemically.

After incubation for 20–24 hours, the plates should be examined carefully for the presence of typical *S. Pullorum* and *S. Gallinarum* colonies. If growth is slight after 24 hours’ incubation, the plates should be reincubated for a further 24 hours and examined again. For biochemical and serological confirmation from each plate, five typical or suspect colonies should be chosen for further examination. If there are fewer than five typical or suspect colonies, all of them should be taken for further examination. Selected colonies should be streaked on to the surface of nutrient agar, in a manner that allows the growth of separate colonies. For biochemical confirmation, only pure cultures taken from nonselective media should be used. The following media should be streaked using an inoculating loop: triple sugar iron (TSI) agar; lysine iron agar (or l-lysine decarboxylation medium); urea agar according to Christensen; tryptone/tryptophan medium for indole reaction; glucose with an inverted Durham tube for acid and gas production; dulcitol, maltose, ornithine decarboxylation medium and semisolid agar, for motility. The reactions shown in Table 1 occur.

Identification kits are commercially available, for example Analytical Profile Index (API) system for Enterobacteriaceae. However, care must be taken when using API because *S. Pullorum* may be misidentified as *Hafnia* spp. Molecular tests using ribotyping techniques and polymerase chain reaction have been developed in research laboratories (9), but are not generally available for confirmation of *S. Gallinarum* and *S. Pullorum*.

For serological confirmation as to serogroup, colonies from nonselective media (nutrient or blood agar) are used. The first stage is elimination of autoagglutinable strains. For this, material taken from a single colony of pure culture is transferred to a glass slide and mixed with a drop of sterile saline. The slide is rocked gently or the drop stirred with a loop for 30–60 seconds and observed for agglutination against a dark background, preferably with the aid of a magnifying glass or dissecting microscope. If the bacteria have clumped into more or less distinctive units, the strain is considered to be autoagglutinable and must not be submitted to the following tests. If the bacterial sample is recognised as non-autoagglutinable, it is tested with a polyvalent ‘O’ (A–G) antiserum. For this purpose, the material from a single colony is dispersed in the drop of polyvalent ‘O’ antiserum on the glass slide to obtain a homogenous and turbid suspension. After gently rocking for 30–60 seconds, the reaction is observed against a dark background for agglutination. Alternatively the slide agglutination test may be carried out with smaller volumes of suspension under a dissecting microscope. In this case a portion of the colony to be checked is added to a loopful of saline on the microscope slide to produce a light suspension to check for
autoagglutination (‘rough strains’). If no agglutination takes place, one or two loops of antisera are added, the drop stirred with a loop and observed for agglutination. Salmonella Pullorum and S. Gallinarum should agglutinate with polyvalent ‘O’ antisera but not with polyvalent flagella (poly ‘H’ phase 1 and phase 2) antisera. If the reaction is positive, the single colony is tested further in the same manner using group-specific sera for S. Pullorum and S. Gallinarum serotypes (‘O9 antiserum). After serogrouping, isolates may be sent to a reference laboratory for serotyping.

Table 1. Biochemical investigation of Salmonella Pullorum and S. Gallinarum

<table>
<thead>
<tr>
<th></th>
<th>Salmonella Pullorum</th>
<th>Salmonella Gallinarum</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSI glucose (acid formation)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TSI glucose (gas formation)</td>
<td>v</td>
<td>−</td>
</tr>
<tr>
<td>TSI lactose</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>TSI saccharose</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>TSI hydrogen sulphide</td>
<td>v</td>
<td>v</td>
</tr>
<tr>
<td>Gas from glucose (medium with Durham tube)</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Urea hydrolysis</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Lysine decarboxylation</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ornithine decarboxylation</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Maltose fermentation</td>
<td>v or late +</td>
<td>+</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

+ = 90% or more positive reaction within 1 or 2 days; − = No reaction (90% or more); v = Variable reactions.

- **Test procedure for culture of visceral, faecal, intestinal and environmental samples for S. Pullorum and S. Gallinarum**
  i) Where possible, begin laboratory procedures on the same day as samples are collected.
  ii) Homogenise the material as much as possible by manual mixing, macerating or stomaching with a small volume of sterile saline if the material is dry.
  iii) Stir the mixture with a small rectal swab or loop and streak thickly on to one-quarter of a brilliant green agar plate. (Swabs from uncontaminated tissues sampled in an aseptic manner can also be streaked on to blood agar.)
  iv) From this deposit of material on the plate, streak the rest of the plate to obtain individual colonies.
  v) Add 5–25 g of the homogenised material to freshly made selenite cysteine broth (see note on liquid enrichment and selective media above) to make a 1:10 sample to broth ratio. Shake or stir to disperse the sample in the broth.
  vi) Incubate the brilliant green agar plates and selenite cysteine broth at 37°C for 24 hours.
  vii) Examine the plate after 24 hours’ culture. Carry out agglutination tests on up to five suspect colonies with polyvalent ‘O’ (A-G) antisera and polyvalent H (phase 1 and phase 2) antisera. If agglutination is unclear subculture suspect colonies on to nutrient agar or blood agar and repeat tests after 24 hours’ incubation of those media.
  viii) If poly ‘O’ is positive then check with ‘O9 antiserum. If ‘O9 is positive and poly ‘H’ is negative, this is indicative of the possible presence of S. Pullorum or S. Gallinarum.
  ix) If there are no positive colonies on the brilliant green agar plate, streak out a 10 µl loop of incubated selenite cysteine broth onto brilliant green agar as in step iv above.
  xi) Incubate the brilliant green agar plates at 37°C for 24 hours and re-incubate the previous (negative) brilliant green agar plates and the selenite cysteine broths for a further 24 hours.
  xii) Repeat examination of plates as in step vii above.
  xiii) If plates are still negative, replate from selenite cysteine broth and incubate second brilliant green agar plate for a further 24 hours and examine as in step vii above.
  xiv) Confirm S. Pullorum and S. Gallinarum using biochemical tests as shown in Table 1. Isolates can be sent to a Salmonella reference laboratory for confirmation of serotype and for phage typing of S. Pullorum.
• Molecular epidemiology

Standard molecular ‘fingerprinting’ techniques used for *Salmonella*, such as plasmid profile analysis or pulsed field gel electrophoresis can be used for investigating outbreaks of *S. Pullorum* or *S. Gallinarum*.

2. Serological tests

Serological tests are best applied as a flock test as results for individual birds will vary according to the stage of infection. It is therefore necessary to take sufficient individual samples to determine infection in the flock. If the test is to be used for detecting individual infected birds for culling, it should be repeated at least twice and preferably until the whole flock has given at least two negative tests.

The tests that are most readily applied include rapid whole blood agglutination, rapid serum agglutination (RST), tube agglutination and micro-agglutination (13). Other invasive *Salmonella* such as *S. Enteritidis* and *S. Typhimurium* may give false-positive results in serological tests for *S. Pullorum*.

Both *S. Pullorum* and *S. Gallinarum* possess ‘O’ antigens 9 and 12 and may also possess O antigen 1. However, in the case of *S. Pullorum*, there is a variation in the ratio of 12₁, 12₂ and 12₃; the standard strain contains more 12₂ than 12₂, while the reverse is true of the variant form. Intermediate forms also exist. (There appears to be no such form variation in the case of *S. Gallinarum*.) As this variation occurs, it is necessary to use a polyvalent antigen in the agglutination tests. The same antigen is used to detect both *S. Pullorum* and *S. Gallinarum*.

a) Rapid whole blood agglutination test

The rapid whole blood agglutination test can be used under field conditions for detecting both *S. Pullorum* and *S. Gallinarum*, and the reactors can be identified immediately. However, it is not reliable in turkeys as the test results in a significant proportion of false-positive results. Chickens can be tested at any age, although some authorities specify a minimum age of 4 months (13, 14) and positive results from chicks less than 4 weeks of age may be due to maternal antibodies.

• Preparation of stained antigen for the rapid whole blood or rapid serum agglutination test

Incubate one standard form strain of *S. Pullorum* (antigenic structure 9, 12₁, 12₂) and one variant form (antigenic structure 9, 12₁, 12₃) at 37°C and harvest separately until final mixing for the complete antigen.

Sow strains on to separate agar slopes, incubate at 37°C for 24 hours, emulsify growth with sterile normal saline and spread an inoculum over an agar plate to produce easily selected discrete colonies. For this the plates are incubated for 48 hours, a number of colonies are marked out and each is tested for agglutination on a slide with 1/500 acriflavine in saline. Smooth-phase colonies do not produce agglutination. Pick off typical colonies that do not produce any agglutination, seed on to agar slopes, and incubate for 24 hours. Emulsify the growth in saline and evenly distribute 2 ml over the surface of the medium (200 ml) in a Roux or similar flask. Incubate the flasks for 60 hours.

For harvesting the bacterial growth, flood the surface of each flask with enough sterile buffered formol saline, pH 6.5 (8.5 g/litre sodium chloride, 10 ml/litre neutral formalin, 4 ml/litre 0.5 M sodium phosphate; made up to 1 litre with distilled water, pH adjusted to 6.5 using 1 M orthophosphoric acid or 1 M sodium hydroxide), to give dense cell suspensions (about 10 ml per flask). Add 12–15 sterile glass beads of 3–5 mm diameter and rock the flasks until all the growth is in even suspension; leave in a vertical position for at least 15 minutes. Check the morphology and purity of the suspensions by preparing and examining Gram-stained films. Bulk the suspension from each flask containing the same strains. To each 100 ml of suspension, add 200 ml of absolute alcohol. Shake the mixture and allow to stand for 36 hours or until precipitation is complete. Check the agglutinability of the standard and variant precipitate by first centrifuging a sample to separate the alcohol, which is removed, dilute with normal saline and test with a known positive and negative serum. If satisfactory, remove the clear supernatant alcohol (centrifugation at 2000 g for 10 minutes may be helpful in precipitation), and add sufficient phosphate buffered saline (PBS) containing 10% (v/v) glycerol to standardise the density to 75 × No. 1 Wellcome opacity tube (or 50 × tube No. 1.0 on the McFarland scale). Add equal volumes of standard and variant strains, and add 1% (v/v) of 3% (w/v) alcholic crystal violet solution to the final mixture, and allow to stand for 48 hours at room temperature. Store in a tightly closed container at 0–4°C for up to 6 months. To assess safety, carry out a culture test on blood agar for nonviability of the unwashed antigen before standardisation. Each bottle of antigen must be tested after alcoholic precipitation and before standardisation against standard titre antisera for *S. Pullorum* and *S. Gallinarum*, and against a negative serum. If possible, also test with known positive and negative serum and blood from positive and negative chickens.

Stained antigen products for the whole blood plate agglutination test are available commercially, and although there seems to be some slight differences in their sensitivity (5), it is unlikely that poultry flocks infected with the different variants of *S. Pullorum* would be missed.
Test procedure

i) Use a clean white tile marked into squares of about 3 x 3 cm. If a tile with 3 x 4 squares is used, up to 12 blood samples can be tested at the same time.

ii) Place 1 drop (about 0.02 ml) of crystal-violet-stained antigen in the centre of each square.

iii) Obtain a sample of fresh whole blood. This is conveniently done by making a stab of a wing vein using a needle with a triangular point.

iv) Place an equal size drop of fresh whole blood next to a drop of antigen.

v) Mix the drops of antigen and blood using a fine glass rod, which is wiped clean between samples.

vi) Use a gentle rocking motion to keep the drops agitated for up to 2 minutes. Several tests may be carried out simultaneously on the same tile, but the drops should not be allowed to dry out during this time. In very warm conditions, larger drops may be required to avoid drying out.

vii) A positive reaction is indicated by easily visible clumping of the antigen within 2 minutes.

viii) A negative reaction is indicated by absence of clumping of the antigen within 2 minutes.

ix) Include known positive and negative control sera on each testing occasion, using them in the same way as the blood.

x) On completion of a set of tests, the tile is washed and dried, ready for further use.

In the absence of positive reactions, any doubtful reactions can only be interpreted in the light of the previous Salmonella testing history of the flock. Where there are positive reactors, any doubtful reactor should be regarded as positive. Also, recently infected birds may not show a typical positive reaction until they are retested after 3–4 weeks.

b) Rapid serum agglutination test

The RST is performed in the same manner, except that serum is substituted for whole blood. For export test purposes an initial screening of sera by RST followed by confirmation of positives by the tube agglutination test is the optimal approach. Ideally serum samples tested by any method should be tested within 72 hours of collection as nonspecific reactions may increase in older samples. Fresh samples can be frozen for later testing if a delay is unavoidable.

c) Tube agglutination test

Fresh serum from chickens, turkeys or other birds is used at an initial dilution of 1/25, obtained by mixing 0.04 ml of serum with 1.0 ml of antigen\(^2\). Positive and negative control sera are included in each test. The antigen is prepared from unstained S. Pullorum or S. Gallinarum cultures diluted to a concentration of No. 1 on the McFarland scale (as described above). The mixture is incubated at 50°C for 18–24 hours before reading. A positive reaction consists of a granular white deposit with a clear supernatant fluid; a negative reaction shows uniform turbidity. Samples positive at a dilution of 1/25 are retested at a higher range of dilutions and a titre of 1/50 is usually considered to be positive, although this figure seems to vary in the literature.

d) Micro-agglutination test

This resembles the tube agglutination test, but requires much smaller volumes of reactants. The test is performed in microtest plates. Sera are first diluted by adding 10 µl of serum to 90 µl of normal saline, and then adding 100 µl of previously standardised stained microtest antigen to give a final dilution of 1/20. By titrating the serum in doubling dilutions and adding an equal volume of standardised stained antigen, an end-point (titre) can be obtained. The plates are sealed and incubated at 37°C for 18–24 or 48 hours. A positive reaction consists of a fine diffuse precipitation, whereas a negative reaction shows a button-like precipitate. Titres of 1/40 are usually considered to be positive but this test is more liable to produce false-positive results with turkey sera.

Other serological tests include micro-antiglobulin (Coombs), immunodiffusion, haemagglutination and enzyme-linked immunosorbent assay (ELISA).

ELISA techniques have been described for detecting antibodies to S. Pullorum and S. Gallinarum. The indirect ELISA using lipopolysaccharide antigen is likely to be the most sensitive and specific serological flock test for Salmonella, including S. Gallinarum and S. Pullorum. It is relatively easy to perform with serum or yolk, and can

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\(^2\) For preparation of small volumes of somatic antigens see Chapter 2.10.3. Salmonellosis.
be used for quantifying the titre of antibody (1, 2, 14). No commercial ELISA kits for S. Pullorum and S. Gallinarum are currently available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Although both live and inactivated vaccines have been prepared for use against S. Gallinarum, the vaccine most widely used is made from the rough 9R strain (6). It has only been employed in chickens. The number of viable organisms per dose is important; these organisms can survive in vaccinated birds for many months and may be transmitted through the egg (and perhaps from bird to bird). Vaccination may reduce flock losses, but will not prevent infection with field strains. In addition, vaccination with 9R may sometimes precipitate high mortality in infected birds (12), and may stimulate the production of transient antibodies. It is usual to vaccinate at 8 weeks and again at 16 weeks of age. Antimicrobials should be avoided before and after vaccination.

Currently available vaccines, however, have only a minor role to play in the control of fowl typhoid. This can best be achieved by biosecurity, hygiene in management, monitoring and removal of infected flocks.

1. Seed management

a) Characteristics of the seed

Live fowl typhoid vaccine is a suspension of suitably attenuated living organisms of a rough strain of S. Gallinarum, e.g. 9R. The organisms in the vaccine give the biochemical reactions characteristic of S. Gallinarum. Colonies of a 24-hour culture prepared from the vaccine on nutrient agar plates are rough when examined by the acriflavine slide test. The culture does not contain the somatic antigens characteristic of the smooth forms of S. Gallinarum.

b) Method of culture

Salmonella Gallinarum is grown on or in a suitable medium, such as nutrient agar or broth, for 24 hours at 37°C.

c) Validation as a vaccine

There is no satisfactory method of assessing the protection afforded by the vaccine in the field. However, experience has shown that the vaccine can provide some benefit in situations where control cannot be achieved by hygiene and management alone. The potency test described below may be used to provide evidence of efficacy.

2. Method of manufacture

The vaccine may be prepared by inoculation of a suitable medium, such as nutrient broth, with a fresh culture of S. Gallinarum (9R) and incubation at 37°C for 24 hours, with or without aeration. The organisms are harvested by sedimentation or centrifugation.

Alternatively the organisms may be grown on and harvested from a solid medium, such as nutrient agar. In either case, the suspension is diluted in PBS solution, pH 7.0, and may be freeze-dried. The dose used per bird is between $5 \times 10^6$ and $5 \times 10^7$ organisms.

3. In-process control

The culture used for inoculation of the production cultures and the harvested cells are examined microscopically using Gram staining to check for purity.

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

Six healthy, susceptible (preferably specific pathogen free [SPF]) chickens, 8–16 weeks of age, are each injected subcutaneously with ten doses of vaccine, and are observed for at least 7 days; no local or systemic reaction should develop.
c) **Potency**
Fifteen healthy chickens, 8–16 weeks of age, of the Light Sussex or Rhode Island Red breeds, or crosses of these, and taken from a stock that is free from *S. Pullorum* infection, are each injected subcutaneously with a quantity of vaccine corresponding to one field dose, i.e. $5 \times 10^7$ viable organisms. After an interval of 21–28 days, the vaccinated chickens and an equal number of similar unvaccinated chickens are deprived of food for approximately 18 hours. The chickens are then challenged by oral administration of 1 ml of a broth suspension containing $5 \times 10^7$ organisms of a virulent strain of *S. Gallinarum* mixed with 300 mg of a powder consisting of chalk (40%), light kaolin (43%) and magnesium trisilicate (17%). All the chickens are observed for 14–21 days. The vaccine passes the test if at the end of this period the number of surviving vaccinated chickens that show no macroscopic lesions of fowl typhoid at post-mortem exceeds by eight or more the number of similarly defined control chickens.

d) **Duration of immunity**
The vaccine should provide protection throughout the laying period, and this can be measured by potency (efficacy) tests at stages during lay. A booster dose during lay may be required, but should not be used during lay in flocks providing eggs for human consumption.

e) **Stability**
The shelf life of the vaccine can be measured by conducting potency tests at periods after manufacture. These should be done on at least six samples. Potency should remain satisfactory for at least 1 year.

f) **Preservatives**
No preservatives are used.

g) **Precautions (hazards)**
The organism is not known to be pathogenic to humans, and there are no special risks associated with the manufacture of either the vaccine or the antigen. However, the vaccine may establish a persistent infection in carrier birds and can precipitate disease in already infected chickens.

5. **Tests on the final product**

a) **Safety**
The safety test described in Section C.4.b. should be used on a representative sample from each batch of final product.

b) **Potency**
The potency test described in Section C.4.c. should be used on a representative sample from each batch of final product.

**REFERENCES**


Chapter 2.7.5. – Fowl typhoid and Pullorum disease


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CHAPTER 2.7.6.

AVIAN INFECTIOUS BRONCHITIS

SUMMARY

Avian infectious bronchitis (IB) is usually defined as an acute, contagious disease of chickens characterised primarily by respiratory signs. However, infections with the causative virus may also lead to nephritis (acute or chronic) and egg production problems in laying hens. The severity of respiratory infections with infectious bronchitis virus (IBV) can be greatly enhanced by the presence of other pathogens of the respiratory tract. Clinical signs are indicative, but not diagnostic, of IB, and confirmation requires the isolation or direct demonstration of the presence of IBV, although serology may also be useful in some circumstances. The widespread use of both live and inactivated vaccines may complicate both virus isolation and serology in the diagnosis of IB. The natural occurrence of antigenic variant strains may overcome any immunity induced by conventional vaccine. Recently coronaviruses have been isolated from turkeys and pheasants that are genetically similar to IBV.

Laboratory diagnosis is made by virus isolation in chicken embryos or in tracheal organ cultures. This can be supplemented by immunofluorescence, electron microscopy, polymerase chain reaction (PCR) techniques, haemagglutination inhibition (HI) tests or enzyme-linked immunosorbent assays (ELISA).

Identification of the agent: IBV may be isolated from tracheal mucosa and lung during the acute phase of the respiratory form of the disease. Faeces, kidneys or caecal tonsillar tissue are better sources of virus at other times.

Chicken embryos originating from specific pathogen free flocks or tracheal organ cultures (TOCs) from 20-day-old embryos are used for virus isolation. The inoculation of the allantoic cavity of chicken embryos of 9–11 days’ incubation with IBV results in embryo stunting or death, usually within three serial passages. TOCs have the advantage that IBV produces stasis of the tracheal cilia on initial inoculation. The virus can be identified by neutralisation tests using specific antiserum. The antigen may be visualised in infected allantoic cells by immunofluorescence, or by electron microscopy after concentration by ultracentrifugation.

Antigenic typing of IBV is difficult and controversial. Within the same laboratory, a limited range of strains can be shown to be antigenically related or different by using various serological methods. The use of monoclonal antibodies may prove to be a useful method of distinguishing vaccine strains from field strains, and for defining serotypes. Genotyping of IB isolates using reverse-transcription PCR is becoming more widely available.

Serological tests: Regular monitoring of sera from flocks for IB antibody titres may help to indicate the level of vaccine response. Because many chicken sera, especially from older birds, contain antibodies that are highly cross-reactive against antigenically unrelated strains, serodiagnosis of suspected disease outbreaks of IB cannot be used with a high degree of confidence. The HI test is rapid, inexpensive, practicable and under some circumstances may allow identification of infection with antigenic variant virus. Commercially produced kits that are very sensitive and suitable for monitoring immune response to vaccine are available for ELISAs. They appear to lack type specificity.

A positive diagnosis of IB is made by virus isolation together with tests to demonstrate a significant rise in specific antibody. Following preparation of monospecific antiserum to a virus isolate, a serological comparison can be made with existing strains to allow antigenic serotype identification.
Requirements for vaccines and diagnostic biologicals: Both live attenuated and oil emulsion inactivated vaccines are available. Live vaccines have been attenuated by serial passage in chicken embryos and confer a better local immunity of the respiratory tract. The use of some live vaccines carries the risk of residual pathogenicity associated with vaccine back-passage in flocks. However, proper mass application will generally result in safe application of live vaccines. The concerns about the use of live vaccines can be avoided by using inactivated vaccines.

Inactivated vaccines have to be given to birds individually, and a single inoculation does not confer protection unless preceded by the administration of a live vaccine. Both types of vaccine are available in combination with Newcastle disease vaccine; in some countries inactivated multivalent vaccines are available that include Newcastle disease, infectious bursal disease, reovirus and egg-drop syndrome 76 viral antigens.

### A. INTRODUCTION

Avian infectious bronchitis (IB) was first described in the United States of America (USA) in the 1930s as an acute respiratory disease mainly of young chickens. A viral aetiology was established, and the agent was termed avian infectious bronchitis virus. Infectious bronchitis virus (IBV) is a member of the genus *Coronavirus*, family *Coronaviridae*, in the order Nidovirales. The virus has a nonsegmented, positive-sense, single-stranded RNA genome.

IBV affects chickens of all ages, which, apart from pheasants and guinea-fowl are the only species reported to be naturally affected. IB occurs worldwide and assumes a variety of clinical forms, the principal one being a classical respiratory syndrome. Infection of the oviduct can lead to permanent damage in young birds lacking maternal antibodies, and, in older birds, can lead to cessation of egg-laying or production of thin-walled and misshapen shells with loss of shell colour. IB can be nephrotropic causing acute nephritis, urolithiasis and mortality (10). After apparent recovery, chronic nephritis can produce sudden death some time later, especially in brown birds. The virus may persist in the intestinal tract and is excreted in the faeces for long periods. This occurs with vaccine strains as well as natural field strains (2).

There have been no reports of human infection with IBV.

### B. DIAGNOSTIC TECHNIQUES

Confirmation of diagnosis is based on demonstration of the virus, sometimes assisted by serology. Extensive use is made of live and inactivated vaccines, which may complicate diagnosis by serological methods as antibodies to vaccination and field infections cannot always be distinguished. Persistence of live vaccine virus may also confuse attempts at recovering the causative agent.

1. Identification of the agent

   a) Sampling

   Samples taken from birds should relate to the disease under investigation. For acute respiratory disease, swabs from the upper respiratory tract of live birds or tracheal and lung tissues from recently killed birds should be kept on ice in transport medium containing penicillin (10,000 International Units [IU]/ml) and streptomycin (10 mg/ml). For birds showing nephritis or egg-production problems, samples may also be selected from the kidneys or oviduct, but the highest success rate of virus recovery has been reported from samples of large intestine, particularly the caecal tonsillar tissue, or faeces (2). Isolates from the intestinal tract, however, may have no relevance to the latest infection or clinical disease, and samples from the respiratory tract must be taken in all investigations.

   Tissue samples from the trachea, kidney, oviduct and caecal tonsils in sterile transport media with antibiotics and dry swabs from the respiratory tract or cloaca can also be submitted to specialist laboratories for reverse-transcription polymerase chain reaction (RT-PCR) analysis (7). For specimens requiring despatch to a diagnostic laboratory it is essential that samples in transport media be kept chilled or frozen throughout transport. Where delays of more than 3 days are expected, the samples should be frozen prior to dispatch and sent on dry ice. Samples should also be selected from fresh carcases for histopathological examination. Blood samples from acutely affected birds should also be submitted for serological analysis.
b) **Culture**

Suspensions of tissues (10–20% w/v) are prepared in sterile phosphate buffered saline (PBS) or nutrient broth for egg inoculation, or in tissue culture medium for tracheal organ culture (TOC) inoculation (10, 16). The suspensions are clarified by low-speed centrifugation and filtration through bacteriological filters (0.2 µ) before inoculation of eggs or TOCs.

Embryonated hens’ eggs and TOCs are widely used to titrate the virus or to make primary isolations of virus. Cell cultures are not used for primary isolation as it is usually necessary to adapt IBV isolates to growth in chicken embryos before cytopathic effects (CPE) of virus infection are seen.

Eggs used in all culture work with IBV must originate from birds that have been neither infected nor vaccinated. Such eggs should preferably be from specific pathogen free (SPF) hens. Most commonly, 0.1–0.2 ml of sample supernatant is inoculated into the allantoic cavity of 9–11-day-old embryos. Embryos should be examined daily thereafter. Any deaths that occur within 24 hours are assumed to be nonspecific and the eggs are discarded. The initial inoculum usually has no effect on the embryo unless the strain is derived from a vaccine and already egg adapted. Normally, the allantoic fluids of all eggs are pooled after harvesting 3–7 days after infection; this pool is diluted 1/5 or 1/10 in antibiotic broth and further passaged into another set of eggs. This is repeated as desired. Typically, a field strain will induce teratological changes in the embryo at the second or third passage consisting of stunted and curled embryos with feather dystrophy and urate deposits in the embryonic mesonephros. Some mortality in later passages may occur. Other viruses, most notably adenoviruses, may also produce embryo lesions indistinguishable from IBV. The allantoic fluid should not agglutinate red blood cells and isolation of IBV must be confirmed by immunological or genotypic testing. Infective allantoic fluids are kept at −60°C or below for long-term storage, or at 4°C after lyophilisation.

TOCs prepared from 20-day-old embryos can be used to isolate IBV directly from field material (16). An automatic tissue-chopper is desirable for the large-scale production of suitable transverse sections or rings of the trachea for this technique (20). The rings are about 0.5–1.0 mm thick, and are maintained in a medium consisting of Eagle’s N-2-hydroxyethylpiperazine N’-2-ethanesulphonic acid (HEPES) in roller drums (15 rev/hour) at 37°C. Infection of tracheal organ cultures usually produces ciliostasis within 24–48 hours. Ciliostasis may be produced by other viruses and suspect IBV cases must be confirmed by immunological or genotypic methods.

c) **Methods for identification**

The virus neutralisation (VN) test in embryonated eggs and the immunodiffusion technique are useful for identification purposes (see below). Fluorescent antibody tests on cells present in the allantoic fluid of infected eggs may also demonstrate the presence of IBV (11) and direct negative-contrast electron microscopy will reveal particles with typical coronavirus morphology in allantoic fluid or TOC fluid concentrates. The specific presence of IBV in infective allantoic fluid may be detected by RT-PCR amplification and use of a DNA probe in a dot-hybridisation assay (27). Direct immunofluorescence staining of infected TOCs for the rapid detection of the presence of IBV has been described (3). Immunohistochemistry, with a group-specific monoclonal antibody, can be used to identify IBV in infected chorioallantoic membranes (35).

d) **Serotype identification**

Antigenic and biological variation among IBV strains is well reported (10, 15, 22, 23, 26), but at present there is no agreed definitive classification system. Nevertheless, antigenic relationships and differences among strains are important, as vaccines based on one particular subtype may show little or no protection against viruses of a different antigenic group. As a result of the regular emergence of antigenic variants, the viruses, and hence the disease situation and vaccines used, may be quite different in different geographical locations. Constant assessment of the viruses present in the field is necessary to produce vaccines that will be efficacious in the face of antigenic variants that may arise. Serotyping of IBV isolates and strains has been done using VN tests in embryonated eggs (22), TOCs (21) and cell cultures (24). Neutralisation of fluorescent foci has also been applied to strain differentiation (18). The haemagglutination inhibition (HI) test has been employed for serotyping IBV (1, 29) and has proved useful providing early response sera are used.

Monoclonal antibodies (MAbs), usually employed in enzyme-linked immunosorbent assays (ELISA), have proven useful in grouping and differentiating strains of IBV (25, 31). The limitations of MAb analysis for IB serotype definition are the lack of availability of MAbs or hybridomas and the need to produce new MAbs with appropriate specificity to keep pace with the ever-growing number of emerging IB-variant serotypes (28).

e) **Genotype identification**

The molecular basis of antigenic variation has been investigated, usually by nucleotide sequencing of the gene coding for the spike (S) protein or, more specifically, nucleotide sequencing of the gene coding for the
S1 subunit of the S protein (5, 33) where most of the epitopes to which neutralising antibodies bind are found (32). An exact correlation with VN results has not been seen, in that while different serotypes generally have large differences (20–50%) in the deduced amino acid sequences of the S1 subunit (33), other viruses that are clearly distinguishable in neutralisation tests show only 2–3% differences in amino acid sequences (5). However, there is in general good agreement between data represented by the S1 sequence and the VN serotype, and it may eventually be possible to select vaccine strains on the basis of sequence data. It has been suggested that the nucleoprotein may play an important role in inducing protection against IB viruses. Recently, it has been shown that coronaviruses isolated from turkeys and pheasants are genetically similar to IBV, having approximately 90% nucleotide identity in the highly conserved region II of the 3' untranslated region (UTR) of the IBV genome (8, 9).

Nucleotide sequencing (of particularly the S gene region) using appropriate primers is the most useful technique for the differentiation of IBV strains and has superseded restriction fragment length polymorphism analysis (RFLP) and the use of DNA probes. Nucleotide sequencing has also produced evidence that recombination between IB strains occurs often (6, 40). RT-PCR is now being used in a number of diagnostic laboratories for sequencing and characterising a wide range of variant serotypes of IBV from many countries (30).

2. Serological tests

A number of tests have been described. Those considered here include VN (22), agar gel immunodiffusion (AGID) (39), HI (1) and ELISA (34). Each test has advantages and disadvantages in terms of practicality, specificity, sensitivity and cost. In general, for routine serological testing, the VN tests are too expensive and impractical, and AGID tests lack sensitivity. HI tests and ELISAs are suitable for routine serology although they differ in their specificity. As ELISAs are available in kit form with detailed instructions for their use, the HI test is described in detail below. Regular monitoring of sera from flocks for IB antibody titres may help to indicate the level of vaccine response. Because many chicken sera, especially from older birds, contain antibodies that are highly cross-reactive against antigenically unrelated strains, serodiagnosis of suspected disease outbreaks of IB cannot be used with a high degree of confidence.

a) Virus neutralisation

In VN tests, all sera should first be heated to 56°C for 30 minutes. Virus is mixed with serum and incubated for 30–60 minutes at 37°C or room temperature. Chicken embryos are most often employed, but antibodies can be measured using TOC or cell culture systems. Two methods have been used to estimate neutralising antibodies. One employs a constant serum concentration reacted with varying dilutions of virus (the alpha method) and the other employs a constant amount of virus and varying dilutions of serum (the beta method).

In the alpha method, tenfold dilutions of egg-adapted virus are reacted with a fixed dilution (usually 1/5) of antiserum, and the mixtures are inoculated into groups of from five to ten eggs. The virus alone is titrated in parallel. End-points are calculated by the Kärber or the Reed and Muench methods. The results are expressed as a neutralisation index (NI) that represents the log_{10} difference in the titres of the virus alone and that of the virus/antiserum mixtures. The NI values may reach 4.5–7.0 in the case of homologous virus/serum mixtures; values of <1.5 are not specific, but a heterologous virus will give a value as low as 1.5.

The beta method is the more widely used neutralisation test for antibody assay with chicken embryos. Two- or four-fold dilutions of antiserum are reacted in equal volumes with a dilution of virus, usually fixed at 100 or 200 EID_{50} (median embryo-infective doses) per 0.05 ml and 0.1 ml of each mixture inoculated into the allantoic cavity of each of from five to ten embryonated eggs. A control titration of the virus is performed simultaneously to confirm that the fixed virus dilution in the virus/serum mixtures was between 10^{1.5} and 10^{2.5} EID_{50}. End-points of the serum titres are determined by the Kärber or Reed and Muench method as before, but here are expressed as reciprocals of log_{2} dilutions. This fixed-virus/varying-serum method is also employed for neutralisation tests in tracheal organ cultures using five tubes per serum dilution, as is conventional with other viruses (21). The results are calculated according to Reed and Muench, and the virus titres are expressed as median ciliostatic doses per unit volume (log_{10} CD_{50}). Serum titres are again expressed as log_{2} dilution reciprocals. This test is more sensitive than others, but technical logistics hamper its more widespread adoption.

b) Haemagglutination inhibition

A standard protocol for a HI test for IBV has been described (1), and the test procedure detailed below is based on that standard. Many strains and isolates of IBV have been shown to agglutinate chicken red blood cells (RBCs) after enzyme treatment. The virus selected to produce antigen may be varied, depending on the requirements of diagnosis.
• **Preparation of antigen**

IBV antigen requires enzyme treatment to acquire haemagglutination (HA) activity. Originally this was shown to be facilitated by commercial phospholipase C type 1 enzyme, and it was recommended that the virus suspension be mixed with an equal volume of this enzyme to a final concentration of 1 unit/ml in the same buffer. However, it was found to be more efficient to use crude filtrate from *Clostridium perfringens* culture, and it seemed most likely that a contaminating enzyme rather than phospholipase was the constituent responsible for the activity. Subsequent work has indicated that this enzyme is most probably neuraminidase (36). Infective allantoic fluid is centrifuged at 30,000 g for 3 hours and the pellet is resuspended at 100-fold concentration in *Clostridium perfringens* type A filtrate and incubated at 37°C for 2 hours.

For HA and HI tests, procedures are best carried out at 4°C.

• **Haemagglutination test**

i) Dispense 0.025 ml of isotonic PBS, pH 7.0–7.4, into each well of a plastic microtitre plate.

ii) Place 0.025 ml of virus antigen in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/3, 1/4, 1/5, 1/6, etc.

iii) Make twofold dilutions of 0.025 ml volumes of the virus antigen across the plate.

iv) Dispense a further 0.025 ml of PBS into each well.

v) Dispense 0.025 ml of 1% (v/v) chicken RBCs to each well.

vi) Mix by tapping the plate gently and allow the RBCs to settle for about 40 minutes at 4°C, when control RBCs should be settled to a distinct button.

vii) HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA in which there is no streaming; this is 100% HA and represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

• **Haemagglutination inhibition test**

The HI test is used in the diagnosis and routine flock monitoring of vaccine responses.

i) Dispense 0.025 ml of PBS into each well of a plastic microtitre plate.

ii) Place 0.025 ml of serum into the first well of the plate.

iii) Make twofold dilutions of 0.025 ml volumes of the serum across the plate.

iv) Add 4 HAU of virus antigen in 0.025 ml to each well and leave for 30 minutes.

v) Add 0.025 ml of 1% (v/v) chicken RBCs to each well and, after gentle mixing, allow the RBCs to settle for about 40 minutes when control RBCs should be settled to a distinct button.

vi) The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed more exactly by tilting the plates. Only those wells in which the RBCs ‘stream’ at the same rate as the control wells (containing 0.025 ml RBC and 0.05 ml PBS only) should be considered to show inhibition.

vii) The validity of results should be assessed against a negative control serum, which should not give a titre >2², and a positive control serum, for which the titre should be within one dilution of the known titre.

viii) Sera are usually regarded as positive if they have a titre of 2⁴ or more. However, it should be noted that even in SPF flocks, a very small proportion of birds may show a nonspecific titre of 2⁴, but usually in birds over 1 year of age.

c) **Enzyme-linked immunosorbent assay**

The ELISA technique is the most sensitive serological method and gives earlier reactions and higher antibody titres than other tests (34). It lacks type or strain specificity, but is suitable for monitoring vaccination responses under field conditions. Commercial kits for ELISAs are available – these are based on several different strategies for the detection of IBV antibodies. Usually, such tests have been evaluated and validated by the manufacturer, and it is therefore important that the instructions specified for their use be followed carefully. The ELISA is widely used to identify IBV-infected flocks (broilers) based on high antibody titres. If IB reoccurs in the next flock on the farm, virus isolation attempts are performed and the virus is genotyped by RFLP or S1 sequencing.
d) Agar gel immunodiffusion

AGID can be used in diagnosis (39). The antigen is prepared from a homogenate of the chorioallantoic membranes of infected chicken embryos. The Beaudette embryo-lethal strain is often employed to produce antigen. The test lacks sensitivity and is liable to yield inconsistent results as the presence and duration of precipitating antibodies may vary with individual birds.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

All viruses in live virus vaccines must be attenuated or naturally apathogenic. At present, many countries only permit live vaccines based on the Massachusetts type. A single inoculation of an inactivated IB vaccine is unlikely to confer full protection to the end of lay unless preceded by a primary response, usually to a live vaccine. Inactivated vaccines have to be administered to birds individually, using intramuscular or subcutaneous injection, whereas live vaccines can be given as aerosols or in the drinking water, or by eyedrop. Live vaccines confer a better local immunity on the respiratory tract and may protect against a wider antigenic spectrum of field strains (16, 17). Live vaccine may not protect for the life of the layer flock as variant serotype challenge is very high on farms with flocks of multiple ages and production drops as early as 40 weeks of age are not uncommon. The use of some live vaccines carries the risk of residual pathogenicity associated with vaccine back-passage in flocks. However, proper mass application techniques (e.g. spray or drinking water) to achieve even coverage/distribution of the vaccine in the flock and avoiding the use of suboptimal fractional doses of the vaccine will generally result in safe application of live vaccines. Concerns about the use of live vaccines can be avoided by using inactivated vaccines.

The more recent oil emulsion inactivated vaccines are now more efficacious, especially when preceded by a live virus vaccine. They also stimulate a more persistent antibody response. There are prospects for genetically engineered vaccines (4), and in-ovo vaccination is under development (38).

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. National and international standards that apply in the country in which IB vaccines are manufactured must be complied with. The licensing authority should provide information and guidance on requirements. These are now often presented in general terms, as applying to all vaccines – avian and mammalian, live and inactivated, or viral and bacterial vaccines. There may also be specific requirements applying to IB vaccines, live and inactivated. As examples, references are given to the European and USA regulations (12–14, 37).

The list of extraneous agents that must be shown to be absent continues to grow. Manufacturers must be familiar with those that currently apply in their country. Recent additions are avian nephritis virus and avian pneumovirus.

For IB vaccines, important differences among countries may arise regarding the challenge virus to be used for potency tests, and its validation. Traditionally, a virulent M-41 strain of the Massachusetts type has been used for challenge tests of both live and inactivated vaccines. Although this type is still common, it is often not the only or the dominant type in many countries and it may be advisable to prepare vaccines from other types. It is logical for challenges to be made by the same type as present in the vaccine. Establishing criteria for validating the challenge virus may be more difficult for non-Massachusetts types, because of their lower virulence in general. Inactivated vaccines are usually expected to protect against drops in egg production. The traditional M-41 challenge, as described in this chapter, should cause a drop of at least 67% in the unvaccinated controls, but when using other types much lower drops may be regarded as satisfactory, depending on published evidence of the effects of these strains in the field. There is also a tendency to relax the criteria for Massachusetts type challenges, and the European Pharmacopoeia now defines a satisfactory drop for Massachusetts types to be at least 35%, and for non-Massachusetts types to be at least 15%, provided that the drop is ‘commensurate with the documented evidence’ (14).

1. Seed management

a) Characteristics of the seed

The seed-lot system should be employed for whatever type of vaccine is produced, and for challenge strains. Each virus must be designated as to strain and origin and must be free from contamination with other strains of IBV. Separate storage facilities should be provided between the strains of virus intended for vaccines or for challenge.

For live virus vaccines, many countries permit only strains of the Massachusetts type. Some countries allow other strains, usually on the basis that those strains are already present in their national flocks. The antigenic type incorporated in both live and inactivated vaccines requires justification if there is doubt as to its existence in a country.
b) **Method of culture**

All seed viruses are grown in the allantoic sac of developing chicken embryos or in suitable cell cultures. The eggs should be from an SPF flock.

c) **Validation as a vaccine**

- **Purity**
  
  Every seed lot must be free from bacterial, fungal, mycoplasmal and viral contamination.

For the detection of extraneous viruses, the seed is first treated with a high-titred monospecific antiserum prepared against the strain under examination or against one of identical type. This mixture is cultured in a variety of ways, designed to confirm the absence of any viruses considered from past experience to be potential contaminants. The antiserum must not contain antibodies to adenovirus, avian encephalomyelitis virus, avian rotavirus, chicken anaemia virus, fowl pox virus, infectious laryngotracheitis virus, influenza A virus, Newcastle disease virus, infectious bursal disease virus, leukosis virus, reovirus, Marek's disease virus, turkey herpesvirus, adeno-associated virus, egg-drop syndrome 76 (EDS76) virus, avian nephritis virus, avian pneumovirus or reticulo-endotheliosis virus. The inoculum given to each unit of the culture system used should contain a quantity of the neutralised IBV component under test that had an initial infectivity of at least ten times the minimum field dose. These systems include:

1. SPF chicken embryos, incubated for 9–11 days, inoculated via both allantoic sac and chorioallantoic membrane (two passages);

2. Chicken embryo fibroblast cultures, for leukosis virus subgroups A and B. The COFAL test (test for avian leukosis using complement fixation) or double-antibody sandwich ELISA for group-specific leukosis antigen is performed on cell extracts harvested at 14 days. An immunofluorescence test for reticulo-endotheliosis virus is done on cover-slip cultures after two passages.

3. SPF chicken kidney cultures that are examined for CPEs, cell inclusions and haemadsorbing agents passaged at intervals of no fewer than 5 days for up to 20 days' total incubation.

4. SPF chickens of minimum vaccination age inoculated intramuscularly with 100 field doses, and on to the conjunctiva with ten field doses; this is repeated 3 weeks later when the chickens are also inoculated both into the foot pad and intranasally with ten field doses. Observations are made for 6 weeks overall, and serum is collected for tests for avian encephalomyelitis, infectious bursal disease, Marek's disease, Newcastle disease and *Salmonella pullorum* infection.

- **Potency**

  Vaccines intended to protect against loss of egg production should be tested for duration of antibody response. Mean HI titres should be >6 log$_2$ up to at least 60 weeks of age. Serological tests should be done at intervals frequent enough to show that titres have not been boosted by extraneous IBV infection.

Vaccines intended for protection of broiler chickens or rearing chickens against the respiratory form of the disease should be similarly tested for duration of antibody responses; in the case of broilers this would be up to the normal age for slaughtering, and in the case of rears up to the age when a booster vaccination would be administered (often at 16–18 weeks of age).

- **Safety**

  Tests on seed virus should include a test for any potential ability to revert to virulence. Live and inactivated vaccine seed must be tested for safety as in Section C.4.b.

- **Efficacy**

  To demonstrate efficacy, a trial vaccine must be made from the master seed and the working seed at five passages from the master seed and subjected to tests that demonstrate their protective effect.

For live vaccines, a minimum of ten SPF chickens aged 3–4 weeks are vaccinated intranasally or by eyedrop with the recommended dose. Ten unvaccinated control birds from the same age and source are retained separately. All birds of both groups are challenge inoculated intranasally or by eyedrop 3–4 weeks later, each with $10^3.0$–$10^3.5$ EID$_{50}$ of the virulent Massachusetts M-41 strain. A swab of the trachea is taken from each bird 4–5 days after challenge and placed in 3 ml of antibiotic broth. Each fluid is tested for IBV by the inoculation (0.2 ml) of five embryonated eggs after 9–11 days of incubation. An alternative test to that of taking swabs is to kill birds at 4–6 days after challenge and examine microscopically the tracheal rings for ciliary activity (19). Failure to resist challenge is indicated by an extensive loss of ciliary motility. The live
vaccine is suitable for use if at least 90% of the challenge vaccinated birds show no evidence of IBV in their trachea, while 80% or more of the control birds should have evidence of the presence of the virus.

To assess an inactivated vaccine intended to protect laying birds, 30 or more SPF chickens are vaccinated as recommended at the earliest permitted age. If a primary vaccination with live vaccine is first undertaken, an additional group of birds is given only the primary vaccination. In both cases, these primary vaccinations should be done at no later than 3 weeks of age. The inactivated vaccine is given 4–6 weeks after the live priming vaccination. A further group of 30 control birds are left unvaccinated. All groups are housed separately until 4 weeks before peak egg production, and then are housed together. Individual egg production is monitored and once it is regular, all birds are challenged, egg production being recorded for a further 4 weeks. The challenge should be sufficient to ensure loss of production during the 3 weeks after challenge. The loss in the control group should be at least 67%; the group that received primary live virus vaccine followed by inactivated vaccine should remain at the previous level, and the group given only a primary vaccination should show an intermediate drop in production. Sera are collected from all birds at vaccination, 4 weeks later, and at challenge; there should be no response in the control birds.

To assess an inactivated vaccine intended to protect birds against respiratory disease, 20 SPF chickens aged 4 weeks are vaccinated as recommended. An additional 20 control birds of the same age and origin are housed with this first group. Antibody responses are determined 4 weeks later; there should be no response in the control birds. All birds are then challenged with $10^3$ CID$_{50}$ (50% chick infective dose) of virulent virus, killed 4–7 days later, and tracheal sections are examined for ciliary motility. At least 80% of the unvaccinated controls should display complete ciliostasis, whereas the tracheal cilia of a similar percentage of the vaccinated birds should remain unaffected.

Both live and inactivated vaccines containing Newcastle disease, infectious bursal disease, reovirus and EDS76 viruses are available in some countries. The efficacy of the different components of these vaccines must each be established independently and then as a combination in case interference between different antigens exists.

2. Method of manufacture

All virus strains destined for live vaccines are cultured in the allantoic sac of SPF chicken embryos or in suitable cell cultures. For inactivated vaccines, hens’ eggs from healthy non-SPF flocks may be used. The pooled fluid is clarified and then titrated for infectivity. For live vaccines this fluid is lyophilised in vials, and for inactivated vaccines it is blended with high-grade mineral oil to form an emulsion to which a preservative is added.

3. In-process control

The required antigen content is based on initial test batches of vaccine of proven efficacy in laboratory and field trials. Infectivity titrations are done in chicken embryos.

Live vaccine should contain not less than $10^{3.5}$ EID$_{50}$ per dose per bird until the expiry date indicated, and not less than $10^{2.5}$ EID$_{50}$ per dose per bird after incubation at 37°C for 7 days at the time of issue. For inactivated vaccine, the inactivating agent and inactivation procedure must be shown under manufacture to be effective on both IBV and potential contaminants. With the use of beta-propiolactone or formalin, any live leukosis viruses and Salmonella species must be eliminated; and with other inactivating agents, the complete range of potential contaminants must be rendered ineffective. Before inactivation procedures, it is important to ensure homogeneity of suspensions, and a test of inactivation should be conducted on each batch of both bulk harvest after inactivation and the final product.

Tests of inactivation should be appropriate to the vaccine concerned and should consist of two passages in cell cultures, embryos or chickens, using inoculations of 0.2 ml and ten replicates per passage.

4. Batch control

a) Sterility

Every batch of live vaccine should be tested for the absence of extraneous agents as for the seed virus (see Chapter I.1.5.).

b) Safety

• For live vaccines

Use no fewer than ten chickens from an SPF flock that are of the minimum age stated on the label for vaccination. Administer by eyedrop to each chicken ten doses of the vaccine reconstituted so as to obtain a
concentration suitable for the test. Observe the chickens for 21 days. For vaccines intended for chickens that are 2 weeks old or more, use the chickens inoculated in the ‘test for extraneous agents using chickens’ (see Section C.1.c.4.). If during the period of observation, more than two chickens die from causes not attributable to the vaccine, repeat the test. The vaccine complies with the test if no chicken shows serious clinical signs, in particular respiratory signs, and no chicken dies from causes attributable to the vaccine.

- For inactivated vaccines

Inject a double dose of vaccine by the recommended route into each of ten 14–28-day-old chickens from an SPF flock. Observe the chickens for 21 days. Ascertain that no abnormal local or systemic reaction occurs.

c) Potency

The potency test is developed from the results of efficacy tests on the master seed virus. Live vaccines are tested for potency by titration of infectivity, and inactivated vaccines by measuring antibody production. The potency test for a batch of inactivated vaccine consists of vaccinating 20 SPF chickens, 4 weeks of age, and showing that their mean HI titre 4 weeks later is not less than 6 log₂.

d) Duration of immunity

Vaccine must be shown to have the required potency to achieve the claimed duration of immunity at the end of the claimed shelf life.

e) Stability

At least three batches should be tested for stability and must give satisfactory results for 3 months beyond the claimed shelf life.

The stability of a live vaccine must be measured by maintenance of an adequate infectivity titre.

The stability of an inactivated vaccine is measured at intervals by batch potency tests. The concentration of preservative and persistence through the shelf life should be assessed. There should be no physical change in the vaccine and it should regain its former emulsion state after one quick shake.

f) Preservatives

There are maximum level requirements for the use of antibiotics, preservatives and residual inactivating agents.

g) Precautions (hazards)

IBV itself is not known to present any danger to staff employed in vaccine manufacture or testing. Extraneous agents may be harmful, however, and the initial stages of handling a new seed virus should be carried out in a safety cabinet. It is a wise precaution with all vaccine production to take steps to minimise exposure of staff to aerosols of foreign proteins. Persons allergic to egg materials must never be employed in this work.

5. Tests on the final product

a) Safety

A safety test must be carried out on each batch of final product, as in Section C.4.b.

b) Potency

A potency test must be carried out on each batch of final product, as in Section C.4.c., at manufacture and at the end of the stated shelf life.

REFERENCES


* * *
CHAPTER 2.7.7.

AVIAN INFECTIOUS LARYNGOTRACHEITIS

SUMMARY

Avian infectious laryngotracheitis (ILT) is a respiratory disease caused by Herpesviridae alphaherpesvirinae gallid herpesvirus 1. It is principally a disease of chickens, although it can also affect pheasants, partridges and peafowl. The clinical signs and pathological reactions may vary from extremely severe, with some birds dying due to asphyxiation, to very mild, indistinguishable from other mild respiratory diseases of chickens. The principal lesion is tracheitis.

Laboratory diagnosis depends on isolation of the virus, demonstration of the presence of the virus or viral antigens, and detection of specific antibodies in the serum. Histopathological examination of the trachea for characteristic intranuclear inclusions may be of value.

Identification of the agent: Virus isolation may be done by inoculation of suspected material on to the dropped chorioallantoic membrane of embryonated eggs, or into avian embryonic cell cultures. These methods are time-consuming but sensitive. Rapid methods include direct electron microscopy on tracheal exudate, immunofluorescence on tracheal exudate or frozen sections, agar gel immunodiffusion (AGID) to detect viral antigens in tracheal samples or infected egg material, and an enzyme-linked immunosorbent assay (ELISA) to demonstrate viral antigen in mucosal scrapings. Molecular techniques using polymerase chain reaction methodology have been reported to be more sensitive than virus isolation for the examination of clinical material and are likely to be used increasingly in future. Virus isolation may be needed if these tests are negative or inconclusive.

Serological tests: Antibodies to ILT virus can be detected by virus neutralisation tests conducted in eggs or in cell cultures, or by AGID reactions, indirect immunofluorescence, or ELISA.

Requirements for vaccines and diagnostic biologicals: Vaccines against ILT are usually prepared from attenuated live virus. Those available at present afford some degree of protection, but are not completely satisfactory.

A. INTRODUCTION

Avian infectious laryngotracheitis (ILT) is a respiratory disease of chickens caused by an alphaherpesvirus. It can also affect pheasants, partridges and peafowl. In the virulent form, the history, clinical signs and very severe tracheal lesions are highly characteristic of the disease, but the mild form may be indistinguishable from other mild respiratory diseases. Laboratory diagnosis depends on the demonstration of the presence of the virus or viral antigens or of specific antibodies in the serum (14).

Clinically, the disease may appear in three forms, namely peracute, subacute, and chronic or mild. In the peracute form, onset of disease is sudden with a rapid spread. The morbidity is high and mortality may exceed 50%. Some birds may die in good body condition before the appearance of signs, which are characteristic and comprise difficulty in breathing with extension of the neck and gasping in an attempt to inhale. There is also gurgling, rattling and coughing when birds try to expel obstructions in the trachea. Clots of blood may be coughed up and can be found on the floor and walls of the house. Post-mortem changes are confined to the upper respiratory tract and are also characteristic, consisting of haemorrhagic tracheitis with blood clots, mucoid rhinitis, and blood-stained mucus along the length of the trachea.

In the subacute form, the onset of illness is slower and respiratory signs may extend over some days before deaths are seen. The morbidity is high but the mortality is lower than in the peracute form, between 10% and 30%. Post-mortem findings are less severe and consist of mucoid exudate with or without blood in the trachea. Yellow caseous diphtheritic membranes may be found adherent to the larynx and upper tracheal mucosa.
Chronic or mild ILT may be seen among survivors of either of the above forms of the disease, although some outbreaks themselves may be entirely mild. Incidence of chronic ILT within a flock may be only 1–2%, with most affected birds dying of suffocation. Signs include spasms of coughing and gasping, with nasal and oral discharge and reduced egg production. On post-mortem examination, diphtheritic and caseous necrotic plaques and plugs are found in the trachea, larynx and mouth. Outbreaks of mild ILT may affect large numbers of birds simultaneously, in which case gross lesions may consist only of conjunctivitis, sinusitis and mucoid tracheitis. Given that transmission of ILT takes place by close contact, transmission is slower in cage houses than where birds are loose-housed, and the path of infection through a cage house may be apparent.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The virus may be isolated in chicken embryo liver (13), chicken embryo kidney (6) or in chicken kidney (18) cell cultures. Of these, monolayers of chicken embryo liver cells have been found to be the most sensitive (8). The virus can also be grown on the dropped chorioallantoic membrane (CAM) of 10–12-day-old specific pathogen free embryonated chicken eggs (9).

The causative herpesvirus may be demonstrated directly in tracheal exudate by electron microscopy (18). Viral antigens may be detected by immunofluorescence (4, 19), agar gel immunodiffusion (AGID) (10), or enzyme-linked immunosorbent assay (ELISA), using tracheal mucosal scrapings (21). Histopathological examination of the trachea for typical herpesvirus intranuclear inclusions may also be helpful (3, 15). Methods of detecting ILT virus using polymerase chain reaction (PCR) have been described and PCR has been reported to be generally more sensitive than virus isolation (2, 11, 12, 20).

a) Virus isolation

When samples are taken from live birds for virus isolation, tracheal swabs are superior to oropharyngeal or conjunctival swabs. These are placed in transport medium containing antibiotics. When selecting material for virus isolation from chronic outbreaks, it is more productive to cull a bird in the early stages of the infection, rather than to attempt to isolate virus from a bird that has died of asphyxiation after a long illness. The quality of sample is further improved if the bird is killed by barbiturate or other injection rather than by cervical dislocation. The whole head and neck from dead birds may be submitted, or only the trachea and larynx after their removal with minimal contamination. Tracheas should be transported in antibiotic broth for virus isolation, but wrapped in moist tissue paper if destined for electron microscopy. Any prolonged storage of infected tissues should be at –70°C or below to minimise loss of virus titre. Repeated freezing and thawing must be avoided as this reduces virus infectivity.

Exudate and epithelial cells are scraped from the tracheas, diluted approximately 1/5 in nutrient broth containing penicillin and streptomycin, and agitated vigorously. The resulting suspension is centrifuged at low speed to remove debris, and 0.1 ml of the supernatant fluid is inoculated on to the dropped CAM of at least three embryonated chicken eggs of 10–12 days' incubation. The eggs are sealed with paraffin wax and incubated at 37°C for up to 7 days. They are candled daily and the CAMs of dead embryos or of those surviving for 7 days are examined for typical pocks. Alternatively, at least two confluent chicken embryo liver or chicken embryo kidney cell monolayers, with their medium removed, are inoculated and allowed to adsorb for 1–2 hours. Cultures are overlaid with fresh medium, incubated for up to 7 days and examined daily under the microscope for evidence of a typical syncytial cell cytopathic effect (CPE).

In each instance, up to three passages of material may be necessary before a specimen is considered to be negative. A virus isolate can be confirmed as ILT virus by a neutralisation test in eggs or cell culture using hyperimmune antiserum to ILT virus. Alternatively, virus particles may be identified rapidly in cell culture fluid or in pocks on CAMs by electron microscopy, and viral antigens may be detected by immunofluorescence in acetone-fixed ILT-virus-infected cell cultures or in frozen sections of CAM.

b) Electron microscopy

To demonstrate the presence of virus by electron microscopy, tracheal exudate or epithelial scrapings from the trachea are smeared on to a microscope slide and mixed with a few drops of distilled water. One drop of suspension is placed on a carbon and formvar-coated grid and left for 2 minutes, after which excess moisture is removed using filter paper. One drop of 4% phosphotungstic acid, pH 6.4, is added and the excess removed after a further 3 minutes. The grid is allowed to dry thoroughly and examined using the electron microscope at a magnification of ×30–45,000 for typical herpesvirus particles.

c) Immunofluorescence

In immunofluorescence tests for viral antigens, epithelial cell scrapings from the trachea are smeared on to a glass slide. Alternatively, snap-frozen 5 µm thick sections of trachea may be used. The preparations are
fixed in acetone at room temperature for 10 minutes. These can be stained directly by applying chicken anti-ILT virus immunoglobulin labelled with fluorescein isothiocyanate (FITC) for 1 hour, followed by rinsing for 15 minutes in a bath of phosphate buffered saline (PBS), pH 7.2, agitated with a magnetic stirrer. Otherwise, they can be stained indirectly by applying an appropriate dilution of chicken anti-ILT serum for 1 hour. The slide is rinsed thoroughly with PBS for 15 minutes as above, and an FITC-labelled anti-chicken immunoglobulin is applied for 30 minutes. After a final rinse, cover-slips are applied over nonfaded mountant.

The preparations are examined for specific intranuclear fluorescence in the epithelial cells using a microscope with epifluorescent ultraviolet illumination. Suitable controls include the use of known uninfected specimens and, for the indirect method, the application of nonimmune chicken serum. Particular care should be taken in the reading of indirect immunofluorescence preparations, as endogenous chicken IgG in the trachea may cause unwanted attachment of FITC-labelled anti-chicken IgG.

d) **Agar gel immunodiffusion**

ILT viral antigens may be demonstrated by AGID tests on tracheal exudate, infected CAMs and infected cell culture material using hyperimmune ILT virus antiserum. The gel is made with Noble agar (1.5%) containing sodium chloride (8%) and sodium azide (0.02%) – as preservative – in distilled water. The ingredients are autoclaved at 15 lb/sq. inch (2.4 bar) for 15 minutes; 5 ml of the molten agar is poured into a 5 cm diameter Petri dish. When the agar has set, a pattern of wells is punched in the agar, consisting of a central well and six surrounding wells. The wells are usually 8 mm in diameter and 4 mm apart. The hyperimmune serum is pipetted into the central well, while the surrounding wells are filled with suspect virus samples under test, but with at least one well containing positive viral antigen. Dishes are incubated in a humid atmosphere at room temperature or at 37°C, and examined 24–48 hours later by oblique illumination for lines of precipitation (reactions of identity). Tests should include uninfected material as negative antigen and known negative antiserum as controls. For economy of materials, the test can be done on a microscale – the agar being poured in a thin layer on a microscope slide and holes punched of 4 mm diameter and 2 mm apart.

e) **Enzyme-linked immunosorbent assay**

When the monoclonal antibody (MAb) ELISA is used for detecting viral antigens (16), tracheal exudate is mixed with an equal volume of PBS containing 1% (v/v) of a detergent, such as Nonidet P40 (BDH Chemicals, Poole, United Kingdom), then vortexed for 30 seconds and centrifuged at 10,000 g for 1 minute. The supernatant fluid is dropped in 50 µl volumes in wells of microtitre plates previously coated with rabbit IgG against ILT virus, diluted 1/200 in 0.05 M carbonate/bicarbonate buffer, pH 9.0, and incubated for 1 hour. Next, 50 µl of MAb against major glycoproteins of ILT virus, diluted 1/50 in PBS, is added to each well, followed by 50 µl of a 1/1000 dilution of affinity-purified goat anti-mouse IgG conjugated to horseradish peroxidase. The substrate, 5-aminosalicylic acid (6.5 mM), is added to the wells in 100 µl volumes. After 30 minutes, the plates are read at 450 nm on a spectrophotometer and the absorbance reading for each well is corrected by subtracting the reading obtained for wells containing diluting buffer instead of tracheal exudate. The positive/negative cut-off point is taken as the mean absorbance value for several negative (i.e. tracheal material without ILT virus) samples plus 3 standard deviations.

f) **Histopathology**

Tracheas for histopathological examination should be placed in formol saline immediately after removal from the birds and embedded in paraffin wax. Intranuclear inclusions may be seen in the epithelial cells of the trachea in longitudinal sections after staining by haematoxylin and eosin. They are the classical Cowdry type A inclusions of herpesviruses, but they may be present for only 3–5 days after infection. In severe cases where most infected cells have detached from the tracheal lining, inclusions may be seen in intact cells among the cellular debris in the lumen of the trachea.

g) **Molecular methods**

Molecular methods for identifying ILT virus DNA in clinical samples have been reported (11, 12, 20). Dot-blot hybridisation assays and cloned virus DNA fragments were shown to be highly sensitive for detecting virus when isolation and ELISA were negative (11, 12).

The PCR has been found to be more sensitive than virus isolation for clinical samples, especially when other contaminant viruses such as adenoviruses are present (20). Alexander & Nagy (2) found that during the middle to the end of the infection phase, PCR and virus isolation were similar in sensitivity, but PCR was superior in the recovery phase.

A problem of detection methods for ILT virus has been that hitherto, they have not been able to differentiate between field strains and vaccine strains. This seems to have been overcome by Chang et al. (5), who used PCR in conjunction with restriction fragment length polymorphism (PCR-RFLP). This approach would also seem to offer promise for a better understanding of ILT virus epidemiology and evolution. Indeed, an analysis of Korean strains of ILT virus by PCR-RFLP, which classified virus according to thymidine kinase and glycoprotein genes, showed that the method could be used to distinguish between strains of high and low virulence (7).
2. Serological tests

Antibodies to ILT virus in chicken serum can be detected by virus neutralisation (VN), AGID, indirect immunofluorescence tests and ELISA (1).

a) Virus neutralisation

VN tests may be conducted on the dropped CAMs of embryonating chicken eggs that have been incubated for 9–11 days, where antibody specifically neutralises pock formation due to ILT virus. Alternatively, the tests can be performed in cell cultures, where antibody specifically neutralises the ILT virus thus preventing CPE. Doubling dilutions of serum are added to equal volumes of a constant concentration of virus. This concentration may either be 100 median egg infectious doses (EID$_{50}$) for egg inoculations, or 100 median tissue culture infectious doses (TCID$_{50}$) for the inoculation of cultures. The mixtures are incubated at 37°C for 1 hour to allow any neutralisation to occur.

When the test is performed in eggs, the virus/serum mixtures are inoculated on to the dropped CAMs, using at least five eggs per dilution. Eggs are sealed and incubated at 37°C for 6–7 days. The end-point is recorded as the highest dilution of the serum where no pocks are present on the CAMs. When the tests are done in cell cultures, serum dilutions are prepared in 96-well microculture plates and virus is then added. After the period allowed for neutralisation, freshly prepared chicken embryo liver or kidney cells are added to each well. The plates are incubated at 37°C in an atmosphere of 5% CO$_2$ and examined daily for CPE; 50% end-points are read after approximately 4 days when the virus control titre indicates that 30–300 TCID$_{50}$ of virus have been used in the test. For the cell culture method of testing, virus neutralisation at 1/8 (initial dilution) or greater is considered positive.

b) Agar gel immunodiffusion

For AGID tests, antigen is prepared from virus-infected CAMs or infected cell cultures. For the former, at least $10^4$ TCID$_{50}$ of ILT virus is inoculated into the allantoic cavity of a batch of 10-day-old embryonating specific pathogen free (SPF) chicken eggs. The CAMs are harvested after 4 days' incubation, and those with large pocks are homogenised and sonicated in a small amount of PBS, pH 7.1. Alternatively, heavily infected monolayers of chicken embryo liver or kidney, or chicken kidney cells are incubated at 37°C until the CPE is maximal. Any remaining attached cells are scraped from the culture vessel into the medium. Total culture harvests may be concentrated up to 100-fold by dialysis against polyethylene glycol (PEG 20,000 or PEG 30,000). For the test, the agar is prepared as described previously for antigen detection, but this time the CAM or cell culture antigen is placed in the central well with test sera in the surrounding wells. Known positive and negative antisera are incorporated in the test, which is read after 24–48 hours' incubation at room temperature or at 37°C. AGID tests are simple, economical to perform, and useful for flock screening, although they are less sensitive than the other methods.

c) Indirect fluorescent antibody test

For indirect fluorescent antibody tests, the antigen consists of ILT-virus-infected cell culture monolayers grown on teflon-coated multipor slides. When CPE is beginning to develop, the cultures are fixed in acetone for 10 minutes. Dilutions of test sera prepared in PBS are applied to each spot culture and the slides are incubated at 37°C for 1 hour. The slides are washed in PBS as described previously, drained and treated with an appropriate dilution of a commercial FITC-labelled rabbit anti-chicken IgG. After incubation at 37°C for 1 hour, the slides are rewashed and cover-slips are applied over a non-fade mountant. They are examined by epifluorescence with ultraviolet illumination, and end-point titres are read as the highest serum dilutions giving specific fluorescent staining. This test is more sensitive than AGID, but the interpretation of results may be subjective.

d) Enzyme-linked immunosorbent assay

The antigen for ELISA is obtained by sonication of heavily infected cell cultures at the time of maximum CPE, which is then absorbed on to the wells of microtitre plates. A negative antigen is provided by uninfected cell culture material treated in the same way. The test consists essentially of the addition of 0.1 ml of 1/10 dilutions of test sera to duplicate wells coated with positive or negative antigen. After incubation at 37°C for 2 hours, the plates are washed four times and a 1/4000 dilution of a rabbit anti-chicken IgG conjugated with peroxidase is added. After incubation at 37°C for 1 hour, the plates are washed again four times. Finally a substrate consisting of 5-aminosalicylic acid is added to each well followed by hydrogen peroxide to a final concentration of 0.0005%, and the absorbance of the fluid in each well is read at 450 nm on a spectrophotometer. The result for each serum is expressed as the difference between the mean absorbance produced with the positive and negative antigens. The positive/negative cut-off point is taken as the mean absorbance value for numerous negative sera plus 3 standard deviations. The test is very sensitive and possibly the best available for surveillance purposes. A fowl laryngotracheitis antibody ELISA kit is also available commercially.
C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

ILT is usually controlled with live vaccines, although inactivated vaccines have also been used for safety reasons. The live virus seed is a suitably attenuated or naturally avirulent strain of ILT virus. Vaccines may be administered by eye-drop, spray or in the drinking water. If administered by spray and a small droplet size is produced and inhaled, clinical disease may be precipitated. Young chickens may require vaccinating in endemic areas, but show more severe reactions to the vaccine. Repeated doses may be required to afford good protection. The level of virulence of the vaccine virus is critical. Strains of low virulence may not be effective, and those of higher virulence may cause severe disease. The spray route of administration requires care over droplet size and uniformity of application. It can be more effective with low virulence strains, but may be more dangerous with high virulence strains. At present, the available vaccines attempt to make a compromise between lack of efficacy and poor safety. Because of persistence of virulent vaccine virus on a site, it may be difficult to discontinue vaccination once it has been started. Subclinical mixed infections of vaccine and field virus, in vaccinated birds, can cause severe disease in unvaccinated in-contacts.

Guidelines for the production of veterinary vaccines are given in Chapter 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 (e.g. ref. 17).

1. Seed management

a) Characteristics of the seed

The master seed virus (MSV) is selected and can be propagated in SPF chicken embryos or tissue cultures derived from such embryos. The MSV is tested in chicken embryos or chickens for the following: 1) purity, 2) Mycoplasma spp., 3) Salmonella spp., 4) avian leukosis virus, 5) haemagglutinating viruses, 6) virus identity, and 7) extraneous pathogens. Additionally, initial tests are performed to demonstrate the safety and efficacy of the chosen master seed. The safety test on the MSV must include tests to show lack of reversion to virulence on serial passage and also safety in birds. Evidence of shed and spread is also required. The MSV is stored in aliquots at –70°C. The MSV should not cause mortality or a severe respiratory reaction in chickens following ocular instillation, although pheasants are more susceptible. Administration by spray is convenient but may cause quite severe respiratory disease in some flocks.

b) Method of culture

In large-scale vaccine production, the virus is propagated in SPF chicken embryos or tissue culture derived from such embryos, up to the fifth passage from the MSV. The acceptable passage level is supported experimentally by the passage level used to prepare the experimental product used in the efficacy study.

c) Validation as a vaccine

A test must be carried out to establish the efficacy of the vaccine in birds of the minimum age for which the product is destined and also for each avian species. This is repeated in further batches of chickens for each of the recommended routes of administration and/or age of bird. Three weeks later (or 10–14 days in the USA), the birds, together with ten controls of the same age and source, are challenged intratracheally or in the orbital sinus with a strain of ILT virus of known high virulence. To be satisfactory, only 5% of the vaccinated birds should die or show severe signs of ILT. No more than four should show mild signs of ILT. At least eight of the controls should die or show severe signs of ILT.

2. Method of manufacture

The vaccine is made by inoculation of the production seed virus into 9–11 day-old chicken embryos or tissue culture prepared from chicken embryos derived from SPF flocks. Eggs are inoculated through a hole in the shell, on to the dropped CAM. They are sealed and incubated at 37°C for 4–6 days. All eggs are candled before harvest and only those with living embryos are used. To harvest the virus, the eggs are chilled, then cleansed and opened aseptically. The CAMs and fluids are pooled in sterile, cooled containers. The CAMs should show the thick grey plaques typical of ILT virus growth. Tissue culture-derived product would be prepared from virus-bearing cell culture fluids, which would also be subsequently pooled and tested.

3. In-process control

The infected tissue or tissue culture homogenate may be tested for purity, potency, and virus content, mixed with a stabiliser (usually beef peptone and sucrose) and then lyophilised and stored at 4°C.
4. Batch control

a) Sterility
Tests for sterility and freedom from contamination of biological materials may be found in Chapter I.1.5.

b) Safety
Using the recommended route of administration, each batch of vaccine is tested in ten SPF chickens, or ten birds of other target species, using ten doses per bird. The birds are observed for at least 21 days for adverse effects attributable to the vaccine.

c) Potency
Once the in-vivo efficacy of the vaccine has been established, the batch potency may be determined by measuring the virus content. Serial dilutions of the vaccine are inoculated on to the dropped CAM of 9–11-day-old SPF chicken embryos, using at least seven eggs per dilution, in a volume of 100 µl. The eggs are incubated for 5 days and the virus titre is calculated by observing characteristic lesions on the CAMs. The virus content should be at or above a release value and above and expiration titre during dating of the product. Both the release and expiration titres are based on the minimum protective dose described above.

d) Duration of immunity
The results of vaccination will depend on many factors, including dose schedule and route of administration. Some degree of protection should be given, over a period of several months.

e) Stability
Stability is tested by taking samples of correctly stored vaccine at intervals and measuring virus content. Tests should be carried out on at least six batches of the vaccine or until a statistically valid number of serials have been evaluated and be continued for 3 months after the claimed shelf-life.

f) Preservatives
Preservatives may not be required, but some antibiotics may be added to the tissue harvest or at serial assembly during manufacture. For products licensed in the USA, any antibiotics added are listed on the label.

g) Precautions (hazards)
Care should be taken over diluting and administering the vaccine, and over the proper disposal of unused vaccine.

5. Tests on the final product

a) Safety
In the USA, 25 susceptible chickens are injected intratracheally and observed for 14 days. Deaths are counted as failures. Four or fewer failures are allowed for satisfactory serials. In the European Union, tests of virus content are carried out. The virus titre shall normally be no higher than one-tenth of the dose at which the vaccine has been shown to be safe.

b) Potency
The test of virus content (see above) may also be used as a measure of potency. It must be no lower than the agreed minimum release titre. Each serial or subserial shall have a virus titre of $10^7$ greater than the minimum protective dose, but not less than $10^{25}$ EID$_{50}$ (or TCID$_{50}$ for tissue culture prepared product)/dose.

c) Tests of final product
The lack of chicken pathogens should be confirmed in embryos or chickens. It should also be confirmed by testing for *Mycoplasma* spp., *Salmonella* spp., avian leukosis virus, and haemagglutinating viruses.

REFERENCES


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CHAPTER 2.7.8.

AVIAN TUBERCULOSIS

SUMMARY

Avian tuberculosis is a bacterial disease of birds caused by the slow-growing nonphotochromogenic, acid-fast, rod-shaped Mycobacterium avium. The disease has a world-wide distribution and occurs in many kinds of birds, but all varieties are not equally susceptible. Tuberculous lesions are mostly found in the intestinal tract, liver and spleen. The organism causes tuberculosis in chickens and other fowl and avian species, but can also infect an extensive range of different animal species such as swine, cattle, deer, sheep, goats, horses, cats, dogs, and exotic species (20, 21).

In humans, M. avium is capable of inducing a progressive disease that is refractory to treatment. All operations involving the handling of open live cultures of M. avium, or of material from infected birds, must be carried out with adequate biohazard containment.

Diagnosis of tuberculosis in birds depends on the demonstration of M. avium in the dead bird, or the detection of an immune response, cellular or humoral, in the live bird (19).

Identification of the agent: Where clinical signs of tuberculosis are seen in the flock, or typical lesions of tuberculosis are present in birds at necropsy, the demonstration of acid-fast bacilli in smears or sections made from affected organs is sufficient for a positive diagnosis. If acid-fast bacilli are not found, but typical signs or lesions are present in the birds, culture of the organism must be attempted. This can be done on artificial media. Any acid-fast organism isolated should be identified by biochemical, nucleic-acid relatedness, serological or chromatographic (thin-layer chromatography of lipids) criteria. In selected cases (namely if the isolate is not serotype 1, 2, or 3), the virulence of the isolate for the species of bird affected should be demonstrated by inoculation.

Tuberculin test and serological tests: These tests are normally used to determine the prevalence of disease in a flock, or to detect infected birds. When used to detect the presence of tuberculosis in a flock, they should be supported by the necropsy of any birds that give positive reactions.

In domestic fowl, the tuberculin test in the wattle has been the test of choice. This test is less useful in other species of bird. A better test, especially for waterfowl, is the whole blood stained-antigen agglutination test (Rozanska). It is more reliable and has the advantage that it will give a result within a few minutes, while the bird is still being held. The tests are not reliable in cage birds.

Requirements for vaccines and diagnostic biologicals: No vaccines are available for use in birds. An antigen preparation stained with 1% malachite green is available for the whole blood agglutination test. Avian tuberculin purified protein derivative is the standard preparation for use in the tuberculin test of domestic poultry.

A. INTRODUCTION

According to the current taxonomy, Mycobacterium avium species contains four subspecies (11). The first subspecies, M. avium subsp. avium of serotypes 1, 2, and 3 and genotype IS901+ (more than two copies) and IS1245+ (three copies of IS1245 in conserved pattern), is fully virulent for birds (5). The representatives (especially animal field isolates) of this subspecies most often cause development of miliary tuberculosis affecting parenchymatous organs: liver, spleen, kidney, lymph nodes and lungs (13). The main sources of this agent is infected wild and domestic birds and wild small terrestrial mammals.
The second subspecies, partly virulent for birds, is *M. a. hominissuis* of serotypes 4 to 6, 8 to 11 and 21 and genotype IS901- and IS1245+, in more than four copies (11). Major sources of that subspecies are various constituents of the external environment: water, soil, dust, invertebrates etc.

The third subspecies, *M. a. paratuberculosis*, causes a serious chronic bacterial disease of the intestinal tract in ruminants and other animals (see Chapter 2.2.6. Paratuberculosis [Johne’s disease]). This subspecies contains specific insertion sequence IS901 in more than 13 copies.

The fourth subspecies, *M. a. silvaticum*, grows only in primary culture in agar enriched with growth stimulator Mycobactin J, but in subculture, it grows without this stimulator. *Mycobacterium a. silvaticum* is diagnosed rather rarely but can be virulent for birds and contains IS901 (5).

Tuberculosis in birds is most prevalent in chickens and in many wild birds raised in captivity. Turkeys are quite susceptible, but duck and geese are comparatively resistant. The practices of allowing poultry to roam at large on the farm (free range) and of keeping the breeders for several years are conducive to the spread of tuberculosis among them. In most cases, infected birds show no clinical signs, but they may eventually become lethargic and emaciated. Under intensive husbandry conditions, sudden death may occur, often associated with severe lesions in the liver; such lesions are easily observed at post-mortem examination.

The primary lesions of tuberculosis in birds are nearly always in the intestinal tract. Such lesions take the form of deep ulcers filled with caseous material containing many organisms, and these are discharged into the lumen and appear in the faeces. Before the intestinal tract is opened, the ulcerated areas appear as tumour-like masses attached to the gut wall, but when the intestine is opened, the true nature of the mass becomes evident. Typical caseous lesions are nearly always found in the liver and spleen, and these organs usually are greatly enlarged because of the formation of new tuberculous tissue. The lungs and the other tissues are ordinarily free from lesions even in advanced cases.

It is essential to bear in mind that *M. avium* is capable of giving rise to a progressive disease in humans that is refractory to treatment, especially in immunocompromised individuals. All manipulations involving the handling of open live cultures or of material from infected birds must be performed with adequate biohazard containment (see Chapter 1.1.6. Human safety in the veterinary microbiology laboratory).

### B. DIAGNOSTIC TECHNIQUES

#### 1. Identification of the agent

If there is a characteristic history of tuberculosis in the flock and typical lesions are found in birds at post-mortem, the detection of acid-fast bacilli in smears or sections from affected organs, stained by the Ziehl–Neelsen method, is normally sufficient to establish the diagnosis. Occasionally a case will occur, presumably as a result of large infecting doses giving rise to acute overwhelming disease, in which affected organs, most obviously the liver, have a ‘morocco leather’ appearance with fine greyish or yellowish mottling. In such cases acid-fast organisms may not be found, but careful inspection will reveal parallel bundles of brownish refractile bacilli. Prolongation of the hot carbol fuchsin stage of Ziehl–Neelsen staining to 10 minutes will usually reveal that these are indeed acid-fast bacilli, with unusually high resistance to penetration of the stain. Recently, DNA probes and polymerase chain reaction (PCR) techniques have been used to identify the agent. Traditionally, *M. avium* is separated from common nonchromogenic slow-growing organisms by their ability to grow at 42°C and biochemical tests such as the Tween hydrolysis test, pyrazinamidase, growth on thiophen-2-carboxylic acid hydrazide (TCH)-containing media and tellurite reduction.

- **Culture**

If there is a characteristic flock history and suggestive lesions are found at necropsy, but no acid-fast bacilli are seen in smears or sections, an attempt must be made to isolate *M. avium* from the necropsy material. Liver or spleen are usually the best organs to use, but if the carcass is decomposed, bone marrow may prove more satisfactory as it could be less contaminated. *Mycobacterium avium* grows best on media such as Lowenstein–Jensen, Herrold’s medium, Middlebrook 7H10 and 7H11 or Coletos, with 1% sodium pyruvate added. It may occasionally be necessary to incorporate mycobactin, as used for the isolation of *M. paratuberculosis* and *M. silvaticum*. Growth may be confined to the edge of the water of condensation. Cultures should be incubated for at least 8 weeks. Typically *M. avium* produces ‘smooth’ colonies, but rough variants do occur.

Typing of mycobacteria to the species and subspecies level requires a specialised laboratory. Conventional biochemical tests for species identification are lengthy and fail to distinguish between *M. avium* and *M. intracellulare*. Thus, a miscellaneous group of mycobacteria that includes both species is usually classified under the denomination of *M. avium* complex (MAC). Seroagglutination, which is based on sugar residue
specification of surface glycopeptidolipids, allows classification of MAC organisms into 28 serovars. More sophisticated typing methods directed at cell-wall-specific targets are currently available, such as enzyme-linked immunosorbent assays with monoclonal antibodies to major serovars, and thin-layer chromatography of lipids. Serovars 1 to 6, 8 to 11 and 21 are currently ascribed to \textit{M. avium}, and serovars 7, 12 to 20 and 25 to \textit{M. intracellular}. However, no consensus was achieved on other serovars, and some isolates cannot be typed (8). Tuberculosis in birds is usually caused by \textit{M. avium} types 1, 2, or 3. If one of these is found, it may be assumed to be the cause of the disease. If the isolate is not one of these, but is still suspected of being the cause of the disease, pathogenicity tests must be carried out. However, it should be borne in mind that superficial tuberculosis lesions in cage birds, especially psittacines, may be caused by \textit{M. tuberculosis}. Hence, if rough colonies of mycobacteria are isolated from such birds, they should be tested for growth at 42°C. If the isolate will not grow at 42°C, \textit{M. tuberculosis} should be suspected.

- **Nucleic acid recognition methods**

Specific and reliable genetic tests for speciation are currently available (16). Commercial nucleic acid hybridisation probes have become a ‘gold standard’ for distinction between \textit{M. avium} and \textit{M. intracellular} cultures\(^1\). A further probe that covers the whole MAC was also developed, as genuine MAC strains have been described that fail to react with specific \textit{M. avium} and \textit{M. intracellular} probes (17). These tests use a chemiluminescent-labelled, single-stranded DNA probe that is complementary to the ribosomal RNA of the target organism. The labelled DNA–RNA hybrids are measured in a luminometer. Various in-house molecular methods have been reported for the identification of mycobacterial cultures, including MAC. The latter include 16S rRNA sequencing (9) to PCR amplification followed by either hybridisation with species-specific probes or restriction enzyme analysis (4, 18). Even though some of these methods would theoretically detect the agent directly in tissue samples, none of them has been validated for this use. Therefore, molecular identification of MAC is currently performed on organisms previously isolated by culture.

Regarding intraspecies genotyping, pulsed-field gel electrophoresis of large DNA restriction fragments have proved to be highly sensitive (10). Also, a number of DNA mobile elements have been identified that may be exploited for this purpose. Insertion sequence IS\(^{1245}\) is virtually \textit{M. avium} specific, was shown to be the most discriminative for the analysis of strain relatedness (2, 6). A standardised method consisting of IS\(^{1245}\) restriction fragment length polymorphism (RFLP) analysis was recently proposed (22). Bird infection was found to be caused by a particular subset of \textit{M. avium} strains that are characterised by specific, highly conserved RFLP patterns with IS\(^{1245}\) and IS\(^{901}\), in addition to serovars 1, 2 or 3 (14).

Recently O’Grady et al. performed and RFLP investigation using probes derived from IS\(^{901}\), IS\(^{1245}\) and IS\(^{1311}\) to study the molecular epidemiology of \textit{M. avium} and \textit{M. intracellular} infection, in particular to gain an understanding of the sources of infection in humans (12).

If specialised typing facilities are not available, the likelihood that the organism isolated is the cause of the disease may be established by pathogenicity tests. It is preferred that these be carried out on the species of bird being investigated, but failing that, domestic fowl or Japanese quail may be used. Young adult birds are best. An inoculum is prepared by putting a small square of aluminium foil and some glass beads in a screw-capped container, which is then sterilised and weighed. A loopful of culture is then placed on the foil and the whole is reweighed. Finally, sufficient sterile normal saline solution is added to suspend the culture at 0.1 mg/ml. Birds are then inoculated intravenously with 1 ml of the suspension. If the organism is virulent, the bird will die in 5–6 weeks or, by that time, the bird will have extensive lesions filled with acid-fast bacilli.

2. **Immunological methods**

a) **Tuberculin test**

The most widely used test in domestic fowl, and the only test for which an international standard for the reagent exists, is the tuberculin test. The tuberculin is the standard avian purified protein derivative (PPD). Birds are tested by intradermal inoculation in the wattle with 0.05 ml or 0.1 ml of tuberculin (containing approximately 2000 International Units [IU]), using a very fine needle of approximately 10 mm × 0.5 mm. The test is read after 48 hours and a positive reaction is any swelling at the site, from a small firm nodule approximately 5 mm in diameter to gross oedema extending into the other wattle and down the neck. With practice, even very small wattles on immature birds can be inoculated successfully. However, in immature birds the comb may be used, although results are not so reliable. Tuberculin testing of the wattle in turkeys is much less reliable than in the domestic fowl. Inoculation in the wing web has been recommended as being more efficient, but this is still not as good as for domestic fowl. Other birds may also be tested in the wing web, but results are not generally satisfactory. The bare ornamental skin areas on Muscovy ducks and some species of pheasant can be used, but reliability is doubtful and interpretation difficult. Testing in the

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1 Accuprobe, Gen-Probe, Gen-Probe Inc., San Diego, California, United States of America.
In pheasants, the tuberculin test can be performed in either of two ways. In the first, 0.05 ml or 0.1 ml of tuberculin is injected into the skin of the lower eyelid. A positive result is indicated by marked swelling at the site of injection after 48 hours. Alternatively, 0.25 ml of tuberculin is injected into the thoracic muscles and the birds are observed for 6–10 hours. Infected birds will show signs of depression and keep aside from the flock, and there may be cases of sudden death. No clinical signs will be provoked in uninfected birds.

b) Stained antigen test

- Preparation of the antigen

An antigen stained with 1% malachite green is used for the rapid whole blood plate agglutination test (15). The strain used for preparation of the stained antigen must be smooth and not autoagglutinate in saline suspension. It must conform to the characteristics of the *M. avium* species.

A strain that will detect infection with any serotype is recommended to be used instead of the specific serotype that is most likely to be encountered (in Europe serotype 2 for domestic fowl, serotype 1 for waterfowl). It may be preferable to use a strain that is highly specific for the serotype it detects. The specificity of strains can be determined only by testing them as antigens, although in general a serotype 2 antigen will always detect serotype 3 infection and vice versa. Serotype 1 strains appear to detect more often a wide spectrum of infection, and will often also detect infections with mycobactin-dependent mycobacteria or *M. silvaticum*. There is no reason not to use a culture containing more than one strain of *M. avium*, provided that it shows the desired properties of sensitivity and specificity. Consistency of results between batches will be easier with the use of pure cultures.

The organism should be grown in a suitable liquid medium, such as Middlebrook 7H9 containing 1% sodium pyruvate for better growth. Good growth should be obtained in approximately 7 days. The liquid culture is used as seed for bulk antigen preparation.

Antigen for agglutination tests is best grown on solid medium, such as Löwenstein–Jensen or 7H11, containing 1% sodium pyruvate instead of glycerol, using Roux flasks or large bottles. The use of solid medium maximises the chance of detecting any contamination, and antigens grown in some liquid media are not agglutinated by specific antibody. Liquid seed culture should be diluted (on the basis of experience) to give discrete colonies on the solid medium. This will usually give the best yield, and again increases the chance of detecting contamination. About 10 ml of inoculum will usually be enough to allow it to wash over the whole surface, and provide sufficient moisture to keep the air in the bottle near 100% humidity.

The bottles are incubated at 37°C, and good growth should be obtained in 14–21 days with most strains. The antigen is harvested by the addition of sterile glass beads and twice the volume of sterile normal saline (containing 0.3% formalin) as was used to inoculate the bottle. The bottle is then shaken gently to wash off all the growth and the washing is collected into a sterile bottle and reincubated at 37°C for 7 days. The killed bacilli are then washed twice in sterile normal saline with 0.2% formalin by centrifugation and resuspension. This sequence is safer than the original method in which the washing was carried out before the incubation that kills the organisms. Finally the organisms are again centrifuged and resuspended in sterile normal saline containing 0.2% formalin and 0.4% sodium citrate, to a concentration of about $10^{10}$ bacteria per ml. This corresponds to a concentration ten times that which matches tube No. 4 on McFarland’s scale.

Cultures for antigen should be inspected for contamination daily for the first 5 days of incubation. The suspension made from the culture washings is also re-examined microscopically (for likely contaminants such as yeasts), and rechecked by culture to ensure that the formalin has killed the mycobacteria.

- Validation of the antigen

Cultures should be checked by Gram staining for the presence of organisms other than mycobacteria.

One or more batches for agglutinating antigen must be tested for efficacy in naturally or artificially infected tuberculous birds by comparison with a standard preparation of known potency. The potency relative to that of the standard preparation must not differ significantly from that declared on the label. Each bottle of antigen must be tested with normal chicken serum (to detect autoagglutination) and *M. avium* positive chicken serum of low and high antibody content. This should be done, where possible, alongside a previous batch of stained antigen. Those bottles that give satisfactory agglutination reactions with the antisera can now be pooled and the antigen stained. This is done by the addition of 3 ml of 1% malachite green solution per 100 ml of suspension. If possible, the stained antigen should now be checked using whole blood just as the unstained antigen was tested with serum. The agglutinating antigen should keep for at least 6 months in
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the refrigerator at 4°C, and much longer if frozen at –20°C or below. If a batch has not been used for a long time it should be rechecked, especially for autoagglutination.

The only safety test needed is the culture test of the unwashed antigen after 7 days of incubation, to ensure that all the bacilli are dead.

- **Test procedure**

The stained-antigen agglutination test has been used with good results, especially in both domestic and ornamental waterfowl. A drop (0.05–0.1 ml) of the antigen is mixed with the same volume of fresh whole blood, obtained by venipuncture, on a white porcelain or enamel tile. The mixture is rocked for 2 minutes and examined for agglutination. The agglutination may be coarse, in which case it is obvious, or quite fine, in which case it may be most clearly seen as an accumulation of the malachite-green-stained antigen around the edge of the drop, leaving the centre a normal blood-red colour. This test is especially useful for screening large flocks for immediate culling, and therefore has advantages over the tuberculin test for the control of the disease, even in domestic fowl. It has also been claimed that in domestic fowl it is more reliable than the tuberculin test.

**Note on limitation of use**

Neither the tuberculin test with avian tuberculin nor the stained-antigen agglutination test is likely to be of any value in cases of *M. tuberculosis* infection in cage birds.

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

No vaccines are available.

**Avian tuberculin** is a preparation made from the heat-treated products of growth of *M. avium*. It is used by intradermal injection to reveal delayed hypersensitivity as a means of identifying birds infected with or sensitised to the same species of tubercle bacillus.

Guidelines for the production of veterinary vaccines are given in Chapter 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**

   Strains of *M. avium* used to prepare seed cultures should be identified as to species by appropriate tests. They should be shown to be free from contaminating organisms and to be capable of yielding a product of satisfactory quality. The strains recommended by the European Union (EU), for example, are D4ER and TB56. Reference may also be made to the World Health Organization (23).

   b) **Method of culture**

   The seed material is kept as a stock of freeze-dried cultures. If the cultures have been grown on solid media, it will be necessary to adapt the organism to grow as a floating culture. This is most easily accomplished by incorporating a piece of potato in the flasks of liquid medium (e.g. Watson Reid’s medium). When the culture has been adapted to liquid medium, it can be maintained by passage at 2–4-week intervals (1, 7).

   The production culture substrate must be shown to be capable of producing a product that conforms to the standards of the European Pharmacopoeia or other international standards (3). It must be free from ingredients known to cause toxic or allergic reactions.

   c) **Validation**

   The strains of *M. avium* used as seed cultures must be shown to be free from contaminating organisms.

   Seed lots must be shown to be efficacious in producing tuberculin with sufficient potency. The necessary tests are described in Section C.4. below.

2. **Method of manufacture**

Avian tuberculin may be made by the following three methods:
a) **Old tuberculin**

The organism is cultivated in glycerol broth medium, killed by heating in flowing steam, and filtered to remove cells. The filtrate is concentrated by heat and sterilised by filtration.

b) **Heat-concentrated synthetic-medium tuberculin**

As for old tuberculin but the glycerol broth medium is replaced by a synthetic medium (modified Dorset-Henley’s synthetic medium).

c) **Purified protein derivative**

As for heat-concentrated synthetic-medium (HCSM) tuberculin but, instead of being concentrated by heat, the protein in the filtrate is precipitated chemically (ammonium sulphate or trichloroacetic acid [TCA] are used), washed and resuspended. PPD tuberculin is recommended as it gives fewer false-positive reactions and can be standardised more precisely. An antimicrobial preservative that does not give rise to false-positive reactions, such as phenol (not more than 0.5% [w/v]), may be added. Mercurial derivatives should not be used. Glycerol (not more than 10% [w/v]) or glucose (2.2% [w/v]) may be added as a stabiliser. The product is dispensed aseptically into sterile neutral glass containers, which are then sealed to prevent contamination. The product may be freeze-dried.

3. **In-process control**

The production flasks, inoculated from suitable seed cultures, are incubated for the appropriate time period. Any flasks showing contamination or grossly abnormal growth should be discarded after autoclaving. As incubation proceeds, the surface growth of many cultures becomes moist and may sink into the medium or to the bottom of the flask. In PPD tuberculins, the pH of the dissolved precipitate (the so-called concentrated tuberculin) should be pH 6.6–6.7. The protein level of the PPD concentrate is determined by the Kjeldahl method. Total nitrogen and trichloroacetic acid precipitable nitrogen are usually compared.

4. **Batch control**

a) **Sterility**

Sterility testing is generally performed according to the European Pharmacopoeia or other guidelines (see also Chapter I.1.5.).

b) **Safety**

Two guinea-pigs, each weighing not less than 250 g and that have not been treated previously with any material that will interfere with the test, are injected subcutaneously with 0.5 ml of the tuberculin under test. No abnormal effects should occur within 7 days.

c) **Residual infectivity**

Tests on tuberculin for living mycobacteria may be performed either on the tuberculin immediately before it is dispensed into final containers or on samples taken from the final containers themselves. A sample of at least 10 ml must be taken and this must be injected intraperitoneally or subcutaneously into at least two guinea-pigs, dividing the volume to be tested equally between the guinea-pigs. It is desirable to take a larger sample, 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea-pigs are observed for at least 42 days, and are examined macroscopically at post-mortem. Any lesions found are examined microscopically and by culture.

Each filled container must be inspected before it is labelled, and any showing abnormalities must be discarded.

d) **Sensitising effect**

To test the sensitising effect, three guinea-pigs that have not previously been treated with any material that could interfere with the test are each injected intradermally on each of three occasions with the equivalent of 500 IU of the preparation under test in a 0.1 ml volume. Each guinea-pig, together with each of three control guinea-pigs that have not been injected previously, is injected intradermally 15–21 days after the third injection with the same dose of the same tuberculin. The reactions of the two groups of guinea-pigs should not be significantly different when measured 24–28 hours later.

e) **Potency**

The potency of avian tuberculin is determined in guinea-pigs sensitised with *M. avium*, by comparison with a standard preparation calibrated in IU.
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Use no fewer than nine albino guinea-pigs, each weighing 400–600 g. Sensitise the guinea-pigs by administering to each, by deep intramuscular injection, a suitable dose of inactivated or live *M. avium*. The test is performed between 4 and 6 weeks later as follows: Shave the guinea-pigs’ flanks so as to provide space for three-to-four injections on each side. Prepare at least three dilutions of the tuberculin under test and at least three dilutions of the standard preparation in isotonic buffer solution containing 0.0005% (w/v) polysorbate 80 (Tween 80). Choose the dilutions so that the reactions produced have diameters of not less than 8 mm and not more than 25 mm. Allocate the dilutions to the injection sites randomly according to a Latin square design. The dilutions are injected intradermally in volumes of 0.1 or 0.2 ml.

After 24–28 hours, the diameters of the reactions are measured and the results are calculated using standard statistical methods, taking the diameters to be directly proportional to the logarithms of the concentrations of the tuberculins. The estimated potency must be not less than 75% and not more than 133% of the potency stated on the label. The test is not valid unless the fiducial limits of error (*p* = 0.95) are not less than 50% and not more than 200% of the estimated potency. If the batch fails a potency test, the test may be repeated one or more times provided that the final estimate of potency and of fiducial limits is based on the combined results of all the tests.

It is recommended that avian tuberculin should contain the equivalent of at least 25,000 IU/ml, giving a dose for practical use of 2500 IU/0.1 ml.

f) Specificity

One or more batches of tuberculin may be tested for specificity together with a standard preparation of bovine tuberculin by comparing the reactions produced in guinea-pigs sensitised with *M. bovis* using a procedure similar to that described in Section C.4.d. In guinea-pigs sensitised with *M. bovis*, the potency of the preparation of avian tuberculin must be shown to be not more than 10% of the potency of the standard preparation of bovine tuberculin used in the potency test.

g) Stability

During storage, liquid avian tuberculin should be protected from the light and held at a temperature of 5°C (±3°C). Freeze-dried preparations may be stored at higher temperatures (but not exceeding 25°C) protected from the light. During use, periods of exposure to higher temperatures or to direct sunlight should be kept at a minimum.

Provided the tuberculins are stored at a temperature of between 2°C and 8°C and protected from light, they may be used up to the end of the following periods subsequent to the last satisfactory potency test: Liquid PPD tuberculins: 2 years; lyophilised PPD tuberculins: 8 years; HCSM tuberculins diluted: 2 years.

h) Preservatives

Antimicrobial preservatives or other substances that may be added to a tuberculin, must have been shown not to impair the safety and effectiveness of the product. The maximum permitted concentrations for phenol is 0.5% (w/v) and for glycerol it is 10% (v/v). The pH should be between 6.5 and 7.5.

i) Precautions (hazards)

Experience both in humans and animals led to the observation that appropriately diluted tuberculin injected intradermally results in a localised reaction at the injection site without generalised manifestations. Even in very sensitive persons, severe, generalised reactions are extremely rare and limited.

5. Tests on the final product

a) Safety

A test for the absence of toxic or irritant properties must be carried out according to the specifications of the European Pharmacopoeia (see also Section C.4.b.).

b) Potency

The potency of tuberculins must be estimated by biological methods. These methods must be used for HCSM and PPD tuberculins; they are based on the comparison of the tuberculins to be tested with standard tuberculins (see also Section C.4.d.).

REFERENCES


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CHAPTER 2.7.9.

DUCK VIRUS HEPATITIS

SUMMARY

Hepatitis in ducks can be caused by at least three different viruses. The more common and internationally widespread is duck hepatitis virus (DHV) type I, an enterovirus, which causes a highly lethal, acute, contagious infection in ducklings under 6 weeks of age and, frequently, under 3 weeks of age. It does not occur in older birds. This infection is often referred to simply as duck virus hepatitis.

DHV type II has been reported in the United Kingdom only. It occurred in ducklings from 10 days to 6 weeks of age, and caused pathological changes similar to those of DHV type I. From electron microscopy studies it is considered to be an astrovirus.

DHV type III has been reported only in the United States of America. It causes similar liver lesions in young ducklings, but is less virulent than DHV type I. It is believed to be a picornavirus, serologically unrelated to type I virus.

Diagnosis of hepatitis in ducklings is based on the characteristic disease pattern in the flock, gross pathological changes, the recovery of virus from dead ducklings, and the reproduction of the disease in susceptible ducklings.

Identification of the agent: It is not possible to distinguish among DHV types I, II and III on the basis of clinical findings and pathology, but distinctions can be made from the responses of ducklings, embryonated eggs and cell cultures to the isolated viruses.

Serological tests: Serological tests have little value in the diagnosis of the acute infections caused by DHV types I, II and III.

Serum neutralisation tests in ovo have been used with all three viruses and in-vitro tests have been developed for DHV type 1. These tests have been used for virus identification, assay of immune responses to vaccination and epidemiological surveys.

Requirements for vaccines and diagnostic biologicals: DHV type I infections can be controlled by the use of live attenuated virus vaccines and an inactivated virus vaccine. They are administered to breeder ducks to confer passive immunity to ducklings. Live attenuated virus vaccines may also actively immunise DHV type-I-susceptible day-old ducklings.

Ducklings susceptible to DHV type I may also be passively protected with a chicken egg yolk antibody preparation.

DHV type III infections can be controlled by the use of a live attenuated virus vaccine given to breeder ducks to confer passive immunity to ducklings.

A. INTRODUCTION

Duck hepatitis is caused by at least three different viruses, namely duck hepatitis virus (DHV) types I, II and III. The most common is DHV type I, which is an enterovirus. DHV type II is considered to be an astrovirus, and DHV type III is considered to be a picornavirus.

These viruses, which cause acute infections, should not be confused with duck hepatitis B virus, a hepadnavirus classified in the same group as mammalian hepatitis B virus. The significance of this infection for the duck is not fully understood.
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B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) DHV type I

DHV type I causes a highly contagious infection of ducks. It is of no known public health significance. The disease is an acute, rapidly spreading, often fatal virus infection of young ducklings. It usually affects ducklings under 6 weeks of age and often much younger. The clinical disease is characterised by lethargy and ataxia. Ducklings lose their balance, fall on their sides and kick spasmodically prior to death. At death the head is usually drawn back in the opisthotonos position. The whole disease sequence is rapid and can take as little as 1–2 hours. Practically all mortality in a flock will occur within 3–4 days, with most deaths on the second day. Gross pathological changes appear chiefly in the liver, which is enlarged and displays distinct punctate and ecchymotic haemorrhages. Spleen enlargement and swelling of the kidneys with some congestion of renal blood vessels may also be apparent. Microscopic changes in the liver are characterised by extensive hepatocyte necrosis and bile duct hyperplasia, together with varying degrees of inflammatory cell response and haemorrhage.

The clinical and pathological observations are highly indicative of DHV type I infection. The virus can readily be recovered from liver tissue by homogenisation as a 20% (w/v) suspension in buffered saline. The suspension is clarified, and can then be treated further (if desired) with 5% chloroform (v/v) for 10–15 minutes at ambient temperature. DHV type I is resistant to this treatment.

The presence of DHV type I is usually confirmed by one or more of the following procedures:

i) By subcutaneous or intramuscular inoculation of the isolate into ducklings between 1 and 7 days of age that are susceptible to DHV type I. The characteristic clinical disease should follow, with deaths occurring within 18–48 hours of inoculation, often in under 24 hours. The ducklings should show the gross pathology attributable to DHV type I. Virus should be re-isolated from the livers.

ii) By inoculation of serial dilutions of the liver homogenate into the allantoic sac of embryonated duck eggs (10–14 days) or chicken eggs (8–10 days). Duck embryos die between 24 and 72 hours later, whereas chicken embryos are more variable and erratic in their response and usually take 5–8 days to die. Gross pathological changes in the embryos include stunting and subcutaneous haemorrhages over the whole body, with oedema particularly of the abdominal and hind limb regions. The embryo livers may be red and yellowish, swollen and may show some necrotic foci. In embryos that take longer to die, the greenish colour of the allantois is more pronounced, and both the liver lesions and stunting become more evident.

iii) By inoculation of primary cultures of duck embryo liver (DEL) cells, which are particularly sensitive (10). Dilutions of the liver homogenate containing DHV type I cause a cytopathic effect (CPE), which is characterised by cell rounding and necrosis. When overlaid with a maintenance medium containing 1% agarose (w/v), the CPE gives rise to plaques approximately 1 mm in diameter.

• Immunological tests

Such tests have not been used extensively for the routine identification of DHV type I infection. Various virus neutralisation (VN) assays have been described, which may assume greater significance if DHV types II and III infections become more widespread. The tests that have been described (2, 10–12) include:

i) Passive subcutaneous immunisation of 1–7-day-old ducklings susceptible to DHV type I with 1–2 ml specific hyperimmune serum or specific egg yolk antibody. These ducklings are then challenged intramuscularly or subcutaneously, 24 hours later with at least 10^3.0 LD_{50} (50% lethal dose) of the virus isolate. A control group of uninoculated ducklings is similarly challenged. Identification of infection is based on 80–100% survival in the passively immune ducklings and 80–100% mortality in the controls.

ii) 1–7-day old DHV type-I-susceptible and DHV type I maternally immune ducklings are challenged intramuscularly or subcutaneously with at least 10^3.0 LD_{50} of the virus isolate. Identification is based on 80–100% losses in the susceptible ducklings and 80–100% survival in the maternally immune ducklings.

iii) Serial tenfold dilutions of the virus isolate are mixed with equal volumes of DHV type-I-specific hyperimmune serum diluted between 1/5 and 1/10. The mixtures are allowed to react at room temperature for 1 hour and are then inoculated (0.2 ml) subcutaneously into susceptible ducklings, also via the allantoic cavity (0.2 ml) of embryonated duck eggs and on to primary DEL cell monolayer cultures. Controls in each case consist of the virus isolate mixed with control serum.
There is little evidence for antigenic variation among DHV type I isolates. However, a variant, DHV type Ia, isolated in the United States of America (USA) only partially reacts with the classical type I virus in cross serum neutralisation tests (7, 13). Other variants have been reported from India and Egypt, but nothing further is known about them.

b) DHV type II

DHV type II infection of ducks has only been reported from the United Kingdom (1, 4). It is an acute, fatal infection of ducklings producing clinical and pathological signs similar to DHV type I. Affected birds may show signs of polydypsia and usually die within 1–2 hours of appearing sick.

Gross pathological changes include multiple haemorrhages, both punctate and confluent bands in the liver, swollen pale kidneys with congested blood vessels, and enlarged spleens. The alimentary tract is often empty although the small intestine may contain mucus, and haemorrhagic areas are occasionally seen. Petechial haemorrhages are also occasionally seen on the heart. Histologically, changes in the liver are similar to those seen in DHV type I infections; the extent of bile duct hyperplasia may be greater than with DHV type I, but this is relative. Electron microscopy of liver and faecal preparations has revealed viral particles 28–30 nm in diameter with a morphology resembling that of astroviruses (4).

The virus may be recovered in 20% (w/v) homogenised liver suspensions in buffered saline. This can be used to inoculate:

i) Susceptible ducklings, in which the response can be variable. A mortality rate of up to 20% may occur within a period of 2–4 days. The gross pathology is similar to that observed in field cases (4). This is in contrast to the findings with DHV type I infection, which is more virulent and rapid in its effect.

ii) Embryonated chicken or duck eggs, either via the amniotic cavity or yolk sac. These may respond, erratically, after four passages, but no deaths may be seen during earlier passages. Embryos take 6–10 days to show evidence of infection; when this occurs there is stunting with green necrotic livers.

Immunological tests

Immunological tests have not been employed routinely as the serological response to infection of both ducklings and duck embryos is poor. However, a neutralisation assay has been applied (4) for virus identification by inoculating chicken embryos via the amniotic cavity with constant-serum/varying-virus mixtures.

Cross protection tests have been performed in 2–4-day old ducklings (4); these are inoculated with antisera to types I or II, then challenged 3 days later with the virus isolate. This technique could distinguish DHV type II from types I and III.

c) DHV type III

DHV type III has been reported in the USA only. Losses of up to 20% occur in ducklings immune to DHV type I (5, 8). DHV type III causes an acute infection of young ducklings with clinical signs similar to those seen in type I infections.

The gross pathology is also similar to type I infection. The liver surface is pale and mottled with many red bands and some petechial haemorrhages. The spleen is paler, but not noticeably enlarged, and the kidneys may show patchy congestion.

The virus can be recovered from homogenised liver suspensions and is resistant to treatment with 5% chloroform. The virus can be isolated by:

i) Inoculating the isolate intramuscularly into susceptible ducklings. The mortality rate may reach 20% with 60% morbidity. No deaths occur in the first 24 hours and all losses ensue between day 2 and day 4 after inoculation. Intravenous inoculation is more effective; type III infection is less virulent than type I.

ii) Inoculating the isolate on to the chorioallantoic membrane (CAM) of 10-day-old embryonated duck eggs. The response is erratic, but some embryo mortality always occurs within 7–10 days. The membranes assume a dry crusty appearance, beneath which they are oedematous. The embryos may be stunted and oedematous with skin haemorrhages. The liver, kidneys and spleen are enlarged.

Attempts to cultivate the virus in hens’ eggs have not been successful.

Attempts to induce a CPE with the virus in tissue cultures have not been successful, but the virus has been detected by direct immunofluorescence in experimentally infected DEL and duck embryo kidney (DEK) cell monolayer cultures (5).
2. Serological tests

These do not apply to diagnosis as the clinical disease is too acute.

All three DHV types have been used in virus neutralisation tests in ovo, but their success depends on the expression of the virus in the assay system used; with type II and III viruses this can be a problem. In-vitro tests have been developed for DHV type I; these include a plaque reduction assay and a microtitre assay (10, 11). The plaque reduction assay may be performed using either primary DEK or DEL cells. Primary cell culture monolayers are prepared in Eagle’s minimal essential medium (MEM) containing 5–10% fetal calf serum (FCS), 2 mM glutamine, 0.17% sodium bicarbonate and gentamicin. Trypsinised cells are seeded into 5 cm diameter Petri dishes, then incubated at 37°C in a 5% CO₂ atmosphere. Monolayers should be nearly confluent at 24–48 hours post-seeding. The monolayers are washed twice with serum-free MEM or Hank’s balanced salt solution to remove all traces of FCS before infecting with DHV type I. Equal volumes of DHV type I suspended in serum-free MEM, adjusted to 200 plaque-forming units (PFU) per 0.1 ml, are mixed with equal volumes of serially diluted duck sera (twofold dilutions in MEM). The serum samples should be heat inactivated at 56°C for 30 minutes before testing. The virus/serum mixtures are incubated at 37°C for 1 hour, then 0.1-ml aliquots are added to the confluent cell monolayers, three dishes per dilution. The plates are left for 30 minutes at room temperature (20–22°C), then overlaid with agarose maintenance medium (MEM containing 2% chicken serum and 0.1–0.2% FCS to which agarose had been added to a final concentration of 1% [w/w]). The plates are then placed at 37°C in a 5% CO₂ atmosphere. The number of plaques produced is recorded after 48 hours’ incubation. Plaques may be observed using an oblique light source, or alternatively monolayers may be fixed with 10% formol-buffered saline and stained with 1% crystal violet. Serum antibody titres are expressed as the reciprocal of the highest serum dilution that reduces the plaque count by 50%.

A microtitre neutralisation assay may be performed using primary DEK cells. Serial twofold dilutions of each serum sample (heat-inactivated) are prepared in 50 µl of serum-free Eagle’s basal medium (BME) in microtitre plates. Approximately 10².⁰ TCID₅₀ (50% tissue culture infective dose) of DHV type I in 50 µl of BME is added to each well and the mixtures are allowed to react at 37°C for 1 hour. Primary DEK cells are suspended in BME supplemented with 10% tryptose phosphate broth, 2 mM L-glutamine, 0.17% sodium bicarbonate and 2–4% chicken serum, and are adjusted to contain 3 × 10⁵ cells/ml. Cells are next added to the plates at 100 µl per well and the plates are then incubated for up to 96 hours at 37°C in a humidified 5% CO₂ atmosphere. Following incubation, cells are fixed with 10% formol-buffered saline and stained with 1% crystal violet. The plates are read macroscopically. The titre for virus neutralising activity is expressed as the reciprocal of the highest dilution of serum at which a monolayer grew, i.e., there is no evidence of CPE and therefore complete virus neutralisation has occurred. A titre of less than 4 log₂ is considered to be negative.

These neutralisation tests have been used to assay humoral immune responses to vaccination and for epidemiological surveys, as well as for virus identification.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

DHV type I can be controlled by the use of a live attenuated virus vaccine. This is given to breeder ducks so that immunity is transferred via the yolk to newly hatched birds. Live vaccine virus can also be used to actively immunise newly hatched DHV type-I-susceptible ducklings (3). An inactivated DHV type I vaccine is also effective when administered to breeder ducks that have been primed with live vaccine or previously field exposed to live DHV type I; progeny from these breeders have maternal immunity (11). Ducks may also be passively protected by inoculation of antibodies in chicken egg-yolk.

An attenuated live virus DHV type II vaccine has been used to protect ducklings only under experimental conditions (4).

DHV type III infections have been controlled by the use of attenuated live virus vaccines given to breeder ducks, so that the immunity is transferred via the yolk sac to the hatching ducklings.

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 type I virus vaccine seed used most commonly in Europe is derived from an isolate passaged in embryonated chicken eggs 53–55 times, that in the USA for live and inactivated vaccines has been passaged 84–89 times.
Chapter 2.7.9. — Duck virus hepatitis

The type II virus vaccine seed originated from an isolate attenuated by 25 serial passages in embryonated chicken eggs (1), and has been employed only experimentally under field conditions (R.E. Gough, personal communication).

The type III vaccine seed has been attenuated by 30 serial passages in embryonated duck eggs inoculated via the CAM.

b) Method of culture

The seed viruses of types I and II are handled similarly. They should be prepared in 8–10-day-old specific pathogen free (SPF) embryonated chicken eggs inoculated via the allantoic route and incubated at 37°C. They can be stored as embryo homogenates in buffered saline at −70°C or below for several years.

The type III seed virus is prepared in 10-day-old SPF duck embryos, inoculated on to the CAM, and incubated for 6–10 days at 37°C. It may be stored as a homogenate of CAM and embryos at −70°C or below.

c) Validation as a vaccine

All seed viruses should be shown to be free from extraneous viruses that are pathogenic for ducks, chickens or turkeys. The seeds should be free from all microbiological and fungal contamination.

In the case of newly hatched ducklings, attenuated live DHV type I replicates rapidly and results in an immunity within 48–72 hours of vaccination. This immunity persists throughout the susceptible period of life (3). However in ducklings protected by vaccination of their parents, the level of maternally derived immunity decreases over the first 2 weeks of life, but such ducklings can be actively re-immunised with attenuated virus given subcutaneously or orally at about 7–10 days of age (6, 9). Alternatively, the immunity can be enhanced by the administration of specific hyperimmune serum or of egg yolk antibody prepared from eggs laid by chickens actively hyperimmunised against DHV type I.

Breeder ducks primed with live DHV type I and then given, intramuscularly, a single dose of inactivated type I vaccine produced maternally immune progeny through a complete laying cycle (11).

2. Method of manufacture

DHV types I and II viruses are treated similarly. The vaccine is produced in 8–10-day-old SPF embryonated chicken eggs inoculated via the allantoic route, and incubated at 37°C. Most embryo deaths occur within 2–3 days in the case of DHV type I, but with type II, the deaths do not occur until 6–10 days after inoculation, although they are harvested at 3–5 days for maximum virus yield. The embryo harvests are homogenised in buffered saline and clarified by low-speed centrifugation. The preparation is diluted as appropriate and dispensed into vials, which are preferably frozen rapidly at −70°C or below. Subsequently, they may be stored satisfactorily between −20°C and −40°C. DHV type I attenuated vaccine is also available as a lyophilised preparation that may be stored at 2–8°C. The reconstituted vaccine may be used with or without the incorporation of aluminium hydroxide in the diluent.

In the case of inactivated DHV type I vaccine, the embryo harvests are homogenised and clarified by low-speed centrifugation and then further purified by treatment with chloroform (final concentration 10% [v/v]). This preparation is then inactivated with freshly prepared binary ethylenimine (BEI). The inactivated virus is then blended with an adjuvant such as LES-STM1; 0.2 % (v/v) formalin is added as a preservative (11).

The type III vaccine is prepared in 10-day-old SPF duck eggs inoculated via the CAM with attenuated DHV type III and incubated at 37°C. Most embryo deaths occur between 6 and 10 days. Eggs containing dying embryos, together with their CAMs, are harvested and homogenised in buffered saline and clarified by low-speed centrifugation. The preparation is diluted as appropriate and dispensed into vials, which are preferably frozen rapidly at −70°C or below.

- Egg yolk antibody

Virulent DHV type I prepared from duckling livers or attenuated virus may be used to hyperimmunise SPF chickens for egg-yolk antibody production. Eggs are collected from the hyperimmunised birds and stored at 4°C until time of production. The yolks are separated, pooled and blended with an antifoaming agent. The mixture is diluted with buffered saline containing no more than 0.2% (v/v) formalin as a preservative. The

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1 A preparation of Salmonella typhimurium (STM), a B cell mitogen, in a lipid emulsion system (LES). Available from Ribi Immunochem Research, Hamilton, Montana 59840, USA.
dispensed product is stored at 4°C and has a shelf life of 1 year. Tests are carried out for sterility in the usual way for the absence of contaminants.

3. In-process control

Any embryo deaths within the first 24 hours of inoculation should be discarded as nonspecific deaths.

The identity of the virus type should be confirmed by a VN test conducted with specific antiserum by a constant-serum/varying-virus method. In the case of types I and II viruses, the tests are performed in embryonated chicken eggs; with type III virus the tests are done in embryonated duck eggs. The antiserum should reduce the titre of the respective virus by at least $10^{2.0}$ ELD<sub>50</sub> (50% embryo lethal dose).

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in the Chapter I.1.5.

b) Safety

A group of 1–3-day-old ducklings susceptible to the type of virus concerned, should be inoculated subcutaneously or intramuscularly (in the case of types I and II), or subcutaneously (in the case of type III), with the attenuated vaccine at ten times the recommended dose, and kept under observation for between 10 and 21 days for any adverse reactions. Attenuated live vaccines should be stable and not revert to virulence on back passages in susceptible ducklings.

A safety test on the inactivated DHV type I vaccine is performed by inoculating the recommended dose (0.5 ml) intramuscularly into a group of day-old ducklings; no adverse effects should be observed during the period of testing.

Safety tests on yolk antibody are done by inoculating 1 ml subcutaneously into each of a group of ducklings, which are then kept under observation for 3 days for signs of adverse effects.

c) Potency

For DHV types I and II viruses, the virus titre of the vaccine should be determined in 8–10-day-old embryonated chicken eggs inoculated into the allantoic cavity and incubated at 37°C. The immunogenicity of the vaccine for ducklings susceptible to type I or II virus can be assessed by inoculating subcutaneously a minimum of $10^{3.3}$ ELD<sub>50</sub> per duckling of the vaccine virus and challenging subcutaneously 72 hours later with $10^{3.0}$ LD<sub>50</sub> per duckling of virulent DHV virus type I or II (3). At least 80% of the vaccinated birds should survive and, in the case of type I, at least 80% of the controls should die; in the case of type II, a 20% mortality in the controls is more realistic.

The immunogenicity of the inactivated vaccine is considered to be satisfactory if a four-fold or greater increase in neutralising antibody titre can be demonstrated following administration to ducklings that have been previously primed with live attenuated DHV type I.

For type III virus, the titre of the vaccine should be determined in 10-day-old embryonated duck eggs inoculated on to the CAM. Immunogenicity tests in ducklings have proved difficult because of the variable pathogenicity of the challenge virus for ducklings.

Potency tests on yolk antibody are done by determining the neutralising index (NI) for the product in embryonated hens' eggs using the constant-yolk/varying-virus method. A minimum NI of $10^{3.0}$ is considered to be satisfactory. The efficacy of the product is determined by inoculating a group of susceptible ducklings with the recommended dose of egg yolk antibody. A second group is left untreated. After 24 hours each group is challenged with virulent DHV type I virus. The product is adjudged efficacious if at least 80% of the treated ducklings survive and at least 80% of the controls die.

d) Duration of immunity

Breeder ducks given live attenuated DHV type I vaccine two or three times at 12, 8 and 4 weeks before coming in to lay, and breeder ducks given live attenuated DHV type III vaccine twice at 12 and 4 weeks before coming in to lay should produce passively immune progeny throughout a breeding season. However, it is usually recommended to revaccinate every 3 months with DHV type I vaccine and every 6 months with DHV type III vaccine after the onset of lay. DHV type I attenuated vaccine can also be supplied as a lyophilised preparation that is blended with a diluent containing aluminium hydroxide, just before
administration. This is given at 7 weeks of age with a second dose 2 weeks before onset of lay. This should provide maternally immune progeny throughout a complete laying cycle. No information on the use of DHV type II vaccine in breeder ducks is available.

Live attenuated DHV type I or type II vaccine given subcutaneously or intramuscularly to 1-day-old ducklings protects against the disease for the duration of their susceptibility. No information is available on the use of DHV type III vaccine to actively immunise 1-day-old ducklings.

Breeder ducks primed with live DHV type I and then given a single dose of inactivated DHV type I vaccine intramuscularly, should produce maternally immune progeny through a complete laying cycle (11).

Egg-yolk antibody offers passive immunisation in the face of an outbreak. The duration of its efficacy is short-lived.

e) Stability
Aqueous preparations of live attenuated DHV type I, II and III vaccines when stored frozen at –70°C or lower should remain stable for at least 1 year. Once thawed these vaccines should be held at 4°C and used within 1 week. Live lyophilised vaccines may be stored at 2–8°C and should retain their potency for at least 1 year.

The inactivated DHV type I vaccine is blended with adjuvant and can be stored at 4°C for at least 20 months without loss of immunogenicity.

Egg-yolk antibody can be stored for up to 1 year at 4°C.

f) Preservatives
No preservatives are added to the live attenuated DHV type I, II and III vaccines.

Formalin (up to 0.2% [v/v]) is added to the DHV type I inactivated vaccine, and to the egg-yolk antibody preparation.

g) Precautions (hazards)
The inactivated DHV type I vaccine should be shaken well to ensure that it is completely blended before use.

5. Tests on the final product
The live attenuated DHV type I and III vaccines are issued as vials of lyophilised or frozen concentrated vaccine virus together with bottles of sterile diluent, on which standard sterility checks have been made (see Section C.4.a.). The DHV type II live attenuated vaccine has only been made experimentally.

a) Safety
No additional testing is performed after the batch testing on any of the products.

b) Potency
No additional testing is performed after the batch testing on any of the products.

REFERENCES


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CHAPTER 2.7.10.

DUCK VIRUS ENTERITIS

SUMMARY

Duck virus enteritis (DVE) or duck plague is an acute contagious infection of ducks, geese and swans (order Anseriformes) caused by a herpesvirus. Diagnosis is based on a combination of assessing the clinical signs, gross pathology and histopathology supported by identification of the virus by either isolation or polymerase chain reaction.

Identification of the agent: The virus may be isolated from the liver, spleen and kidneys of birds dying from this infection. Virus may be recovered by infecting susceptible ducklings, in which the disease can be reproduced; by inoculating embryonated Muscovy duck eggs on the chorioallantoic membrane; or by inoculating cell cultures of duck embryo or Muscovy duck embryo origin. The identity of the virus can be confirmed by neutralisation tests using specific antiserum to inhibit pathological changes in the duck embryos or the cytopathological effects in the cell cultures, or by direct or indirect immunofluorescence tests on infected cell cultures. Alternatively the DVE DNA may be detected by the polymerase chain reaction from the oesophagus, liver and spleen of DVE-infected birds as well as from Muscovy duck embryos or cells used for virus isolation.

Serological tests: Immunological tests have little value in the diagnosis of acute DVE infection. Serum neutralisation tests in ovo and in vitro have been used to monitor exposure to DVE in wildfowl.

Requirements for biological products: A live attenuated virus vaccine is available to control DVE in birds over 2 weeks of age. Ducks are vaccinated subcutaneously or intramuscularly for active immunity. Vaccine virus is not thought to spread from vaccinated to unvaccinated stock. An inactivated vaccine has been reported to be efficacious in laboratory tests, but has not been developed or licensed for large-scale use.

A. INTRODUCTION

Duck virus enteritis (DVE) is an acute, sometimes chronic, contagious virus infection that occurs naturally only in ducks, geese and swans, all members of the family Anatidae of the order Anseriformes. The aetiological agent, a herpesvirus, is a member of the alphaherpesvirinae subfamily of the Herpesviridae. DVE may also be referred to as duck plague, anatid herpes, eendenpest, entenpest and peste du canard. The infection has not been reported in other avian species, mammals or humans.

In domestic ducks and ducklings, DVE has been reported in birds ranging from 7 days of age to mature breeders. In susceptible flocks the first signs are often sudden, high and persistent mortality with a significant drop in egg production. In chronically infected partially immune flocks only occasional deaths occur. Recovered birds may be carriers and may shed the virus in the faeces or on the surface of eggs over a period of years. Recently, DVE limited solely to Muscovy ducks has been observed in the USA (2, 5).

Clinical signs and gross pathology associated with a DVE outbreak vary with the species, age and sex of the affected birds, and the virulence of the virus. In breeder ducks the range of signs include eye watering and pasted eye-lids associated with photophobia, polydypsia, loss of appetite, ataxia, watery diarrhoea and nasal discharge. Birds often have ruffled feathers and soiled vents. Sick birds may maintain an upright stance by using their wings for support, but their overall appearance is one of weakness and depression. In ducklings 2–7 weeks of age, losses may be lower than in older birds and the signs associated with DVE infection include dehydration, loss of weight, a blue colouration of the beaks and blood-stained vents.

At necropsy, there is little evidence of emaciation in adult ducks that have died. In mature males, prolapse of the penis may occur. In mature females, haemorrhages may be observed in ovarian follicles. The gross lesions are characterised by vascular damage, with tissue haemorrhages and free blood in the body cavities, eruptions, or
annular haemorrhages and diphtheroid lesions of the mucosal surfaces of the digestive tract, lesions of the lymphoid organs and retrograde changes of the parenchymatous organs. Collectively, these lesions are pathognomonic for DVE. The pathology and histopathology of DVE in white Pekin ducks has been reviewed (19). Microscopic lesions are characterised by vascular damage and its consequences in visceral organs. Eosinophilic intranuclear inclusions and cytoplasmic inclusions in epithelial cells of the digestive tract are typically present.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Primary isolation of the virus is best achieved from samples of liver, spleen or kidney tissue, which have been homogenised in buffered saline containing antibiotics and clarified by low-speed centrifugation (1800 g). Isolation may be attempted by inoculating such homogenates into cell cultures, ducklings or duck embryos.

a) Cell cultures

Cell culture is reported as the method of choice for isolation of DVE virus, but may not always be successful. If attempted, isolations may be made in primary duck embryo fibroblasts (DEF) (22) or, preferably primary Muscovy duck embryo fibroblasts (MDEF) (9, 14) or Muscovy duck embryo liver (MDEL) cells. Cells are thought to be even more sensitive (R.E. Gough, pers. comm.). Cell monolayers grown in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum (FCS), 2 mM glutamine, and 0.17% sodium bicarbonate and gentamicin are washed with serum-free MEM and then inoculated with the clarified sample homogenate suspected to contain DVE virus. After incubation for 1 hour at 37°C to allow for virus adsorption, the cultures are maintained on MEM containing 2% FCS, 2 mM glutamine, and 0.17% sodium bicarbonate and gentamicin, and incubated in an atmosphere containing 5% CO2. The cytopathic effect (CPE) is characterised by the appearance of rounded clumped cells that enlarge and become necrotic 2–4 days later. Cultures should be stained with a fluorescent antibody conjugate using a direct or indirect method to identify the virus (see Section B.1.d.). Cells can also be fixed and then stained with haematoxylin and eosin to show syncytial formation, intranuclear inclusions and marked cytoplasmic granulation. It has been reported (1) that the isolation of DVE in MDEF cells is favoured by incubation at temperatures between 39.5°C and 41.5°C. However, an elevated temperature does not appear to be essential for isolation, which is often carried out at 37°C. More than one passage in cell culture may be necessary to isolate the virus. This virus isolation method in cell cultures may be modified to a plaque assay by overlaying the cell monolayer with maintenance medium containing 1% agarose. As the virus can be cell associated, sequential passaging should be carried out by trypsinising potentially infected cells and replanting them, as well as inoculating fresh cells with infected culture supernatant from the previous passage.

b) Ducklings

When inoculated intramuscularly, 1-day-old susceptible ducklings die within 3–12 days; uninoculated ducklings, housed separately, should be maintained as controls at the same time. Muscovy ducklings (Cairina moschata) are more susceptible than white Pekin ducklings. Both macroscopic and microscopic lesions typical of DVE should be seen on post-mortem examination. The diagnosis may be confirmed either by vaccinating ducklings against DVE and challenging them subsequently with the virus isolate or by immunofluorescence. However, virulent strains of the virus exist, against which the vaccine may be ineffective (13). In the author's experience of natural infections occurring in Muscovy ducks, this method of virus isolation has proved more sensitive than cell culture methods.

c) Duck embryos

Primary virus isolations can be made by inoculation on to the chorioallantoic membrane (CAM) of 9–14 day embryonated Muscovy duck eggs. The embryos may die, showing characteristic extensive haemorrhages 4–10 days after inoculation. Two to four serial blind passages of the homogenised CAMs may be necessary before an isolation can be effected. This method is not as sensitive as that using susceptible day-old ducklings.

Embryonated chicken eggs are not very susceptible to infection with field strains of DVE. The virus can nevertheless be adapted to chicken embryos by serial passages. Pekin duck embryos vary in their susceptibility to strains of DVE virus.

d) Immunological methods

Serological tests used to confirm the identity of newly isolated virus include neutralisation assays performed in either embryonated eggs or cell cultures. A plaque assay for DVE in duck embryo cell cultures has been described (4). In the author's laboratory a microtitre assay using primary MDEF or MDEL cells is used. Provided a hyperimmune antiserum of sufficiently high titre is used, a fluorescent antibody test (direct or
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indirect) for DVE in DEF, MDEF or MDEL cells is the next most sensitive assay after isolation in 1–9-day old ducklings (8). A reverse passive haemagglutination test for DVE has been described (6), but it is reported to be less sensitive than immunofluorescence and plaque assays. An avidin–biotin–peroxidase method of immunoperoxidase staining to detect DVE antigen in formalin-fixed, paraffin-embedded sections of liver and spleen from experimentally infected birds has been described (12); this method could have diagnostic potential. The identity of the virus may also be confirmed by negative stain electron microscopy, but this alone is not positive confirmation that the herpesvirus observed is DVE virus. Immunoelectron microscopy has been developed recently using DVE hyperimmune serum (15).

e) Nucleic acid recognition methods

Recently, detection of DVE virus by polymerase chain reaction (PCR) has been reported (10, 11, 16, 17). Primers have been identified that are able to amplify DNA from DVE virus present in various tissues, including oesophagus, liver and spleen, from an original outbreak and after passage from Muscovy duck embryos. The following is an example protocol for PCR methods for detection of DVE virus; other protocols exist.

• PCR method

This DNA extraction procedure can be used on disrupted cell suspensions from DVE-infected tissue culture, 10% ground tissue suspensions, or cloacal swab material in transport medium. This method is used to prepare duck plague DNA for the known positive PCR controls.

• Extraction of viral DNA

Note: All product transfers in steps i to v are performed in a biological safety cabinet.

i) For a 10% ground tissue suspension, add 400 µl to a 1.5 ml microfuge tube and microfuge at 16,000 g for 5 minutes. Transfer the supernatant to a new tube and go to step ii.

ii) For tissue culture suspensions and cloacal swab material, add 400 µl of the sample, or supernatant from step i above, to a 1.5 ml tube and microfuge at 16,000–20,000 g for 45 minutes to pellet the virus.

iii) Discard the supernatant and resuspend the pellet with 200 µl of Tris/ethylene diamine tetra-acetic acid (EDTA) buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA).

iv) Add 10 µl of a 5 µg/µl proteinase K solution to give a final concentration of 0.2 µg/µl, mix thoroughly, and incubate at 56°C for 1 hour.

v) Add 25 µl of 10% sodium dodecyl sulfate (SDS) solution to give a final SDS concentration of 1%, mix thoroughly, and incubate at 37°C for 1 hour.

vi) Add 15 µl of 5 M NaCl to give a final concentration of 0.3 M and mix thoroughly.

vii) Add 300 µl of fresh phenol buffered with Tris/HCl, pH 8.0, to the tube, and mix by inverting 50 times.

viii) Microfuge the tube at 16,000 g for 5 minutes and transfer the top aqueous phase (sample) to a new tube.

ix) Repeat the phenol extraction steps vii and viii once more.

x) Add 500 µl of ether to the tube, mix thoroughly, and microfuge at 16,000 g for 1 minute.

xi) Discard the top aqueous phase (ether) and repeat the ether extraction step (step x) once more.

xii) Heat the tube with the lid open at 56°C for about 15 minutes or until the smell of ether is gone.

xiii) Split the tube contents in two and add 2.25 times the sample volume of 100% ethanol to each tube, mix the tube contents by inverting the tube several times, and leave at room temperature (22°C) for 30 minutes.

xiv) Microfuge the tube at 16,000 g for 45 minutes and discard the supernatant.

xv) Add 200 µl of 70% ethanol to gently wash the pellet and then microfuge at 16,000 g for 15 minutes.

xvi) Discard the supernatant and dry the pellet at 56°C for 30–45 minutes with the tube lid open.

1 Provided by Dr W.R. Hansen, US Geological Survey, Biological Resources Division, National Wildlife Health Center, 6006, Schroeder Road, Madison, WI 53711, USA. This procedure uses the following commercial items: GeneAmp PCR Reagent Kits containing dNTPs, 10x amplification buffer for hot start PCR, Taq DNA polymerase, Lambda PCR control reagents, and Ampliwi wax beads (Applied Biosystems), and a 100 base pair molecular size ladder (Invitrogen)
xvii) Resuspend the DNA in 30 µl distilled water that is RNAase and DNAase free.

xviii) Store the sample tube at 4°C until tested (few days) or at –20°C for long-term storage.

- Polymerase chain reaction

Lower reaction mixtures for the DVE PCR and the lambda control are prepared in advance in a biosafety cabinet using the kit manufacturer’s recommended methods for a hot start PCR. The lower reaction mixture is dispensed into tubes, sealed with Ampliwax at 80°C, as recommended by the manufacturer, and stored at 4°C for 1–2 months.

PCR primers for DVE DNA-directed DNA polymerase gene

Primer 1 sequence : 5’-GAA-GGC-GGG-TAT-GTA-ATG-TA-3’ (forward)

Primer 2 sequence; 5’-CAA-GGC-TCT-ATT-CGG-TAA-TG-3’ (reverse)

i) The upper reaction mixture is prepared according to the kit manufacturer’s recommendations the day of the test, and distributed to each sample tube including DVE and lambda control tubes.

ii) Add 10 µl of DNA suspension from the stored sample tubes to the PCR lower reaction tubes with corresponding labels.

iii) Place known DVE DNA diluted to 1 pg/10 µl into one control tube and 10 µl of distilled water into the no DNA control tube. Add 10 µl of lambda DNA supplied in the kit and 10 µl of water to the corresponding lambda control tubes.

iv) Place all the tubes in a thermal cycler that is programmed as follows:

One cycle: Hold 94°C for 2 minutes
Hold 37°C for 1 minute
Hold 72°C for 2 minutes

35 cycles: Hold 94°C for 1 minute
Hold 55°C for 1 minute
Hold 72°C for 3 minutes

One cycle: Hold 72°C for 7 minutes
Hold 4°C until stored

PCR tubes are stored at 4°C until the samples are examined for amplification products.

- Electrophoretic analysis of PCR products

i) A fresh 1 x TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3) is prepared from a 10x stock for agarose preparation and for use in the electrophoresis chamber.

ii) A 1% agarose solution is prepared in TAE buffer, heated to dissolve the agarose, and, when cool, poured into a gel former with a comb.

iii) The solidified gel is placed into the electrophoresis chamber and TAE running buffer is added.

iv) PCR test samples, including the DVE and lambda controls, are mixed 1/10 with 1 µl of loading buffer (0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol, 0.01 M Tris/HCl, pH 8.0, and 50% [v/v] glycerol) and 10 µl of each is added to individual wells of the gel. The 100 bp molecular size markers are added to each side of the gel.

v) Run the gel for 1 hour at 120 volts and then stain in a 1% ethidium bromide solution for 20 minutes. Destain the gel for 45 minutes in deionised water and view the gel on a UV-illuminated light box. Photograph the gel to record results.

- Interpretation of the results

A 500 bp amplification band in the lambda control sample indicates the PCR ran successfully. A 446 bp band in the DVE known DNA control indicates the DVE primers are working. A 446 bp band in the unknown test sample indicates DVE viral DNA was present. No amplification products will be present in the DVE or lambda no DNA controls. If bands appear in these negative control products, cross-contamination occurred during the test set-up and the test must be repeated.

f) Strain variation

Although strains of DVE differ considerably in virulence, there is little reported evidence of serological variation.
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2. Serological tests

Serological tests have little value in the diagnosis of acute DVE infections, but assays based on serum neutralisation in embryonated eggs and cell cultures have been used to monitor antibodies following exposure to DVE in wildfowl. The humoral response to natural infection with DVE virus is often low and antibodies may be short-lived (7); it is assumed that cell-mediated immunity also plays a role in the infection (18). However, detection of neutralising antibodies to DVE virus in serum is possible. Virus neutralisation (VN) (21) assays using a constant-serum/varying-virus method may be performed in chicken or duck embryos by using embryo-adapted virus, or in cell cultures. Neutralisation indices (NI) (21) between 0 and 1.5 were detected in domestic and wild waterfowl that had not been exposed to DVE; a NI of 1.75 or greater was considered to be evidence of prior exposure to DVE virus (3). Alternatively, sera may be screened using a constant-virus/varying-serum method. In the author’s laboratory a microtitre neutralisation assay using primary MDEF or DEF is used. Serial twofold dilutions of each serum sample (heat-inactivated at 56°C) are prepared in 50 µl of serum-free MEM in microtitre plates. Approximately $10^{2.0} \text{TCID}_{50}$ (50% tissue culture infective dose) of DVE virus in 50 µl of MEM is added to each well and the mixtures are allowed to react at 37°C for 1 hour. A suspension of primary MDEF or DEF in MEM supplemented with 2 mM L-glutamine, 0.17% sodium bicarbonate and 10% FCS, are adjusted to contain $3 \times 10^5$ cells per ml. Cells are next added to the plates at 100 µl per well and the plates are then incubated for up to 96 hours at 37°C in a humidified 5% CO₂ atmosphere. Following incubation, cells are observed daily by light microscopy and finally fixed with 10% formol-buffered saline and stained with 1% crystal violet. The plates are read macroscopically. The titre for virus neutralising activity is expressed as the reciprocal of the highest dilution of serum at which a monolayer grew, i.e. there is no evidence of CPE and therefore complete virus neutralisation has occurred. A titre of less than $3 \log_2$ is usually considered to be negative. A VN titre of 8 or greater is considered to be significant and is evidence of exposure to DVE virus (7). VN antibody may also be detected using cell cultures by mixing sera at a single dilution, e.g. 1/10, with 100–200 TCID₅₀ virus and then testing inoculated cell cultures for non-neutralised virus by immunofluorescence. Although this method is not quantitative, it can be useful for screening large numbers of sera. These latter methods, using constant-virus/varying-serum, are much more economical on sera than the NI methods.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

A live attenuated virus vaccine can be used to control DVE in birds over 2 weeks of age (18, 19). Fattening or breeding ducks may be vaccinated subcutaneously or intramuscularly to produce an active immunity. The vaccine virus is not thought to spread by contact from vaccinated to unvaccinated ducks, as the unvaccinated birds remain susceptible to infection.

An inactivated vaccine has been reported to be as efficacious as modified live vaccine (20). This vaccine has been tested only under laboratory conditions; it has not been tested on a large scale and is not licensed.

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
      DVE vaccine can be prepared from a strain of the virus that has been attenuated by serial passage in embryonated chicken eggs. In the USA the vaccine strain seed was originally imported from Holland and has been serially passaged 41–46 times.
   b) Method of culture
      The seed virus should be prepared in 8–11-day-old specific pathogen free (SPF) embryonated chicken eggs by inoculating on to the CAM followed by incubation at 37°C. The seed may be stored at −70°C or lower in the form of a homogenate of the embryo CAM in buffered saline.
   c) Validation as a vaccine
      The seed virus should be shown to be free from extraneous viruses pathogenic to ducks, chickens and turkeys. It should also be free from bacterial, fungal and mycoplasmal contaminants.
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The identity of the virus should be confirmed by a VN test conducted with specific antiserum using the constant-serum/varying-virus method. This test should be performed in embryonated chicken eggs. The antiserum should reduce the virus titre by at least $10^{1.75} \text{ ELD}_{50}$ (50% embryo lethal dose).

The immunogenicity of the vaccine can be assessed in DVE-susceptible ducks or ducklings by inoculating the recommended vaccine dose intramuscularly and challenging intramuscularly 21 days later with virulent DVE virus. The vaccinated birds should survive challenge while unvaccinated control birds should die. This test should be carried out on the master seed but need not be done routinely on each vaccine batch produced. For release of subsequent batches, the titre of the virus should be a sufficient indication of vaccine potency.

Once frozen at –70°C or lower the vaccine stores well for at least 1 year with little loss in titre. Once issued, the vaccine should not be refrozen, it should be kept at 4°C and used as soon as possible.

2. Method of manufacture

The vaccine is produced in 8–11-day-old SPF embryonated chicken eggs inoculated on to the CAM and incubated at 37°C. Most embryo deaths occur between 48 and 96 hours after inoculation. The embryos, their CAMs and chorioallantoic fluids are harvested, pooled and homogenised in buffered saline and clarified by low-speed centrifugation (1800 $g$). The preparation is diluted as appropriate, and a stabiliser is incorporated. It is then dispensed into vials and preferably frozen rapidly to –70°C or lower.

3. In-process control

Eggs that have been inoculated should be candled 24 hours later to identify any embryos that have died from nonspecific causes.

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

A group of 1-day-old ducklings susceptible to DVE should be inoculated subcutaneously or intramuscularly with the vaccine at 10 times the recommended dose, and observed for 7–14 days for any signs of adverse reactions.

The final product should be free from contamination by bacteria, fungi, and mycoplasma as well as extraneous viruses potentially pathogenic to poultry.

c) Potency

The virus titre of the vaccine should be determined in 9–11-day-old embryonated chicken eggs inoculated on to the CAM and incubated at 37°C. The vaccine should contain a minimum of $10^{3.0} \text{ ELD}_{50}$ per dose at time of use.

d) Duration of immunity

Immunity in vaccinated ducks should last throughout a breeding season. Annual re-vaccination is recommended (19).

e) Stability

When stored at –70°C or lower the vaccine is stable for at least 1 year. Potency testing should be repeated after this time on an aliquot of vaccine to determine whether virus titre has been maintained. Once thawed the vaccine should not be refrozen, it should be maintained at 4°C in a refrigerator but for no longer than 1 week. Lyophilised vaccine should be stored at 4–8°C and used before the stated expiry date.

f) Preservatives

No preservatives are added to the vaccine.
g) Precautions (hazards)

None.

5. Tests on final product

The vaccine is issued as a vial of frozen concentrated vaccine virus together with a bottle of sterile diluent (phosphate buffered saline), on which standard sterility checks have been made (see Section C.4.a.).

a) Safety

No additional testing is performed after the batch testing.

b) Potency

No additional testing is performed after the batch testing.

REFERENCES


* * *
Fowl cholera (avian pasteurellosis) is a commonly occurring avian disease that can affect all types of birds and is distributed world-wide. Fowl cholera outbreaks often manifest as acute fatal septicaemia. Diagnosis depends on isolation and identification of the causative bacterium, Pasteurella multocida. Presumptive diagnosis may be based on the occurrence of typical signs and lesions and/or on the microscopic demonstration of myriad bacteria in blood smears, or impression smears of tissues such as liver or spleen. Mild or chronic forms of the disease also occur where the disease is endemic, with localised infection primarily of the respiratory and skeletal systems.

**Identification of the agent:** Pasteurella multocida is readily isolated, often in pure culture, from visceral organs such as lung, liver and spleen, bone marrow, gonads or heart blood of birds that succumb to the acute bacteraemic form of the disease, or from the caseous exudate characteristic of chronic fowl cholera lesions. It is a facultative anaerobic bacterium that grows best at 37°C. Primary isolation is usually accomplished using media such as dextrose starch agar, blood agar, and trypticase–soy agar. Isolation may be improved by the addition of 5% heat-inactivated serum. Colonies range from 1 to 3 mm in diameter after 18–24 hours of incubation and are discrete, circular, convex, translucent, and butyrous. The cells are cocco- to bacillary or short rod-shaped, 0.2–0.4 × 0.6–2.5 µm in size, stain Gram negative, and generally occur singly or in pairs. Bipolar staining is evident with Wright or Giemsa stains.

Identification of *P. multocida* is based on the results of biochemical tests, which include carbohydrate fermentation, enzyme production, and selected metabolite production.

Serological characterisation of strains of *P. multocida* includes capsular serogrouping and somatic serotyping. DNA fingerprinting can differentiate among *P. multocida* having the same capsular serogroup and somatic serotype. These characterisations require a specialised laboratory with appropriate diagnostic reagents.

**Serological tests:** Serological tests are rarely used for diagnosis of fowl cholera. The ease of obtaining a definitive diagnosis through isolation and identification of the causative organism generally precludes the need for serodiagnosis.

**Requirements for vaccines and diagnostic biologicals:** The *P. multocida* vaccines in general use are bacterins, containing aluminium hydroxide or oil as adjuvant, prepared from multiple serotypes. Two doses of the killed vaccine are typically required. Live culture vaccines tend to impart greater protective immunity, but are used less frequently because of potential post-vaccinal sequelae such as pneumonitis and arthritis. Multivalent vaccines typically incorporate somatic serotypes 1, 3, and 4 as they among the more commonly isolated avian serotypes. Safety and potency testing of bacterins usually use the host animal. Final containers of live cultures are tested for potency by bacterial counts.

**A. INTRODUCTION**

Fowl cholera is a contagious bacterial disease of domesticated and wild avian species caused by infection with *Pasteurella multocida*. It typically occurs as a fulminating disease with massive bacteraemia and high morbidity and mortality. Chronic infections also occur with clinical signs and lesions related to localised infections. The
pulmonary system and tissues associated with the musculoskeletal system are often the seats of chronic infection. Common synonyms for fowl cholera are avian pasteurellosis and avian haemorrhagic septicaemia. Fowl cholera is not considered to have zoonotic potential as avian isolates are generally nonpathogenic in infection. Common synonyms for fowl cholera are avian pasteurellosis and avian haemorrhagic septicaemia.

Infections often involve joints, foot pads, tendon sheaths, sternal bursa, conjunctivae, wattles, pharynx, lungs, air sacs, middle ears, bone marrow, and meninges. Lesions resulting from these infections are usually characterised by bacterial colonisation with necrosis, fibrinosuppurative exudate, and degrees of fibroplasia. All avian species are susceptible to \( P. \) multocida, although turkeys may be the most severely affected. Often the first sign of disease is dead birds. Other signs include: fever, anorexia, depression, mucus discharge from the mouth, diarrhoea, ruffled feathers, drop in egg production coupled with smaller eggs, increased respiratory rate, and cyanosis at the time of death. Lesions that are often observed include: congested organs with serosal haemorrhages, enlarged liver and spleen, multiple small necrotic areas in the liver and/or spleen, pneumonia, and mild ascites and pericardial oedema. Birds that survive the acute septicaemic stage or those infected with organisms of low virulence may develop chronic fowl cholera, characterised by localised infections. These infections often involve joints, foot pads, tendon sheaths, sternal bursa, conjunctivae, wattles, pharynx, lungs, air sacs, middle ears, bone marrow, and meninges. Lesions resulting from these infections are usually characterised by bacterial colonisation with necrosis, fibrinosuppurative exudate, and degrees of fibroplasia.

B. DIAGNOSTIC TECHNIQUES

Fowl cholera (avian pasteurellosis) is a commonly occurring avian disease that can affect all types of birds and is often fatal (3, 7). In the peracute form, fowl cholera is one of the most virulent and infectious diseases of poultry. Diagnosis depends on identification of the causative bacterium, \( P. \) multocida, following isolation from birds with signs and lesions consistent with this disease. Presumptive diagnosis may be based on the observance of typical signs and lesions and/or on the microscopic demonstration of bacteria showing bipolar staining in smears of tissues, such as blood, liver, or spleen. Mild forms of the disease may occur.

Diagnosis depends on isolation and identification of the causative organism.

1. Identification of the agent

\( Pasteurella \) multocida is a facultative anaerobic bacterium that grows best at 35–37°C. Primary isolation is usually accomplished using media such as blood agar, trypticase–soy agar or dextrose starch agar, and isolation may be improved by supplementing these media with 5% heat-inactivated serum. Maintenance media usually do not require supplemental serum. Colonies range from 1 to 3 mm in diameter after 18–24 hours of incubation. They usually are discrete, circular, convex, translucent, and butyraseous. Capsulated organisms usually produce larger colonies than those of noncapsulated organisms. Watery mucoid colonies, often observed with mammalian respiratory tract isolates, are very rare with avian isolates. The cells are cocccobacillary or short rod-shaped, usually 0.2–0.4 by 0.6–2.5 \( \mu \)m in size, stain Gram negative, and generally occur singly or in pairs. Recently isolated organisms or those found in tissue smears show bipolar staining with Wright or Giemsa stains or methylene blue, and are usually encapsulated.

Isolation of the organism from visceral organs, such as liver, bone marrow, spleen, or heart blood of birds that succumb to the acute form of the disease, and from exudative lesions of birds with the chronic form of the disease, is generally easily accomplished. Isolation from those chronically affected birds that have no evidence of disease other than emaciation and lethargy is often difficult. In this condition or when host decomposition has occurred, bone marrow is the tissue of choice for isolation attempts. The surface of the tissue to be cultured is seared with a hot spatula and a specimen is obtained by inserting a sterile cotton swab, wire or plastic loop through the heat-sterilised surface. The specimen is inoculated directly on to agar medium or into tryptose or another broth medium, incubated for a few hours, transferred to agar medium, and incubated again.

Identification is based primarily on the results of biochemical tests. Carbohydrate fermentation reactions are essential. Those carbohydrates that are fermented include: glucose, mannose, galactose, fructose, and sucrose. Those not fermented include: rhamnose, cellobiose, raffinose, inulin, erythritol, adonitol, m-inositol, and salicin. Mannitol is usually fermented. Arabinose, maltose, lactose, and dextrin are usually not fermented. Variable reactions occur with xylose, trehalose, glycerol, and sorbitol. \( Pasteurella \) multocida does not cause haemolysis, is not motile and rarely grows on MacConkey agar. It produces catalase, oxidase, and ornithine decarboxylase, but does not produce urease, lysine decarboxylase, beta-galactosidase, or arginine dihydrolase. Phosphatase production is variable. Nitrate is reduced; indole and hydrogen sulphide are produced, and methyl red and Voges–Proskauer tests are negative. Detection of hydrogen sulphide production may require lead acetate-laden paper strips suspended above a modified \( H_2S \) liquid medium (8). Commercial biochemical test kits are available.
Differentiation of *P. multocida* from other avian *Pasteurella* spp. and *Riemerella (Pasteurella)* anatipestifer can usually be accomplished using the tests and results indicated in Table 1. Laboratory experience has shown that *P. multocida* is most easily identified by its colony morphology and appearance in Gram stains. Positive reactions to indole and ornithine decarboxylase are the most useful biochemical indications.

**Table 1. Tests used to differentiate Pasteurella multocida from other avian Pasteurella species and Riemerella anatipestifer**

<table>
<thead>
<tr>
<th>Test*</th>
<th>Pasteurella multocida</th>
<th>Pasteurella haemolytica</th>
<th>Pasteurella gallinarum</th>
<th>Riemerella anatipestifer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemolysis on blood agar</td>
<td>–*</td>
<td>+</td>
<td>–</td>
<td>v</td>
</tr>
<tr>
<td>Growth on MacConkey’s agar</td>
<td>–</td>
<td>+u</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Indole production</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Gelatin liquefaction</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+u</td>
</tr>
<tr>
<td>Catalase production</td>
<td>+</td>
<td>+u</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urease production</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>v</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Lactose fermentation</td>
<td>–u</td>
<td>+u</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sacrose fermentation</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Maltose fermentation</td>
<td>–u</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Test reaction results: – = no reaction; + = reaction; v = variable reactions; –u = usually no reaction; +u usually a reaction.*

Antigenic characterisation of *P. multocida* is accomplished by capsular serogrouping and somatic serotyping. Capsular serogroups are determined by a passive haemagglutination test (1, 2). Serogroups A, B, D, E, and F have been reported; all but serogroup E have been isolated from avian hosts. A nonserological disk diffusion test that uses specific mucopolysaccharidases to differentiate serogroups A, D, and F has been developed (6).

Somatic serotypes are usually determined by an agar gel immunodiffusion (AGID) test (4, 5). Serotypes 1 through 16 have been reported; all 16 serotypes have been isolated from avian hosts (8). The most effective characterisation involves determination of both serogroup and serotype. These determinations require a specialised laboratory with appropriate diagnostic reagents. To determine the serotype, the laboratory prepares the unknown bacterial culture as antigen for the AGID test and then must test it against all 16 serotype-specific antisera. Antigens present in a single isolate may react with multiple serotype-specific antisera resulting in bi- or trinomial serotypes, as illustrated by the 3, 4 and 3, 4, 12 strains (8).

DNA fingerprinting of *P. multocida* by restriction endonuclease analysis (REA) has proved valuable in epidemiological investigations of fowl cholera in poultry flocks. Isolates of *P. multocida* having both capsular serogroup and somatic serotype in common may be distinguished by REA. Ethidium-bromide-stained agarose gels are analysed following electrophoresis of DNA digested with either HhaI or HpaII endonuclease (10).

### 2. Serological tests

Serological tests for the presence of specific antibodies are not used for diagnosis of fowl cholera. The ease of obtaining a definitive diagnosis by isolation and identification of the causative organism precludes the need for serodiagnosis. Serological tests, such as agglutination, AGID, and passive haemagglutination, have been used experimentally to demonstrate antibody against *P. multocida* in serum from avian hosts; none were highly sensitive. Determinations of antibody titres using enzyme-linked immunosorbent assays have been used with varying degrees of success in attempts to monitor seroconversion in vaccinated poultry, but not for diagnosis.

### C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Fowl cholera may be caused by any of 16 Heddleston serotypes of *P. multocida*, although certain serotypes appear to be more often associated with disease. The *P. multocida* vaccines in general use are bacterins, containing aluminium hydroxide or oil adjuvant, prepared from inactivated cells of serotypes selected on the basis of epidemiological information. Commercial bacterins are usually composed of serotypes 1, 3, and 4.
Vaccination plays a significant role in the control of this disease. Live vaccines containing modified \textit{P. multocida} are not generally used except in North America.

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.

Bacterin is normally administered by intramuscular injection in the leg or breast muscles, or subcutaneously at the back of the neck. Two doses are typically administered at 2–4-week intervals. As with most killed vaccines, full immunity cannot be expected until approximately 2 weeks after the second dose of a primary vaccination course. Live vaccines are typically administered in the drinking water. Vaccination of diseased birds or those in poor nutritional status should be avoided as a satisfactory immune response may not be generated in such circumstances.

1. **Method of manufacture**

The general method for production of \textit{P. multocida} bacterins are presented here. Production cultures of each bacterial isolate to be included in the final product are prepared. The cultures are typically started in small vessels and subpassaged into progressively larger volumes of media until the desired production volume is achieved. Each production culture is inactivated by formalin or other acceptable means. All of the component cultures are mixed, and usually blended, with an adjuvant prior to filling sterile final containers.

The following section is based on the requirements for \textit{P. multocida} bacterins and vaccines as found in Title 9, United States Code of Federal Regulations. Other countries may have slightly different requirements.

2. **Master seed management**

a) **Characteristics of the seed**

All strains of \textit{P. multocida} to be incorporated into a bacterin or vaccine must be well characterised, of known serotype, pure, safe and immunogenic. The culture(s) that is evaluated and characterised is designated by lot number and called a master seed. All cultures used in the production of licensed bacterins or vaccines must be derived from an approved master seed(s) and must be within an accepted number of passages from the master seed lot.

b) **Validation as a vaccine**

i) **Efficacy**

Products prepared from candidate master seeds must be shown to be effective against challenge infection. Efficacy must be demonstrated in each animal species (chickens, turkeys, ducks, psittacines) and by each route of administration for which the product will be recommended, and protection must be demonstrated against each challenge serotype for which protection is claimed. The lot of product used to demonstrate efficacy must be produced from the highest allowable passage of master seed.

For live avian \textit{Pasteurella} vaccines, 20 vaccinates and 10 controls are used in each efficacy trial. Birds are challenged not less than 14 days after vaccination and are observed for 10 days after challenge. A satisfactory test requires that at least eight of the controls die and at least 16 of the vaccinates survive.

The arithmetic mean count of colony-forming units in the lot of product that is used to demonstrate efficacy is used as the minimum standard (immunogenicity standard) for all subsequent production lots of vaccine.

Efficacy of bacterins must be demonstrated similarly prior to licensure. However, no immunogenicity standards are derived from the lot that was used to demonstrate initial efficacy; each production lot is satisfactorily tested in a vaccination-challenge trial prior to release for sale and distribution.

ii) **Safety**

The safety of master seeds used in the production of live vaccines must be evaluated prior to licensing. Safety must be tested in each animal species (chickens, turkeys, ducks, psittacines) for which the product is recommended. Each of 10 birds is given an equivalent of 10 vaccine doses and observed for 10 days. At least 8 of 10 birds must show no unfavourable reactions attributable to the master seed. Additionally, the master seeds must be tested for reversion to virulence and evaluated for excretion from the host and transmission to other target species.

The safety of each production lot is tested by methods described in Section C.4.c.
Chapter 2.7.11. – Fowl cholera (avian pasteurellosis)

3. In-process control

The purity of the cultures is determined at each stage of production prior to inactivation. This may be achieved by microscopic examination (e.g. phase–contrast microscopy, Gram stain) and/or by culture. Killed cultures are tested for completeness of inactivation. Analytical assays to determine the levels of formaldehyde or other preservatives are done on bulk vaccine and must be within specified limits. During manufacturing, production parameters must be tightly controlled to ensure that all serials (batches) are produced in the same manner as that used to produce the serials used in immunogenicity studies.

4. Batch control

a) Sterility

Sterility tests are done on filled vaccine. Each lot must pass sterility requirements, for example those detailed in the 9 CFR Part 113.26 or 113.27 (9). (See also Chapter I.1.5.)

b) Safety

Safety testing is conducted on each bulk or filled vaccine lot. Live vaccines are tested according to the method described in C.1.c.ii, except that only one representative animal species is required. Bacterins are administered according to label recommendations, and the birds are observed for 14 days; at least 18 of 20 birds must show no unfavourable reactions attributable to the bacterin.

c) Potency

Each production lot of bacterin or live vaccine must be tested for potency by a test that is related to, and considered predictive of, efficacy. Potency tests are performed on the product in its final form.

Bacterins are tested for potency in a vaccination-challenge trial. Separate groups of birds (20 vaccinates, 10 controls) must be challenged with each of the serotypes of \textit{P. multocida} for which protection is claimed. Bacterins are administered according to the dose and route recommended on the label. Two doses are administered 3 weeks apart, and all birds are challenged 2 weeks after the second dose. The birds are observed for 14 days after challenge. For a satisfactory test, at least 14 of 20 vaccinates must survive and at least 8 of 10 controls must die.

The potency of live vaccine lots is determined by a bacterial count performed on reconstituted lyophilised product in its final container. The mean bacterial count of any vaccine lot at the time of preparation must be sufficiently high to ensure that at any time prior to product expiration, the count is at least twice the immunogenicity standard. (The European Pharmacopoeia requires a count that is at least equal to the immunogenicity standard).

d) Stability

The acceptability of the shelf life of a vaccine is confirmed by testing the product for potency at the end of the approved shelf life. At least three lots of vaccine are tested and must pass established potency requirements. Vaccines are stored at 2–7°C and protected from freezing. Partly used packs should be discarded at the end of a day's operations.

e) Preservatives

Any preservatives must be added within specified limits. Preservatives are generally added to vaccines to limit the growth of any contaminants introduced when the rubber cap is pierced with a needle. Ideally, multidose vaccination equipment should be used whereby the vaccine pack is entered only once with a sterile needle.

f) Precautions (hazards)

Vaccines prepared with aluminium-based adjuvants may cause temporary nodules at the site of injection. Operator self-injection poses no immediate problems, but medical advice should be sought as there is a risk of infection via a contaminated needle.

Vaccines prepared with oil based adjuvants may cause more severe reactions at the site of injection, which may manifest as large nodules. Care should be taken to administer these vaccines correctly. Operator self injection requires immediate medical attention, involving prompt incision and irrigation of the site.
5. Tests on final product
   
a) Safety
   See Section C.4.b.

b) Potency
   See Section C.4.c.

REFERENCES


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* *
CHAPTER 2.7.12.

FOWLPOX

SUMMARY

Fowlpox is a disease of chickens and turkeys caused by a DNA virus of the genus Avipoxvirus of the family Poxviridae. Its distribution is world-wide. It is slow-spreading and characterised by the formation of proliferative lesions and scabs on the skin, and diphtheritic lesions in the upper parts of the digestive and respiratory tracts. In the case of the cutaneous form, the mortality rate is usually low and affected birds are more likely to recover than those with the diphtheritic form. In the diphtheritic form, proliferative lesions involving the nasal passages, larynx or trachea can result in respiratory distress and death from suffocation.

Fowlpox causes a transient drop in egg production and a reduced growth rate in young birds.

Identification of the agent: Fowlpox should be suspected where skin eruptions occur on exposed areas. Histological examination of cutaneous or diphtheritic lesions reveals epithelial hyperplasia with intracytoplasmic inclusions in affected cells. Elementary bodies may be detected in smears from lesions by the use of the Gimenez method. Electron microscopy of lesions will detect virus particles with the characteristic poxvirus morphology by negative staining or in ultrathin sections of the lesion.

The diphtheritic form of fowlpox involving the trachea must be differentiated from infectious laryngotracheitis, which is caused by a herpesvirus and is characterised by the presence of intranuclear inclusion bodies.

Virus isolation is done by inoculation on to chorioallantoic membranes of 9–12-day-old developing chicken embryos or avian cell cultures.

Serological tests: Immune responses to fowlpox virus may be demonstrated by the use of virus neutralisation, agar gel immunodiffusion, immunofluorescence, or passive hemagglutination tests, enzyme-linked immunosorbent assay and by immunoblotting.

Requirements for vaccines and diagnostic biologicals: Modified live fowlpox or pigeon pox virus vaccines of chicken embryo or avian cell culture origin are available commercially. The use of vaccines is indicated in areas where the disease is endemic, or on premises where infection has been diagnosed.

A. INTRODUCTION

Fowlpox has a world-wide distribution and is caused by a DNA virus of the genus Avipoxvirus of the family Poxviridae (14, 18). Its incidence is variable in different areas because of differences in climate, management and hygiene or the practice of regular vaccination. It can cause drops in egg production, or retarded growth in younger birds.

Fowlpox is a slow-spreading virus disease of chickens and turkeys, characterised in the cutaneous form (dry pox) by the development of proliferative lesions, ranging from small nodules to spherical wart-like masses on the skin of the comb, wattle and other unfeathered areas. In the diphtheritic form (wet pox), slightly elevated white opaque nodules develop on the mucous membranes. They rapidly increase in size to become a yellowish diphtheritic membrane. Lesions occur on the mucous membranes of the mouth, oesophagus, larynx or trachea. The mortality rate is higher in the diphtheritic form than in the cutaneous form, sometimes nearing 50% (19), particularly in young birds. Integration of reticuloendotheliosis virus (REV) sequences has been observed in the genome of fowlpox virus (10). It is interesting that this insertion event occurred over 50 years ago (7). While most field strains contain REV provirus, vaccine strains have only remnants of long terminal repeats (13). Virulence is enhanced by
the presence of REV provirus in the genome of field strains of fowlpox virus. Complete sequence of the genome of a vaccine-like strain of fowlpox virus has been determined (1).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Fowlpox virus multiplies in the cytoplasm of epithelial cells with the formation of large intracytoplasmic inclusion bodies (Bollinger bodies) that contain smaller elementary bodies (Borrel bodies). The inclusions can be demonstrated in sections of cutaneous and diphtheritic lesions by the use of haematoxylin and eosin (H&E), acridine orange or Giemsa stains (17). The elementary bodies can be detected in smears from lesions, for example by the Gimenez method (16), which is described below. Electron microscopy can be used to demonstrate viral particles of typical poxvirus morphology by negative staining or in ultrathin sections of infected tissues (3).

a) A smear technique for fowlpox

i) Place a drop of distilled water and the lesion (cutaneous or diphtheritic) on a clean slide. Prepare a thin smear by pressing the lesion with another clean slide and rotating the upper slide several times.

ii) Air dry and gently fix the smear over a flame.

iii) Stain the smear for 5–10 minutes with freshly prepared primary stain (8 ml stock solution of basic fuchsin mixed with 10 ml of phosphate buffer, pH 7.5, and filtered through Whatman filter paper No. 1).

iv) Wash thoroughly with tap water.

v) Counterstain with malachite green (0.8% in distilled water) for 30–60 seconds.

vi) Wash the smear with tap water and then dry.

vii) Examine the smear under oil immersion. The elementary bodies appear red and are approximately 0.2–0.3 µm in size.

b) Virus isolation

Fowlpox virus can be isolated by the inoculation of suspected material into embryonated chicken eggs. Approximately 0.1 ml of tissue suspension of skin or diphtheritic lesions, with the appropriate concentration of antibiotics, is inoculated onto the chorioallantoic membranes (CAMs) of 9–12-day-old developing chicken embryos. These are incubated at 37°C for 5–7 days, and then examined for focal white pock lesions or generalised thickening of the CAMs. Histopathological examination of the CAM lesions will reveal eosinophilic intracytoplasmic inclusion bodies following staining with H&E (17, 19).

Primary chicken embryo fibroblasts, chicken embryo kidney cells, chicken embryo dermis cells, or the permanent quail cell line QT-35, can also be used to propagate fowlpox virus (6, 10). The adaptation of virus strains to cell cultures is an important requirement for plaque formation, as not all strains will form plaques initially.

c) Molecular methods

Restriction endonuclease analysis is a useful method for comparing closely related DNA genomes and can be used for comparison of field isolates and vaccine strains of fowlpox virus (6, 10).

Cloned genomic fragments of fowlpox virus can be used effectively as nucleic acid probes for diagnosis of fowlpox. Viral DNA isolated from lesions can be detected by hybridisation either with radioactively or nonradioactively labelled genomic probes. This method is especially useful for differentiation of fowlpox from infectious laryngotracheitis when tracheal lesions are present (5).

1 Stock solution: A solution of basic fuchsin (5 g) in 95% ethanol (100 ml) is slowly added to a second solution of crystalline phenol (10 g) in distilled water (900 ml). This stock solution, kept in a tightly screw-capped glass bottle, is incubated for 48 hours at 37°C, and then stored at room temperature.

2 Phosphate buffer, pH 7.5: NaH₂PO₄·H₂O (2.47 g) and Na₂HPO₄ (11.65 g) are added to distilled water (1000 ml) and stored at 4°C.
Genomic DNA sequences of various sizes can be amplified by the polymerase chain reaction (PCR) using specific primers (8). Recently a nested PCR was described for the detection of fowlpox (4). This technique is useful when there is only an extremely small amount of viral DNA in the sample.

2. Serological tests

Although both cell-mediated immunity (CMI) and humoral immunity play an important role in poxvirus infections, routine use of the CMI test is not convenient. Therefore, serological tests, such as virus neutralisation (VN), agar gel immunodiffusion (AGID), passive haemagglutination and fluorescent antibody tests as well as the enzyme-linked immunosorbent assay (ELISA), are used to measure specific humoral antibody responses. Evidence of successful immunisation with vaccine can be determined by examining a flock 7–10 days after vaccination for ‘takes’. A take consists of a swelling of the skin or a scab at the site where the vaccine was applied and its presence is evidence of successful immunisation.

a) Virus neutralisation

After virus/serum interaction, the residual virus activity may be assayed in embryonating chicken eggs or in cell cultures (9). This technically demanding test may not be convenient for routine diagnosis. Only some selected strains of the virus have plaque-forming ability in chicken embryo cells. Neutralising antibodies develop within 1–2 weeks of infection.

b) Agar gel immunodiffusion

Precipitating antibodies can be detected by reacting test sera against viral antigens. The antigen can be derived by sonication and homogenisation of infected skin or CAM lesions as well as by treatment of infected cell cultures as described in Section B.2.f. below. The lysed suspension is centrifuged and the supernatant is used as antigen. Gel-diffusion medium is prepared with 1% agar, 8% sodium chloride and 0.01% thiomersol. The viral antigen is placed in the central well and the test sera are placed in the peripheral wells. It is important to include a positive and negative control serum. The plates are incubated at room temperature. Precipitation lines develop in 24–48 hours after incubation of the antigen with antibody to homologous or closely related strains. The test is less sensitive than the ELISA (2) or the passive haemagglutination test (18).

c) Passive haemagglutination

Tanned sheep or horse red blood cells are sensitised with a partially purified fowlpox viral antigen (18). The antigen is prepared from infected CAMs or cells as described in Section B.2.f. below. Passive haemagglutination is more sensitive than AGID. The test will give cross-reactions among avian pox viruses.

d) Fluorescent antibody tests

Direct or indirect immunofluorescence tests will reveal specific intracytoplasmic fluorescence in infected cells. The latter test is commonly used and involves two steps: the antibody against fowlpox virus is reacted with the antigen in the infected cells, followed by a secondary fluorescein-isothiocyanate-labelled antibody against chicken gamma globulin (e.g. goat anti-chicken). Such labelled antibodies are available commercially. In this regard, formalin-fixed tissue sections can be used effectively for fluorescent antibody tests.

e) Immunoperoxidase

Specific staining of cytoplasmic inclusions is achieved when horseradish-peroxidase-conjugated specific polyclonal antibody against fowlpox virus is reacted with the hydrated sections of fowlpox-infected fixed tissues (CAM and skin) or cell culture. Similar results are obtained when either polyclonal or monoclonal antibodies are used in an indirect test. An advantage of the technique is that the sections can be examined with the light microscope and can be stored for an extended period without loss of colour (17).

f) Enzyme-linked immunosorbent assay

ELISAs have been developed to detect humoral antibodies to fowlpox virus. They are capable of detecting antibody 7–10 days after infection (2), but commercial kits for this test are not available.

Fowlpox virus antigens are prepared either from infected QT-35 cell monolayers or CAM lesions. Infected QT cells are pelleted (700 g for 10 minutes at 4°C), washed with isotonic buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 5 mM ethylene diamine tetra-acetic acid [EDTA]) followed by lysis in hypotonic buffer (10 mM Tris, pH 8.0, 10 mM KCl, 5 mM EDTA) containing 0.1% Triton X-100 and 0.025% beta-mercaptoethanol. Nuclei and cellular debris are removed by low-speed centrifugation (900 g for 5 minutes at 4°C) and the resulting supernatant is used as a source of fowlpox virus antigens for ELISA or immunoblotting. To isolate
viral antigen from CAM lesions, initial grinding of the lesions with subsequent detergent treatment as described earlier would be required. Virus propagated in chicken embryo fibroblasts and chicken embryo dermis cells has also been used for antigen. The antigen preparation is as described for QT cells.

Wells of microtitre plates are coated with 1 µg of soluble fowlpox virus antigen in 100 µl of coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) and incubated overnight at 4°C (2, 19). Each well is then rinsed once with wash solution (0.29 M NaCl, 0.05% Tween 20) and then blocked with phosphate buffered saline (PBS, pH 7.4) containing 3% bovine serum albumin (BSA) for 1 hour at 37°C. After one wash, serial dilutions of the test sera in PBS containing 1% BSA are added to the wells. After rocking for 2 hours at 37°C, the wells are washed three times prior to the addition of 100 µl/well horseradish-peroxidase-conjugated goat anti-chicken IgG (H+L) antibodies\(^3\) at a recommended dilution in PBS. After 2 hours' incubation at 37°C and three subsequent washes, 100 µl of the peroxidase substrate TMB\(^3\) is added to each well. Reactions are terminated by the addition of 1 M phosphoric acid and absorbance at 450 nm is recorded using an ELISA plate reader\(^4\).

\( g) \) Immunoblotting

Antigenic variations that occur between strains of fowlpox virus can be evaluated by means of immunoblotting. In this method, viral antigens separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) are reacted either with polyclonal or monoclonal antibodies against fowlpox virus (6, 12, 14).

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

Early studies indicated the feasibility of protecting chickens from fowlpox by the use of pigeon pox or fowlpox viruses (20). Vaccination is indicated in areas where fowlpox is endemic or on premises where infection has been diagnosed previously. Live fowl and pigeon pox virus vaccines, and also fowlpox vectored vaccines that protect against pox, are available commercially. These vaccines are derived from chicken embryos or avian cell cultures.

Guidelines for the production of veterinary vaccines are given in Chapter 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.

Passively acquired immunity should be taken into consideration during vaccination of progeny from flocks that have either had a recent natural infection or been recently vaccinated. As passive immunity (for 2–3 weeks) may interfere with vaccine virus multiplication, such progeny should be vaccinated only after the decline of passively acquired antibody. Fowlpox vaccine is applied by a wing web stab method.

1. **Seed management**

a) **Characteristics of the seed**

A master seed virus (MSV) must be established and used according to a seed-lot system. A record must be kept of its origin, passage history and characteristics. Viruses used may be either fowlpox or pigeon pox viruses. The MSV must be propagated in suitable premises with materials that meet approved standards, and must be tested for freedom from contamination as well as for identity and purity.

b) **Method of culture**

The MSV may be propagated in specific pathogen free (SPF) chicken embryos, using the CAMs, or in avian cell cultures, such as primary chicken embryo fibroblasts, chicken embryo kidney or chicken embryo dermis.

c) **Validation as a vaccine**

i) **Purity**

The MSV may be neutralised with a specific hyperimmune serum before testing for purity. Because of difficulty in neutralising avian pox virus, it is acceptable to treat the MSV by centrifugation at 1000 \( \text{g} \) for 20 minutes, followed by filtration through a 0.2 µm filter. The neutralised or filtered MSV is then used in

\(^3\) Kirkegaard and Perry Laboratories, Gaithersberg, Maryland, United States of America.
\(^4\) Dynatech, Chantilly, Virginia, United States of America.
tests to demonstrate freedom from extraneous agents. These tests should be done in embryonating eggs or avian cell cultures, to demonstrate absence of extraneous virus replication, and in SPF chickens, to demonstrate freedom from antibodies to extraneous agents.

ii) *Safety*

Vaccines should be prepared only from virus that is a stable attenuated strain or a naturally occurring isolate of low virulence.

The vaccine must be shown to be safe by the recommended route of administration, which is wing web stab, in all ages of susceptible birds. A suitable test is to take ten SPF chickens and inoculate each by piercing the wing web with a needle dipped in the vaccine. The birds are observed for 7–10 days for evidence of ‘takes’ and for the absence of adverse effects attributable to the vaccine. A ‘take’ consists of swelling of the skin or a scab at the site where the vaccine was applied and is evidence of successful vaccination. The safety test should be repeated after at least six serial passages of the virus in SPF chickens to show that there has been no reversion to virulence.

iii) *Efficacy*

Data should be obtained using the highest passage level and the lowest titre of virus to be used in the final product: 20 SPF chickens of the minimum age indicated for vaccination should receive one dose of vaccine by the recommended method. The chickens, together with 20 unvaccinated chickens of the same age and source, should be challenged 3 weeks later by scarification with a virulent strain of fowlpox virus. The birds should be observed for 3 weeks. Ninety per cent of the control birds should develop lesions due to the challenge virus and at least 90% of the vaccinated birds should remain free from such lesions.

2. **Method of manufacture**

Vaccine is manufactured on a seed-lot system from the validated MSV. This must be done in approved premises designed to avoid the risk of contamination. All media and cell cultures must be tested to ensure freedom from contamination.

3. **In-process control**

During the process of validation as a vaccine, the efficacy data must be compared to the virus content of the vaccine. A suitable potency can thus be established. The vaccine should be filled into final containers to ensure that each container has sufficient virus to achieve the specified potency.

4. **Batch control**

a) *Sterility*

Tests for sterility and freedom from contamination of biological materials may be found in the Chapter I.1.5.

b) *Safety*

The safety test described in Section C.1.c.ii above, except the requirement for six passages in SPF chickens, should be used on each batch of vaccine.

c) *Potency*

Virus content tests should be carried out using each of at least three containers. The dilutions should span 0–100% infection range, using five-fold dilution steps and at least seven replicates per dilution. Tests should be done in parallel with a standard vaccine, if available. Each lot of vaccine should be titrated in the diluent provided for its use. The virus titre should not normally be higher than 1/10 of the dose at which the vaccine has been shown to be safe and must not be lower than the release titre determined in the test for efficacy. A suitable potency for an attenuated live fowlpox vaccine is likely to be in the region of $10^5$ EID$_{50}$ (50% embryo infective dose) per ml.

d) *Duration of immunity*

The efficacy test given in Section C.1.c.iii may be used to determine the duration of immunity (approximately 6–12 months) by testing at intervals after vaccination, using separate groups of birds for each test.
e) **Stability**

Evidence of stability must be presented to justify the shelf life. This should be based on virus titrations carried out at intervals until 3 months beyond the requested shelf life on at least six batches of vaccine kept under recommended storage conditions.

f) **Preservatives**

Preservatives are not used in live vaccines.

g) **Precautions (hazards)**

It is usually recommended not to vaccinate birds that are in lay. Avoid human contact with the live vaccine. Standard fowlpox vaccine is not to be used in pigeons, though they can be vaccinated with pigeon pox vaccine. In many countries, pigeon pox vaccine has been superseded by attenuated live fowlpox vaccine designed for use in day-old chicks. These products have been safely used in pigeons in the absence of an available pigeon pox vaccine.

5. **Tests on the final product**

a) **Safety**

The safety test described in Section C.1.c.ii above is used on each batch.

b) **Potency**

The potency test described in Section C.4.c. above is used on each batch.

**REFERENCES**


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SECTION 2.8.
LAGOMORPH DISEASES IN LIST B

CHAPTER 2.8.1.
MYXOMATOSIS

SUMMARY
Myxomatosis is a disease of the European rabbit caused by the myxoma virus, a member of the Poxviridae. The diagnosis of myxomatosis, regardless of its clinical form, depends on the isolation and identification of the virus or the demonstration of its antigens. The presence of a humoral immune response facilitates a retrospective diagnosis of a mild form of the disease, and can provide an indication of the prevalence of infection in a rabbit population. The disease is characterised by gross myxomatous skin lesions.

Identification of the agent: When skin lesions are present on a dead rabbit, the viral antigen may be demonstrated by immunodiffusion tests on lesion fragments. Monolayer cell cultures of rabbit kidney inoculated with lesion material will show the characteristic cytopathic effects of poxviruses. The presence of virus can be confirmed by immunofluorescence and negative-staining electron microscopy.

The inoculation of rabbits with suspect material takes longer to identify infection, but this will serve to confirm the presence of infective virus and indicate its pathogenicity.

Serological tests: Identification and titration of specific antibodies arising from natural infection or from immunisation are done by traditional complement fixation or by a recently developed and more sensitive enzyme-linked immunosorbent assay (ELISA), which is not affected by pro- or anti-complementary factors. The difficulty in obtaining blood samples from representative members of a population can be obviated by collecting blood dried on filter paper; this can later be extracted and examined by the indirect fluorescent antibody test or ELISA. Microcapillary blood sampling can also be used for the ELISA.

Qualitative agar gel immunodiffusion tests have the advantage of detecting both antigen and humoral antibodies.

Requirements for vaccines and diagnostic biologicals: Modified live virus vaccines prepared from fibroma virus or modified myxoma virus strains are available for immunisation of rabbits.

A. INTRODUCTION
Myxomatosis is a virus disease of the European rabbit (Oryctolagus cuniculus) caused by the myxoma virus, a member of the Poxviridae. The virus has a characteristic tropism for skin, causing a nodular skin form, or has an oculo-respiratory tract tropism, the nonmyxomatous form. It affects rabbits of all ages, and strains, which vary in virulence, give rise to inflammatory skin lesions, generalisation and, possibly, eventual death of the animal by secondary bacterial infections or through inanition. The virus is transmitted to susceptible rabbits by biting insects, such as fleas or mosquitoes. Limited transmission from rabbit to rabbit is possible if they are closely confined.

A primary myxoma lesion arises at the site of infection after 2–5 days, followed by conjunctivitis (which is a sign of systemic infection), inflammation of the anogenital region, and the formation of secondary skin lesions at
various other sites. In the oculo-respiratory form, the primary lesion is usually an inflammatory pink macule followed by oculo-nasal catarrh. Virulent strains can kill a rabbit within 10–15 days. There is no health risk to humans working with the myxoma virus.

B. DIAGNOSTIC TECHNIQUES

As the signs of the disease become less distinct with the attenuation of virus strains, the submission of samples for laboratory diagnosis becomes more important. The different techniques available vary in their ability to detect myxoma virus in typical myxomatous lesions, oedema of the eyelids or genital oedema. With atypical lesions, these techniques distinguish myxoma virus infection from that due to Shope’s fibroma virus.

1. Identification of the agent

A portion of lesion is excised with scissors and separated from the epidermis and superficial dermis. This is washed with phosphate buffered saline (PBS) with antibiotics as defined below and homogenised with ground glass at a dilution rate of 1 g tissue/4.5–9.0 ml of PBS. Cells are disrupted by two freeze–thaw cycles, or by ultrasonication to liberate virions and viral antigens. This suspension is centrifuged for 5–10 minutes at 1500 g. The supernatant fluid is used for the tests.

a) Culture

Isolation of the virus in cell culture is accomplished using primary cultures of rabbit kidney (RK) cells, or with established cell lines, such as RK-13, in minimal essential medium (MEM) containing 5% calf serum, 100 international units (IU)/ml penicillin; 100 µg/ml streptomycin; 100 µg/ml gentamycin; 50 IU/ml nystatin (mycostatin); and 5 µg/ml amphotericin (fungizone). The inoculum consists of the supernatant fluid from a homogenised lesion or oculo-respiratory discharge in Opti-MEM with 2% calf serum and antibiotics. This is removed from the cell layer after 2 hours. The cell layer is washed in a small volume of medium and then replenished with maintenance medium (Opti-MEM).

A cytopathic effect (CPE) typical of poxviruses (10) usually develops after 24–48 hours (37°C and 5% CO₂), but with some strains, it may take up to 7 days for CPE to be observed. Groups of cells with a confluent cytoplasm form syncytia that vary in size from 2 to 50 or even 100 nuclei together, according to the strain of virus. The nuclei of some cells change, the chromatin forming basophilic aggregations that vary in size and number and give the culture a leopard-skin appearance. Eosinophilic intracytoplasmic inclusions remain discrete, if present at all. Affected cells round up, contract and become pyknotic. They then lyse and become detached from the glass or plastic support. Later, all cells are affected and the cell monolayer detaches completely.

Shope’s fibroma virus at first produces well-defined voluminous masses of rounded cells, which proliferate and pile up (10). At the edge, cells just becoming infected show discrete nuclear changes and acidophilic cytoplasmic inclusions that are numerous at an early stage. The cell layer is destroyed after several days.

b) Immunological methods

Agar gel immunodiffusion (AGID) tests are simple and rapid to perform – results can be obtained within 24 hours. Agar plates are prepared with Noble agar (0.6 g), ethylene diamine tetra-acetic acid (EDTA) (2.5 g), sodium chloride (4.5 g), and distilled water (500 ml) containing thiomersal (merthiolate) at 1/100,000 dilution. Standard antiserum (see below), and the test sample are placed in opposing wells that are 6 mm in diameter and 5 mm apart. Another technique is to deposit a small portion of the lesion directly into the agar, 5 mm away from a filter paper disk impregnated with the antiserum. A number of lines of precipitation, usually up to three, appear within 48 hours, indicating the presence of myxoma viral antigens. Only one line forms in the presence of heterologous reactions with Shope’s fibroma virus.

Indirect fluorescent antibody (IFA) tests can be applied to cultures from 24 hours onwards. IFA tests reveal intracytoplasmic multiplication of virus, without being able to distinguish myxoma virus from fibroma virus. The inoculation of chicken embryo cells (trypsinised at day 11 of egg incubation) does not result in CPE, but it is useful for detecting the viral antigens by IFA tests.

c) Electron microscopy

Negative-staining electron microscopy (EM) can be applied to a portion of skin lesion. The technique is simple and rapid to perform, giving results in 1 hour. About 1 mm³ of the tissue is laid in a watch glass and 3 drops of distilled water are added. After 1–2 minutes at room temperature an EM grid coated with formvar and carbon is laid over the liquid. After 1 minute any excess liquid is removed with filter paper and immediately a 2% aqueous solution of ammonium molybdate, pH 7.0, is dropped on to the grid. After 10 seconds the excess liquid is removed with filter paper and the grid is prepared for the electron
microscope. In a positive case, typical poxvirus particles can be seen. Myxoma virus cannot be distinguished from fibroma virus using this method.

d) Inoculation tests

Rabbit intradermal inoculation also offers a means of identifying the virus through its special characteristics and pathogenicity, namely cutaneous tropism (nodular form) and oculo-respiratory tropism (non-myxomatous form). It should be avoided if possible but has the advantage of being a gauge of virulence, from the type of inflammation in lesions (local or systemic infection) to the extent of lesions and survival time, and can distinguish fibroma virus (with its simple fibromatous local lesion) from myxoma virus (capable of causing generalised infection in adults). Rabbits should be of a domestic breed, weighing approximately 2 kg, unvaccinated and previously tested for the absence of antibodies (10).

The inoculum may be the supernatant fluid from a homogenised lesion (with antibiotics) or the product of a cell culture. Between 0.1 and 0.2 ml is administered intradermally behind the ear or into the dorso-lumbar region, which has previously been depilated. The inoculum may be assayed by injecting serial dilutions in saline buffer at one site for each dilution. A primary lesion will appear at the sites within 2–5 days, followed by conjunctivitis. Using five sites for each dilution allows a 50% infective dose (ID₅₀) to be obtained. If the animal survives, the disease can be confirmed serologically after 15 days.

2. Serological tests

Antibodies develop within 8–13 days. In the nonlethal forms and in vaccinated rabbits, the titre is highest after 20–60 days; it declines thereafter, disappearing after 6–8 months in the absence of reinfection (serological response evaluated by use of the complement fixation [CF] test) (12).

Various serological tests may be used, but CF, IFA and enzyme-linked immunosorbent assay (ELISA) (in order of increasing sensitivity) are the most appropriate tests for international trade and other applications. These tests require standard antigens and antisera. The antigen can be prepared from the Lausanne strain, or some antigenically related strain, propagated in rabbits or cell cultures.

• Preparation of standard reagents

Myxomatous lesions are removed from rabbits at 6–7 days after inoculation and homogenised in veronal buffer to a dilution of 1/5. The antigen is the supernatant fluid that is obtained following centrifugation. Any anticomplementary activity is abolished by adding 0.6% chloroform. The antigen fluid can be frozen at −30°C or −70°C for stock purposes or used directly in CF tests after titration against a standard antiserum.

Antigen is made from cell cultures using the RK-13 lines. The virus is harvested as a suspension of cells 48 hours after infection, and is centrifuged. The supernatant fluid is retained. The cell deposit is homogenised (gently) or ultrasonicated (for 3 minutes continuously or for 5 minutes, 50% pulse 100 watts) resuspended and recentrifuged, and the supernatant is added to the former fluid. The final supernatant fluid is the antigen, and is stored at −20°C or −70°C (for longer conservation). It is titrated in cell cultures before use in virus neutralisation (VN) tests.

For the standard antiserum, an adult serologically negative rabbit is vaccinated with an attenuated strain of myxoma virus, or with the Shope's fibroma virus. After 3–4 weeks, the rabbit is inoculated with myxoma lesion material derived from the Lausanne strain of virus. Serum is obtained 3 weeks later and titrated by the CF test. If the titre is 1/640 or more, the animal is bled out and the serum is stored at −20°C.

• Titration of standard reagents

i) Inactivate standard antiserum for 30 minutes in a water bath at 56°C. No international standard serum for myxomatosis is available, but internal positive standards should be prepared and titrated in the appropriate range, using the CF test. After this, the following procedure is used to standardise batches of antigen.

ii) Make doubling dilutions of standard serum in calcium/magnesium/veronal buffer (CMV), pH 7.2, from 1/2 to 1/4096, using a 96-well round-bottomed microtitre plate, one well per dilution and 25 µl volume in each line.

iii) Using tubes, make doubling dilutions of antigen in CMV, from 1/10 to 1/1280.

iv) Add 25 µl per well of the same dilution of antigen, (transferred from the tubes) to each dilution on one line of the plate, from 1/10 to 1/1280, vertically.

v) Add 25 µl (6 H₅₀ units [50% haemolysis]) per well of complement.

vi) Incubate the plate, covered with a plastic film, for 1 hour at 37°C or 14 hours at 4°C.
vii) Add 50 µl per well of the haemolytic system (2.5% sheep red blood cells [RBCs] and an equal volume of anti-sheep RBC serum 1/2000, both in CMV).

viii) Cover the plate again and incubate for 30 minutes at 37°C.

ix) Read the highest dilution of antigen giving complete haemolysis (H 100) with the highest dilution of standard serum. There is 1 antigenic unit (AgU) in 25 µl of antigen of this dilution.

VN tests (12) can be carried out in flat-bottomed cell-culture grade microtitre plates using the constant virus/varying serum method with 100 TCID50 (50% tissue culture infective dose) of virus.

CF tests (12) are done in tubes or in microtitre plates (4) by conventional methods, recording 100% or 50% haemolysis. This is the standard method at the present time.

a) Complement fixation test
i) Titrate the complement in haemolysis tubes, in the presence of 1 AgU, in order to determine the H 50 unit.

ii) Inactivate the positive and negative control sera in a water bath for 30 minutes at 56°C.

iii) Make doubling dilutions of test and control sera in CMV, from 1/4 to 1/1024, using a 96-well round-bottomed microtitre plate and 25 µl per well. Use the first well for the initial 1/4 dilution and the second as a serum control (anticomplementary control at 1/4 dilution). Prepare antigen, complement, and RBC control wells (two wells of each).

iv) Add 1 AgU in 25 µl per well (except to serum, complement and RBC control wells), then add 6 H 50 of complement in 25 µl per well (except to RBC control wells).

v) Incubate the plates, covered with plastic film, for 14 hours (overnight) at 4°C.

vi) Add 50 µl haemolytic antiseraum per well.

vii) Cover the plates again and incubate for 30 minutes at 37°C.

viii) Prepare H100, H75, H50, H25 haemolysis controls using complement controls (H100) and CMV.

ix) Read after centrifugation or passive sedimentation at 4°C. The test sera results are determined as the highest dilution of serum that gives at least 50% haemolysis inhibition.

x) A negative serum should give haemolysis inhibition <50% at 1/4 dilution.

b) Indirect fluorescent antibody test
IFAT test (7) is carried out using chicken embryo cell cultures in flat-bottomed wells of microtitre plates: 200 µl of a cell suspension, diluted 1/1000 in medium, is distributed into all wells and a confluent cell sheet is formed within 24 hours. The medium is discarded and 100 µl of viral suspension containing 104 TCID50 is added to each well. After 2 hours, 100 µl of MEM containing 2% calf serum is added. After 48 hours of incubation, the plates are washed with PBS and fixed with acetone containing 15% water for 30 minutes at −20°C. The plates are then dried at 37°C for 15 minutes. The plates can be stored at −30°C or −70°C for 3 months. Sera are tested by IFA using anti-rabbit IgG conjugated to fluorescein isothiocyanate. The test results may be qualitative with sera diluted 1/10 or 1/20, or quantitative with serial dilutions of serum.

c) Enzyme-linked immunosorbent assay
A recently developed ELISA (6) uses a semi-purified myxomatosis virus (French hypervirulent T1 strain antigenically related to Lausanne strain) produced in RK-13. The virus is harvested as a suspension of cells 48 hours after infection, and is centrifuged. The cell deposit is homogenised in TL20 (20 mM Tris, pH 8.6, 150 mM NaCl, and 1 mM EDTA), disrupted in ground glass and centrifuged at 1200 g at 4°C for 10 minutes.

The supernatant fluid is laid down on an equal volume of a 36% sucrose cushion in TL20 and centrifuged at 200,000 g for 2 hours in an SW 41 rotor at 4°C. The deposit is homogenised in 4–12 ml TL20 and again laid down on a 36% sucrose cushion.

The new deposit is homogenised in 1 ml TL20 and run into a linear 30–65% saccharose gradient by centrifugation at 200,000 g for 3 hours. The viral disc is harvested and diluted in 10 ml TL20 and then centrifuged at 130,000 g for 1 hour to concentrate the virus.

The deposit is homogenised in 0.2 ml TL20 and quantified by the Bradford method (colorimetric reaction with Coomassie brilliant blue) (3) or spectrophotometry (viral proteins account for around three-fifths of total protein). It can be stored at −30°C before use.
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i) Coat probind assay plates for 16 hours (overnight at 37°C) with 1 µg per well viral proteins in 100 µl PBS, pH 7.6, leaving one column blank (i.e. coat with PBS only). Note that different batches of antigen vary in activity, and should be titrated against known standards to select antigen with high optical densities (OD).

ii) After three washes in PBS, block free binding sites by incubation in 25 mg/ml gelatin in PBS for 1 hour at 37°C.

iii) Wash the plate three times in PBS–0.01% Tween 20, and add 100 µl serial twofold dilutions of serum in PBS–Tween. Include positive and negative serum standards in each plate.

iv) After 60 minutes’ incubation at 37°C and three washes in PBS–Tween, add 100 µl of a dilution in PBS–Tween of goat anti-rabbit IgG serum (typically 1/3000) conjugated to alkaline phosphatase for 1 hour at 37°C.

v) After four washes in PBS–Tween add, as substrate, 100 µl of disodium p-nitrophenyl phosphate at a concentration of 1 mg/ml in 10% diethanolamine.

vi) After 12 minutes in the dark at room temperature, the enzymatic reaction is stopped by the addition of 50 µl of 2 N NaOH.

vii) Read OD in a spectrophotometer at a wavelength of 405 nm.

viii) Express the serum sample titre as the inverse of the highest dilution for which the OD is greater than three times the OD of the negative serum standard.

The detection of specific myxoma virus antibodies by ELISA has been shown to be a highly sensitive and specific method for kinetic studies in experimental infection (2). Evaluation of the test has shown its great value for diagnostic application in wild rabbit populations (6).

For epidemiological surveys, the IFA test and the indirect ELISA can also be carried out using blood dried on blotting or filter paper: discs are cut (paper punch size) and two discs are placed in each well, to which is added 100 µl PBS to extract the serum. The dilution is about 1/30 and this can be used in another well for testing (7). Blood samples collected in anticoagulant-coated capillary tubes can be used for the ELISA. The sample is washed in the diluting solution to obtain the required dilution (11).

d) Agar gel immunodiffusion test

Agar gel immunodiffusion (AGID) (15) is qualitative and can detect antigen or antibody. Agar is prepared as described previously (Section B.1.) using 6 ml per 10 cm Petri dish. Strips of filter paper containing the standard antigen and antiserum, and discs containing test sera are arranged on the surface of the agar (discs between the strips). The plates are incubated in a humid atmosphere at 37°C and read after 24–48 hours. Three precipitation lines should appear. If the test sera contain antibody, at least one of the three lines is bent towards the antigen band; otherwise it remains straight. If sera contain antigen, at least one of the lines is bent towards the standard serum strip. The test can also be carried out in a more conventional manner using liquid reagents in wells cut in the agar.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Two types of live vaccine are used for vaccination against myxomatosis: a heterologous vaccine prepared from Shope’s fibroma virus (5, 9, 14), and a homologous vaccine prepared from an attenuated strain of myxoma virus (1, 8, 13, 16–18). They are administered subcutaneously or intradermally.

Recently a new recombinant myxoma virus expressing rabbit haemorrhagic disease virus (RHDV) capsid protein and conferring double protection against myxomatosis and RHDV (2) has been developed, but is not yet 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.

A master seed virus (MSV) must be established and used according to a seed-lot system. A record must be kept of its origin, passage history and characteristics.
1. **Seed management**

   a) **Characteristics of the seed**

   The viruses employed are fibroma virus or myxoma virus. The strains of fibroma virus are usually the original Shope’s OA strain (1932), Boerlage’s strain or various closely related strains. The strains of myxoma virus are modified by passing in embryonated chicken eggs, rabbit kidney cells at decreasing temperatures, or chicken embryo cells. The strains usually result from having been cloned several times.

   b) **Method of culture**

   *Fibroma strains* are maintained by passage in specific pathogen free (SPF) rabbits or in unvaccinated rabbits from a stock known to be free from myxomatosis. Skin on the backs of healthy adult rabbits is shaved, and multiple sites are inoculated with a 1% suspension of virulent material. Fibromas are fully developed within 8–10 days, at which time the rabbits are killed and the tumours are removed aseptically and homogenised with distilled water. The suspension is stored at −30°C or −70°C in 50% buffered glycerol, or as a 5% dilution in a protein solution. The production of the fibroma virus is also possible in rabbit dermal cell line.

   *Myxoma virus* is grown on chicken embryo cell culture obtained from flocks free from specified pathogens, or on suitable cell lines (rabbit dermal cell line).

   c) **Validation as a vaccine**

   i) **Identity**

   Specific antigenic characteristics of the fibroma virus strains are verified by AGID using sera against fibroma and myxomatosis.

   The identity of myxoma virus is confirmed by neutralisation tests in RK-13 cells, or in a suitable cell line using a monospecific antiserum.

   ii) **Purity**

   The Master seed must be free from bacterial, fungal, mycoplasmal and viral contamination.

   iii) **Safety**

   Samples for safety testing are taken from a batch produced according to the manufacturing process. The dose to be used shall contain the maximum titre or potency established by the manufacturer (release titre).

   Several tests are performed, at the Master Seed level, to demonstrate different aspects of safety. The safety of 10 times the normal dose must be demonstrated. Also, it is necessary to examine the dissemination of vaccine virus within the vaccinated animal, the ability of vaccine virus to spread from the vaccinated animal to in-contact animals and to test whether there is reversion-to-virulence of the vaccine virus, following serial passage in rabbits.

   The pathogenicity of the fibroma virus strains is tested by inoculating rabbits with serial dilutions of supernatant fluids obtained by centrifugation of tumour preparations. Macroscopic and histopathological features and the course of development of fibromas are tested in SPF rabbits periodicaly. (Numerous serial passages in rabbits may induce mutation to the inflammatory IA strain, which produces severe lesions that are more inflammatory than neoplastic.)

   The residual pathogenicity of the myxoma virus strains is tested by intradermal inoculation into SPF rabbits or unvaccinated rabbits free from myxomatosis. These rabbits should not develop more than a local reaction with perhaps small secondary lesions on the head that disappear within a few days.

   For both strains, the rectal temperature and the body weight should be recorded as additional parameters.

   iv) **Efficacy**

   Different trials must be undertaken from representative batches of final product containing the minimum titre or potency. The protective effect is demonstrated as follows:

   A minimum of ten adult rabbits are inoculated with a dose of fibroma vaccine, and three rabbits serve as unvaccinated controls. After 14 days, all rabbits are inoculated, intradermally into the eyelids, with a pathogenic strain of myxoma virus (example: 0.1 ml inoculum containing 10^3 ID_{50} [median infectious dose]). During the following 21 days, the controls will die from myxomatosis, and at least seven of the ten vaccinated rabbits must present no signs of generalised infection.
Similarly, *myxoma vaccine* is tested in ten rabbits with three controls. After 14 days, all the rabbits are challenged with a sufficient quantity of virulent strain (example: 0.1 ml of the Lausanne virus strain containing $10^3$ ID$_{50}$). After 21 days, seven of ten rabbits must have survived, while controls must have died from myxomatosis.

The manufacturer shall have established a minimum titre or potency taking into account loss in potency during the shelf life.

2. Method of manufacture

*Fibroma virus* is produced by multiple intradermal inoculations of seed virus into the skin on the back of a number of rabbits. Production is also possible in rabbit dermal cell line. Only the second (and perhaps the third) passage can be used if modification of the virus is to be avoided. The product of fibroma homogenate can be stored by freezing or used immediately. After clarification by centrifugation, the supernatant fluid is mixed with a stabiliser containing antibiotics and is distributed into ampoules or bottles for lyophilisation. Kaolin may be added as an adjuvant (40 mg/ml), in which case the vaccine is administered subcutaneously.

*Myxoma virus* is produced in chicken embryo cells (derived from SPF eggs) or a suitable cell line, limiting the passage number to a maximum of five. Virus is harvested after 2–6 days. The viral suspension may be stored at −70°C. The vaccine is prepared by diluting in specified proportions the viral preparation with a stabiliser for lyophilisation. After homogenisation, the product is distributed into bottles for lyophilisation, the bottles being sealed under vacuum or in sterile nitrogen.

Each virus can also be produced in RK-13 cells.

3. In-process control

The *fibroma virus* titre is measured by calculating the ID$_{50}$ after intradermal inoculation of serial dilutions of the clarified supernatant fluid into several sites (e.g. five) on up to six rabbits. A dilution of a standard preparation of fibroma virus is also inoculated into each rabbit to confirm the animal’s correct response to inoculation. The titration can also be performed in a rabbit cell line. In each case the titre should correlate with the required potency as defined by the test for efficacy, see Section C.1.c.

The identity of *myxoma virus* is checked in RK cells. Titration of each virus can also be done in RK-13 cells (TCID$_{50}$).

Testing for contaminating viruses is done by inoculating a confluent cell sheet of Vero cells. Vaccine, adjusted to the equivalent of 20 doses/ml, is neutralised with an equal volume of monospecific hyperimmune serum for 30 minutes at 37°C. The mixture is filtered through a 0.22 µm membrane filter, and 1 ml volumes are inoculated into five 25 ml bottles of cell cultures. These are kept under observation for 7 days. After harvesting, the cells are suspended in medium and subjected to several freeze–thaw cycles, followed by centrifugation and filtration, and the material is inoculated into fresh cultures and observed for 7 days. There should be no evidence of CPE or haemadsorption to chicken RBCs.

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

After rehydration, ten doses of the lyophilised *fibroma vaccine* are injected subcutaneously into each of three susceptible rabbits, which are then observed for 21 days. Local reactions should be slight, with no generalisation and no effect on general health.

*Myxoma vaccine* is tested using ten doses injected intradermally into the ears of three susceptible rabbits, which are then observed for 21 days. The primary myxoma lesion should remain mild.

c) Potency

The batch potency is determined by measurement of virus content. Serial dilutions of the vaccine are inoculated into suitable cell cultures. One dose of vaccine shall contain not less than the minimum titre established in Section C.1.c.

If the vaccine strain is not adapted to cultures, an efficacy test in rabbits shall be carried out (see Section C.1.c.).
d) **Duration of immunity**

Several batches of ten susceptible rabbits are vaccinated. One batch is tested by challenge infection (as in the batch potency test), at 1, 2, 3, etc., months post-vaccination for fibroma virus, and at 1, 3, 6, and 9 months for myxoma virus. The duration of immunity is deduced from the time during which at least seven of the ten rabbits prove to be resistant to infection.

e) **Stability**

Titrations of vaccine virus are carried out at intervals until 3 months beyond the requested shelf life on at least three batches of vaccine.

5. **Tests on the final product**

a) **Safety**

See Section C.4.b.

b) **Potency**

See Section C.4.c.

**REFERENCES**


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CHAPTER 2.8.2.

TULAREMIA

SUMMARY

Tularemia is a zoonosis caused by Francisella tularensis. The causative organism is a Gram-negative coccoid rod, 0.2–0.5 µm × 0.7–1.0 µm, non-motile and non-spore-forming organism that is an obligate aerobe with optimal growth at 37°C. It is oxidase-negative, weakly catalase-positive and requires cysteine for growth. It occurs naturally in lagomorphs (rabbits and hares), and in rodents, especially microtine rodents (such as voles, vole rats and muskrats), and beavers. A wide range of other mammals and several species of birds have also been reported to be infected. Among domestic animals, the cat seems to be able to act as a carrier of the bacterium.

Two types of F. tularensis are recognised on the basis of cultural characteristics, epidemiology, and virulence in some hosts. Tularemia is largely confined to the Northern Hemisphere and is not normally found in the tropics or the Southern Hemisphere. Francisella tularensis tularensis (Type A) is associated with lagomorphs in North America. It is transmitted primarily by ticks and biting flies, is highly virulent for humans and domestic rabbits, and ferments glycerol. Francisella tularensis palaearctica (Type B) occurs mainly in aquatic rodents (beavers, muskrats) in northern North America, and in hares and small rodents in northern Eurasia. It may be water- or arthropod-borne, is less virulent to humans and rabbits, and does not ferment glycerol. In addition to vector transmission, tularemia may be spread by direct contact with contaminated animals or environmental fomites by inhalation, or by ingestion of the poorly cooked flesh of infected animals or contaminated water.

The disease is characterised by fever, depression and septicaemia. In humans, there may be ulcers or abscesses at the site of inoculation (this is rarely seen in animals), and swelling of the regional lymph nodes. On post-mortem examination, lesions may include caseous necrosis of lymph nodes and multiple greyish-white foci of necrosis in the spleen, liver, bone marrow and lungs. The spleen is usually enlarged.

It is important to understand that there is a high risk of direct infection of humans by direct contact with this organism. Special precautions, including the wearing of gloves, masks and eyeshields, are therefore recommended when handling infective materials. The facility should meet the requirements for Containment Group 3 pathogens (see Chapter I.1.6. Human safety in the veterinary microbiological laboratory).

Identification of the agent: The bacterium can be demonstrated in impression smears or in fixed specimens of organs, such as liver, spleen, bone marrow, kidney and lung, as well as in blood smears. Immunological methods, such as the fluorescent antibody test (FAT), are the most reliable way to identify the bacterium. With Gram staining, the bacteria appear as very small punctiform Gram-negative rods, often difficult to distinguish as bacteria. They can also be stained with May–Grunwald–Giemsa or phenol thionin.

The organism is highly fastidious. For growth it is necessary to use Francis medium, McCoy and Chapin medium, or glucose cysteine agar supplemented with blood. The colonies are small, round and transparent, and do not appear before 48 hours' incubation at 37°C. On Francis medium, the colonies may be confluent and have a milky appearance. If transportation is necessary, samples should be inoculated into sterile nutrient broth and stored at 4–10°C for a few hours or at −70°C if transit is likely to be prolonged.

Mice or guinea-pigs can be experimentally inoculated with infected tissue material or with cultures to aid in the diagnosis of tularemia. Infection is fatal within 2–10 days depending on the virulence of the organism. The FAT demonstrates F. tularensis in pathological specimens.
Serological tests: Serological tests are useful diagnostic aids in human infection, but are of limited value in sensitive animal species as these species usually die before developing antibodies. Epidemiological surveys can be conducted in domestic animals in relatively resistant species, such as sheep, cattle, pigs, dogs and wild ungulates, as these species develop antibodies. Relatively resistant species of rodents and lagomorphs can also be used in epidemiological surveys. Serological surveys can and are conducted in a number of wildlife species, such as moose, that are exposed to F. tularensis.

Requirements for vaccines and diagnostic biologicals: A live attenuated vaccine strain is available for vaccination of humans at high risk of exposure to virulent F. tularensis. However, the vaccine is only available for restricted use. The outcome of vaccination can be estimated by the demonstration of specific antibodies and the ability of lymphocytes to proliferate in the presence of F. tularensis antigen.

A. INTRODUCTION

Tularemia is a zoonosis caused by Francisella tularensis. It occurs naturally in lagomorphs (rabbits and hares) and rodents, especially microtine rodents such as voles, vole rats and muskrats, as well as in beavers. In addition, a wide variety of other mammals, birds, amphibians and invertebrates have been reported to be infected (14, 15). Tularemia occurs endemically in the Northern Hemisphere. The disease can occur as epizootic outbreaks in many countries in North America, Europe and Japan, while it occurs only as sporadic cases in some other countries in Europe and Asia. It is rarely reported from the tropics or the Southern Hemisphere. Some epizootic outbreaks occur as a result of importation of subclinically infected lagomorphs.

Two types of F. tularensis are recognised on the basis of culture characteristics, epidemiology, and virulence. Francisella tularensis tularensis (Type A) is mainly associated with lagomorphs in North America. It is primarily transmitted by ticks or biting flies, or by direct contact with infected lagomorphs. It is highly virulent for humans and domestic rabbits, and ferments glycerol. Francisella tularensis palaearctica (Type B) occurs mainly in aquatic rodents (beavers, muskrats) and voles in northern North America, and in lagomorphs (hares) and rodents in northern Eurasia. It is primarily transmitted by direct contact or by mosquitoes, but may be transmitted through inhalation or through infected water or food. It is less virulent for humans and domestic rabbits, and does not ferment glycerol (1, 9, 10, 12).

In sensitive animals, clinical signs of severe depression are followed by a fatal septicaemia. The course of the disease is approximately 2–10 days in susceptible species, and animals are usually dead when presented for diagnosis. Most domestic species do not usually manifest signs of tularemia infection, but they do develop specific antibodies to the organism following infection. Outbreaks with high mortality caused by the Type A organism have occurred in sheep (1, 12). Among domestic animals, the cat seems to be able to act as a carrier of the bacterium (4).

At necropsy, animals that have died from acute tularemia are usually in good body condition. There are signs of septicaemia characterised by whitish foci of necrosis randomly distributed in the liver, bone marrow and spleen. In addition, the spleen is usually enlarged. Necrotic foci vary in size, and in some cases may be barely visible to the naked eye. The lungs are usually congested and oedematous, and there may be areas of consolidation and fibrinous pneumonia or pleuritis. Fibrin may be present in the abdominal cavity. Foci of caseous necrosis are often present in one or more lymph node(s). The lymph nodes that are most often affected are those in the abdominal and pleural cavities and lymph nodes draining the extremities. In less sensitive species, the histological picture can resemble that of tuberculosis with chronic granulomas in liver, spleen, lungs and kidneys.

There is a high risk of human infection from F. tularensis, as the infective dose is extremely low and infected animals excrete bacteria in urine and faeces. Infection can occur by simple contact. Suitable precautions, such as the wearing of gloves, masks and eyeshields during any manipulation of pathological specimens or cultures, must be taken in order to avoid human infection. The facility should meet at least the requirements for Containment Group 3 pathogens as outlined in 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 the OIE Reference Laboratory. Experimentally inoculated animals and their excreta are especially hazardous to humans.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Francisella tularensis can be demonstrated in smear preparations or in histological sections. It can also be identified by culture or animal inoculation. However, F. tularensis may be difficult to isolate from dead animals
and carcasses due to overgrowth of other bacteria. As the post-mortem picture is variable, diagnosis is sometimes difficult and immunological or immunohistochemical methods are preferable, although reagents may be difficult to obtain. It can sometimes be recommended, therefore, that fixed specimens be analysed at laboratories equipped with proper reagents or methods, such as the OIE Reference Laboratory (see Part 3 of this Terrestrial Manual).

a) **Smear preparations**

Smear preparations are made on microscope slides as impression smears of organs such as the liver, spleen, bone marrow, kidney, lung or blood. The bacteria are abundant in such smears, but may be overlooked because of their very small size (0.2–0.7 µm). The bacteria can be demonstrated by direct or indirect fluorescent antibody staining. This is a safe, rapid and specific diagnostic tool (8, 11, 13).

Gram staining of smears reveals a scattering of small, punctiform Gram-negative bacteria near the limit of visibility. The use of oil microscopy increases the visibility of the bacteria. The bacteria may be difficult to distinguish from precipitates of stain.

b) **Histological sections**

Bacteria can be demonstrated in sections using immunohistochemical methods, such as the fluorescent antibody test (FAT) (11). The test is normally performed on specimens from liver, spleen or bone marrow, fixed in neutral buffered formalin and paraffin embedded. Slides are treated with rabbit anti-tularemia serum, washed and thereafter treated with sheep fluorescein-isothiocyanate-conjugated anti-rabbit serum. The samples are examined under a fluorescence microscope. Large numbers of bacteria can be seen in necrotic lesions and in the blood.

c) **Culture**

*Francisella tularensis* will not grow on ordinary media, although an occasional strain can sometimes, on initial isolation, grow on blood agar. Incubation is at 37°C. Heart blood, liver, spleen and bone marrow from moribund animals should be used for culture. It is necessary to use special culture media, such as:

i) **Francis medium**: Peptone agar containing 0.1% cystine (or cysteine) and 1% glucose, to which is added, before solidification, 8–10% defibrinated rabbit, horse or human blood.

ii) **McCoy and Chapin medium**: This consists of 60 g egg yolk and 40 ml normal saline solution, carefully mixed and coagulated by heating to 75°C.

iii) **Modified Thayer–Martin agar**: Glucose cysteine agar (GCA)-medium base supplemented with haemoglobin and Iso VitaleX.

Media can be stored for up to 8–10 days at 4°C. Colonies that form on McCoy medium are small, prominent, round and transparent. A more abundant growth is obtained on Francis medium and modified Thayer–Martin agar, with confluent colonies that have a milky appearance and a mucoid consistency. On either medium, colonies do not appear until after 48 hours’ incubation at 37°C.

iv) **GCA agar with thiamine (BBL)**: When used with added blood, the medium is commonly referred to as GBCA and can be substituted for the original, noncommercial medium described by Down et al. (3). Suspend 58 g of the dry material in 1 litre of distilled or demineralised water, and mix thoroughly. Heat with frequent agitation and boil for 1 minute. Dispense into tubes and sterilise by autoclaving at 118–121°C for 15 minutes. For larger volumes (up to several litres) of culture medium, autoclave at the same temperatures for 30 minutes. Cool to 45–48°C. Aseptically add 25 ml of packed human blood cells or 50 ml of defibrinated rabbit or sheep blood. Mix thoroughly and pour into plates. Incubate at 37°C for 24 hours before use to decrease surface moisture and to test for sterility (3).

The following selective medium can be used in addition to the non-selective media: **Cystine heart agar** (DIFCO) with 5% rabbit blood, and penicillin (100,000 units), Polymyxin B sulphate (100, 000 units), and cycloheximide (0.1 ml of a 1% stock solution) per litre.

Differential criteria for the identification of *F. tularensis* include absence of growth on ordinary media, distinctive cellular morphology, and specific fluorescent antibody and slide agglutination reactions. The bacteria are nonmotile, nonsporulating, bipolar staining, and of uniform appearance in 24-hour cultures, but pleomorphic in older cultures.

*Francisella tularensis* can be identified in stained smears, by agglutination with tularemia hyperimmune antiserum, or by animal inoculation. In areas of North America where both types of *F. tularensis* may occur, Type A may be distinguished from Type B by the fact that Type A ferments glycerol.
The bacteria can also be identified by hybridisation with probes specific to the 16S rRNA of *F. tularensis*, *F. tularensis* Type A, and *F. tularensis* Type B (6), or by polymerase chain reaction (PCR) with primers targeting specific regions of the 16 rDNA molecules. The PCR will allow identification at the genus, species and subspecies level (7).

d) **Capillary tube precipitation test on pathological samples**

Tissues, such as spleen, liver or bone marrow, are ground with sterile sand in three-to-five times their volume of normal saline. The suspension is transferred to a tube and two volumes of ethyl ether are added. After shaking, the mixture is allowed to stand for 4–5 hours at room temperature. It is again shaken and then allowed to stand overnight.

The aqueous phase is drawn off and centrifuged at 2000 g for 30 minutes. The supernatant fluid, containing the antigen, is drawn off and distributed into capillary tubes to which tularemia antiserum is added.

The tubes are incubated at 37°C for 3 hours, then kept at 4°C overnight. A positive result is the formation of a ring of precipitate.

e) **Animal inoculation**

Animal inoculation is extremely hazardous and is not recommended for routine identification. It should only be undertaken where proper biosafety facilities and cages are available (see Chapter I.1.6. Human safety in the veterinary microbiological laboratory). The use of animals for diagnostic purposes must be carefully considered.

Laboratory animals are inoculated with culture material to confirm the nature of an isolate. Pathological specimens may be inoculated for the direct detection of *F. tularensis*. Mice are more sensitive than guinea-pigs, although the latter may be preferred for diagnostic purposes as the lesions are more definitive than in mice. Furthermore, mice usually die before lesions can form.

In infected animals, the only evidence at the onset of infection is a slight anaemia with a lymphocytosis and monocytosis.

Intraperitoneal injection is sufficient for passage of pure cultures. All routes of administration in mice, such as subcutaneous, percutaneous, or intravenous, will lead to an infection that is invariably fatal within 2–10 days.

Inoculation in guinea-pigs may be made into a foot-pad, provoking a lymphadenitis that is detectable after 3–4 days. If this reaction is pronounced, the animal may be killed at 5–6 days. Use of the percutaneous route, by smearing a shaved area of skin with the material, allows the selective isolation of *F. tularensis* from material that contains a mixture of organisms.

Post-mortem examination reveals hypertrophy and periadenitis of regional lymph nodes, and oedema at the inoculation site, which is sometimes haemorrhagic. The spleen contains a scattering of minute nodules and is hypertrophied. The liver is also hypertrophied, but foci of necrosis may not be evident. Smears of blood, spleen and liver may be extremely rich in the bacterium.

The regional lymph nodes show a lymphadenitis, oedema that distends the node capsule, a lymphocytic infiltration and peripheral micro-abscessation. Blood vessels are congested and thrombotic. Later, areas of necrosis become confluent to form multiple abscesses. If the animal survives long enough, these may become surrounded by an epithelioid reaction with giant cells, plasma cells and macrophages.

2. **Serological tests**

Serology is currently carried out for diagnosis of tularemia in humans, but is of limited value in sensitive animal species, which usually die before specific antibodies can develop. Serology may be employed, either on sera or on lung extracts (13), in epidemiological surveys of animals that are resistant to infection, such as sheep, cattle, pigs, moose, dogs or birds (13, 15). As there is no antigenic difference between Type A and Type B, the less virulent *F. tularensis palaearctica* could be used as antigen in all serological tests.

a) **Tube agglutination**

The most commonly used serological test is the tube agglutination test. The antigen is a culture of *F. tularensis* on Francis medium. The culture is harvested after 5–6 days. Younger cultures yield a poorer antigen. The colonies are suspended in 96% alcohol, giving a thick suspension that can be stored for 1–7 days at room temperature. The sediment is washed with normal saline and resuspended in an equal
volume of normal saline. Crystal violet powder is added to a final concentration of 0.25%. The bacteria are stained by adding crystal violet and incubating at 37°C for at least 24 hours and at most 7 days.

After the supernatant fluid has been discarded, the deposit is suspended in normal saline with or without thimerosal (merthiolate) at a final concentration of 1/10,000, or formaldehyde at a final concentration of 0.5%. The suspension is calibrated with positive and negative sera, and adjusted by adding normal saline to provide an antigen that when tested on a slide gives readily visible stained agglutination reactions against a clear fluid background.

The test is performed in tubes containing a fixed amount of antigen (0.9 ml) and different dilutions of serum commencing with 1/10, 1/20, 1/40, etc. The results are read after 20 minutes of shaking, or after 1 hour in a water bath at 37°C followed by overnight storage at room temperature. The agglutinated sediment is visible to the naked eye or, preferably, by using a hand lens. The positive tubes are those that have a clear supernatant fluid. Possible cross-reactions with *Brucella abortus*, *B. melitensis* and *Legionella* sp. have to be taken into consideration.

b) **Enzyme-linked immunosorbent assay**

Another serological test, the enzyme-linked immunosorbent assay (ELISA), also allows an early diagnosis of tularemia (2). This method is now widely used for clinical purposes. Different antigens, whole bacteria as well as subcellular components (5), have been used as recall antigens against immunoglobulins IgA, IgM and IgG; 2 weeks after the onset of tularemia, specific antibodies can be detected in the serum. For routine diagnosis, whole heat-killed (65°C for 30 minutes) bacteria can be used as antigen. Bacteria can be coated to plastic plates, using the usual procedures (2) followed by serial dilutions of serum to be tested. Positive reactions can be visualised by anti-antibodies labelled with enzyme. The test should also be read in a photometer with positive and negative sera as controls.

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

Vaccines have been produced with the aim of protecting humans, but as all vaccine development involves testing in animals, it is clear that some of the vaccines could be used to protect animals.

Before 1940, attempts to vaccinate against tularemia were performed by use of whole killed bacteria or bacterial extracts. None of these vaccines induced protection against highly virulent strains of *F. tularensis*. Instead, viable attenuated vaccines were developed. Attenuation was performed by repeated culture of bacteria on various media with or without antiserum. Such live attenuated vaccines have been used in mass vaccinations of people in the former Soviet Union since 1946, either as monocultures or as a mixture of strains.

An attenuated live vaccine strain of *F. tularensis* biovar palaearctica is available and can be used for restricted vaccination of individuals at high risk (16).

**REFERENCES**


Chapter 2.8.2. – Tularemia


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**NB:** There is an OIE Reference Laboratory for Tularemia (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 2.8.3.

Rabbit Haemorrhagic Disease

SUMMARY

Rabbit haemorrhagic disease (RHD) is a highly contagious and acute fatal disease of the European rabbit (Oryctolagus cuniculus), caused by a calicivirus. A similar disease, termed European brown hare syndrome (EBHS), has been described in the hare (Lepus europaeus). The aetiological agent is a different calicivirus, antigenically related to the RHD virus (RHDV). RHD is characterised by high morbidity and high mortality (40–90%), and spreads very rapidly by direct and indirect transmission. Infection can occur by nasal, conjunctival or oral routes. Transmission of RHD is facilitated by the high stability of the virus in the environment. The incubation period varies from 1 to 3 days, and death usually occurs 12–36 hours after the onset of fever. The clinical manifestations have been described mainly in the acute infection (nervous and respiratory signs, apathy and anorexia). Clear and specific pathological lesions, both gross and microscopic, are present. There is primary liver necrosis and a massive disseminated intravascular coagulopathy in all organs and tissues. The most severe lesions are in the liver, trachea and lungs. Petechial haemorrhages are evident in almost all organs and are accompanied by poor blood coagulation.

Identification of the agent: The liver contains the highest viral titre and is the most suitable organ for viral identification. As no satisfactory growth conditions or sensitive cell substrates have been established, in-vitro isolation cannot be employed. The haemagglutination test using human Group O red blood cells was the first test applied for routine laboratory diagnosis of RHD. However, other tests (negative-staining immunoelectron microscopy, sandwich enzyme-linked immunosorbent assay [ELISA], immunohistological staining, polymerase chain reaction and Western blot) have shown a higher level of specificity and sensitivity.

Serological tests: Characterisation and titration of specific antibodies arising from natural infection or from immunisation are performed using the haemagglutination inhibition test or the ELISA, either as an indirect or competitive reaction. The following reagents are prepared: antigen from infected rabbit liver, anti-RHDV serum from convalescent or hyperimmunised rabbits, and negative serum from rabbits fully sensitive to RHDV infection. Monoclonal antibodies have been produced in several laboratories. Some laboratories have produced a recombinant antigen, VP6O structural protein expressed in baculovirus, which can also be used as a diagnostic reagent.

Requirements for vaccines and diagnostic biologicals: Indirect control of the disease is achieved by vaccination using a killed vaccine prepared from clarified liver suspensions of experimentally infected rabbits and subsequently inactivated and adjuvanted. Vaccinated animals quickly produce solid immunity against RHDV infection (within 5–10 days) and experimental data indicate that protection lasts for a long period (over 1 year).

A. INTRODUCTION

Rabbit haemorrhagic disease (RHD) is a highly contagious and acute fatal disease of wild and domestic European rabbits (Oryctolagus cuniculus).

RHD was first reported in 1984 in the People’s Republic of China (19); currently it is endemic in East Asia, Europe and Oceania. Outbreaks have also been recorded in Central America (Mexico and Cuba), Saudi Arabia and West and North Africa. In 2000 and 2001 three independent outbreaks were recorded in the United States of America.
The causative agent of RHD is a calicivirus that is 32–35 nm in diameter and has a single major capsid polypeptide (60 kDa), a positively stranded RNA genome of 7437 kb and a sub-genomic RNA of 2.2 (9, 20–22). The RHD virus (RHDV) capsid protein (VP60) folds in two distinct domains held together by a hinge region: the N-terminal 200–250 residues constitute the inner domain and the C-terminal residues beyond 200–250 constitute the protruding domain. In the overall picture of the capsid, these domains form the inner shell and the outer shell, respectively, which is characterised by arch-like structures. This structure also correlates with the antigenic characteristics of RHDV, in fact the main antigenic determinants are located on the C-terminal end of the VP60 (4, 5, 23, 27).

Since 1991, a second type of virus particle was identified as the main component in approximately 5% of the RHDV-positive specimens, i.e. those taken from rabbits showing a protracted course of the disease (8). The characteristics of this particle are: i) a smooth surface and a diameter smaller than RHDV; ii) its protein is of 28–30 kDa; iii) it is reactive with RHDV convalescent rabbit sera and with those anti-RHDV monoclonal antibodies (MAbs) reactive against the N-terminal end of the RHDV VP60; and iv) it is haemaglutination (HA) negative. This smaller viral particle only corresponds to the inner shell of RHDV, and two hypotheses have been proposed to explain its origin. Granzow et al. (15) assumed that it arises from a truncated RHDV genome or defective expression. However, Barbieri et al. (2) observed the following: i) a strict correlation between the higher prevalence of smooth RHDV (s-RHDV) particles in the organs and the appearance of specific anti-RHDV IgM at 3–4 days post-infection; ii) the presence of large amounts of s-RHDV only in the liver and spleen and not in the bloodstream, as occurs during the vireamatic phase of acute RHD; iii) the finding of fragments of the VP60 having different molecular weight (41–30 kDa) during transition from RHDV to s-RHDV. They concluded therefore that the genesis of the particle is due to a degradative process that is probably the consequence of the physiological clearance of the RHDV-IgM immuno-complex formed in large amounts at the beginning of the humoral response. Apart from its origin, the identification of this second particle in the liver of a rabbit can be considered to be a marker of the subacute/chronic form of RHD that usually evolves between 4 and 8 days post-infection and is followed either by the death of the rabbit or, more often, by its recovery (2).

All known RHD viral isolates appear to belong to one serotype. The complete sequence of geographically different RHD strains has been reported. Comparison reveals close overall homology in terms of genome sequence with few or no predicted changes in amino acid composition (differences between 2% and 5%). Nevertheless, isolates that exhibit temperature-dependant differences in haemoagglutinating characteristics (3) have been described, and more recently a consistent genetic and antigenic RHDV variant has been identified simultaneously in Italy (4) and Germany (23). This RHDV variant, named RHDVa, presents amino acid changes in the surface-exposed E region (aa 344–434) that contains the main antigenic epitopes of calicivirus, three times higher than in all previously sequenced RHDV isolates. A group of related MAbs that protected against infection by RHDV were negative when tested by enzyme-linked immunosorbent assay (ELISA) against RHDVa antigen. However, rabbits experimentally vaccinated with the currently available RHDV vaccine were protected against challenge with RHDVa, even if with a lower efficiency (4, 23).

Another virus, provisionally called rabbit calicivirus (RCV) and related to the RHDV, has been identified in healthy rabbits (6, 7). It is significantly different from the previously characterised RHDV isolates in terms of pathogenicity, viral titre, tissue tropism, and primary sequence of the structural protein. It is avirulent, replicates in the intestine at a low titre and has about a 92% genomic similarity to RHDV. The results of cross-protection experiments would indicate that the new virus will not infect hares. In addition, the antigenic data and sequence comparisons have demonstrated that it is more closely related to RHDV than to the European brown hare syndrome virus (EBHSV).

RHDV is very stable and resistant in the environment; the viral infectivity is not reduced by treatment with ether or chloroform and trypsin, by exposure to pH 3.0, or by heating to 50°C for 1 hour. The virus survives at least 225 days in an organ suspension kept at 4°C, at least 105 days in the dried state on clot at room temperature, and at least 2 days at 60°C, both in organ suspension and in the dried state (24). It also retains its infectivity at low temperatures, and remains quite stable during freezing and thawing. RHDV is inactivated by 10% sodium hydroxide, by 1.0–1.4% formaldehyde and by 0.2–0.5% beta-propiolactone at 4°C. Such treatments do not alter the immunogenicity of the virus.

The European rabbit (Oryctolagus cuniculus) is the only species known to be affected by RHD. No other lagomorphs, such as the Volcano rabbit of Mexico (Romerolagus diazi), the black-tail jackrabbit (Lepus californicus) and the cottontail (Sylvilagus floridanus) of North America, have been shown to be susceptible (16). A similar disease, termed European brown hare syndrome (EBHS), has been described in the hare (Lepus europaeus), but its aetiological agent, which is also a calicivirus, is different from RHDV, although it is related antigenically. Alignment of the RNA sequences of the EBHSV and RHDV genomes shows 71% nucleotide identity, and amino acid alignment shows 78% identity and 87% similarity (27). Cross infection does not occur by experimental infection of rabbits with EBHSV and hares with RHDV (18). Recent studies aimed at finding the susceptibility of cottontail to EBHSV revealed a diffuse seroprevalence of the virus in a wild population of cottontail rabbits and the possibility of inducing clinical disease and mortality in a low number of experimentally infected cottontails (Lavazza, unpublished data).
RHD is characterised by high morbidity and a mortality rate between 40% and 90%. Infection occurs in rabbits of all ages, but clinical disease is observed only in adults and young animals older than 40–50 days. The pathogenic mechanism of resistance in young animals is still unclear (8).

The clinical evolution of the disease can be peracute, acute, subacute or chronic. The clinical manifestations have been described mainly in the acute infection, as there are usually no clinical signs in the peracute form, and the subacute form is characterised by similar but milder signs. The incubation period varies between 1 and 3 days; death may occur 12–36 hours after the onset of fever (>40°C). During an outbreak, a limited number of rabbits (5–10%) may show a chronic or subclinical evolution of the disease. These animals often die 1 or 2 weeks later, probably due to a liver dysfunction.

The gross pathological lesions are variable and may be subtle. Liver necrosis and splenomegaly are the primary lesions. However, a massive coagulopathy is usually the cause of haemorrhages in a variety of organs and sudden death. In subacute and chronic disease, an icteric discoloration on the ears, conjunctiva and subcutis is clearly evident.

The clinical signs and the gross and microscopic lesions observed in hares affected by EBHS are very similar to those described in RHD in rabbits. At necropsy, oedema and congestion of tracheal mucosa with foamy haemorrhagic contents, liver degeneration, enlargement of the spleen and generalised jaundice are the principal findings (8). The disease in hares lasts slightly longer and causes a lower mortality rate (around 50%) than RHD in rabbits; the peak of mortality in experimentally infected hares is commonly observed between 60 and 90 hours post-infection.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The liver contains the highest viral titre (from $10^3 \text{LD}_{50}$ [50% lethal dose] to $10^{6.5} \text{LD}_{50}$) and is the organ of choice for viral identification for both RHDV and EBHSV. The amount of virus present in other parts of the body is directly proportional to vascularisation; thus spleen and serum are quite rich in virus and can serve as alternative diagnostic materials. In particular, higher levels of subviral particles could be detected in the spleen than in the liver of those animals that died from a subacute/chronic form of the disease (2). The initial treatment of the diagnostic samples is almost identical irrespective of the diagnostic method to be applied, with the exception of immunostaining techniques. An organ fragment is mechanically homogenised in 5–20% (w/v) phosphate buffered saline solution (PBS), pH 7.2, filtered through cheesecloth and clarified by centrifugation at 5000 $g$ for 5 minutes. At this stage, the supernatant can be directly examined by the HA test or ELISA. If the sample is to be observed by electron microscopy (EM), it is advisable to perform a second centrifugation at 12,000 $g$ for 15 minutes, before the final ultracentrifugation.

As no satisfactory growth conditions and sensitive cell substrates have been established, in-vitro isolation of RHDV or EBHSV cannot be included among the virological methods. Inoculation of tissue suspensions from infected rabbits failed to produce disease and no replication of the virus was detected by reverse-transcription polymerase chain reaction (RT-PCR) in 28 different vertebrate species other than rabbits (24). Rabbit inoculation therefore remains the only way of isolating, propagating and titrating the infectivity of the virus.

Large quantities of viral antigen are prepared for diagnostic reagents and to produce inactivated tissue-derived vaccines. Experimental infection is not practical as a routine diagnostic method, although it could be useful in the case of samples that give equivocal test results (e.g. HA negative/ELISA positive) or for the initial diagnosis of the disease in countries where RHD is not known to exist.

To perform successful experimental trials, the rabbits involved must be fully susceptible to the virus, i.e. they should be over 40–50 days and have no specific antibodies, even at low titres. RHD can be reproduced by using filtered and antibiotic-treated liver suspensions, inoculated either by the intramuscular, intravenous or oro-nasal route. When the disease is clinically evident, the signs and post-mortem lesions are similar to those described after natural infection. A rise in body temperature is registered between 18 and 24 hours post-infection, followed, in around 70–90% of cases, by death between 24 and 72 hours post-infection. A few individuals may survive until 6 days after infection. Animals that overcome the disease show only a transient hyperthermia, depression and anorexia, but present a striking seroconversion that can be detected easily 4–6 days post-infection.

a) Haemagglutination test

HA was the first test to be used for routine laboratory diagnosis of RHD (19). It should be performed with human Group O red blood cells (RBCs), freshly collected, stored overnight in Alsever’s solution, and washed in 0.85% PBS at pH 6.5 (range 6–7.2). HA is less evident or non-existent when RBCs of other species are used. Washed RBCs are suspended at 0.75% in PBS. A twofold dilution of the clarified
supernatant of a 10% tissue homogenate of liver or spleen is incubated with an equal volume of washed RBCs in a sealed round-bottom microtitre plate at, preferably, 4°C. After 1 hour (range from 20 minutes to 2 hours) of incubation, agglutination at an end-point dilution of $>1/160$ is considered to be positive. Lower titres should be regarded as suspicious, and should be checked using other methods. Around 10% of samples found to be positive by ELISA or EM give negative results in HA (HA false-negative). Some RHD isolates may exhibit temperature-dependant differences in haemoagglutinating characteristics (3) and could show HA activity only when the test is performed at 4°C. Nevertheless, the HA false negativity is mainly detected in organs of rabbits showing a subacute/chronic form of the disease and it depends on the characteristics of the smooth, truncated s-RHDV particles.

Hare organs rarely give a significant titre when the RHDV HA protocol is used. To demonstrate HA activity in organs from EBHSV-infected rabbits, a modified procedure should be adopted: all steps are carried out at 4°C, the organ suspension is treated with an equal volume of chloroform, and RBCs are used at a pH not higher than 6.5 (8). Even using this method, only about 50% of the samples give positive results. This is because the disease of hares is often subacute or chronic and therefore the virus has the antigenic and structural characteristics typical of the s-RHDV particles (8).

Because of the practical difficulty of obtaining, keeping and the risk from working with human Group O blood cells, and because of the difficulty of obtaining consistent results, this test has been replace by the virus-detection ELISA.

b) Electron microscopy

Negative-staining EM can be performed using the so-called ‘drop method’. A formvar/carbon-coated grid is placed on a drop of organ suspension (prepared as described in Section B.1.a.), and left for 5 minutes. After removing excess fluid with the edge of a torn piece of filter paper, the grid is put to float on a drop of 2% sodium phosphotungstate (NaPT), pH 6.8, for 1.5 minutes. Excess stain is removed and the grid is finally observed at ×25,000 magnification.

Due to the lower sensitivity of the drop method, it is advisable to ultracentrifuge the sample in order to concentrate the viral particles. The pellet obtained after ultracentrifugation (at least 100,000 g for 30 minutes or, alternatively, using Beckman Airfuge at 21 psi for 5 minutes) is resuspended in PBS or distilled water, put on to a grid for a few minutes, and then stained as described. RHD virions are visible as uncoated particles, 32–35 nm in diameter, presenting an inner shell (25–27 nm in diameter), delineated by a rim from which radiate ten short regularly distributed peripheral projections. Smooth (s-RHDV) particles are identified by the complete loss of external portions, becoming perfectly hexagonal and smaller, with only the capsid rim visible (2, 8, 14).

For diagnostic purposes and especially when other methods give doubtful results, the best EM method is an immuno-EM technique (IEM). This method uses either a hyperimmune anti-RHDV serum, obtained from rabbit or other species, or specific MAbs, which are incubated with an equal volume of the sample for 1 hour at 37°C before ultracentrifugation. The immunological reaction induces the clumping of the viral particles into aggregates that are quickly and easily identified by EM. Immunogold methods can also be applied to better identify virions and viral proteins.

EBHSV can also be identified in diagnostic samples by EM examination. In addition, the IEM method using convalescent anti-EBHSV serum or specific anti-EBHSV MAbs can be used to identify EBHSV. By using antisera that is specific for EBHSV and RHDV, it is possible to differentiate between the two viruses.

c) Enzyme-linked immunosorbent assay

Virus detection by ELISA relies on a ‘sandwich’ technique and several variations of this have been described. One procedure uses the reagents, solutions, times and temperature that are used in the competitive ELISA (C-ELISA) for serology (see Section B.2.b.), except that the Tween 20 concentration is twofold (0.1% w/v). The microplate used should be of high adsorption capability (e.g. Nunc Maxisorp immunoplate). The liver homogenate is a 10% (w/v) suspension in standard PBS; 50 µl/well is the standard volume to use in each step. The ELISA buffer used for all steps is PBS with 1% yeast extract (or bovine serum albumin [BSA]), and 0.1% Tween 20, pH 7.4. All incubation steps are for 50–60 minutes at 37°C with gentle agitation. After all steps three washes of 3–5 minutes must be performed using PBS with 0.05% Tween 20. A positive and negative RHD rabbit liver homogenate must be used as controls. The horseradish peroxidase (HRPO) conjugate could be purified IgG from a specific polyclonal serum or MAbs (see Section B.2.b.). Anti-RHDV MAbs have been produced in several laboratories and can be used instead of rabbit polyclonal sera. More recently, MAbs recognising specific epitopes expressed only by the RHDVα variant were also produced (Capucci, pers. data).

To better characterise the antigenicity of the RHD isolates by sandwich ELISA, it is advisable to test each sample in four replicas, and then to use four different HRPO conjugates, i.e. two MAbs recognising the
same antigenic determinant present on the virus surface and expressed alternatively by the ‘classical’ strain or by the RHDV var variant, a polyclonal hyperimmune anti-RHDV serum (which could identify potential ‘new variant’ or correlated calicivirus, such as EBHSV) and a pool of MAbs recognising internal epitopes that can detect smooth, degraded s-RHDV particles as well as EBHSV. An alternative antigen-capture ELISA has been described using a sheep anti-RHDV as the capture antibody and an MAb for detection of RHDV (11).

**Test procedure (example)**

For steps that are not specifically indicated see the procedure of the C-ELISA for serology (Section B.2.b.).

i) Coat the plate with anti-RHDV hyperimmune serum and the negative RHDV serum. The latter serves as control for nonspecific reactions (false-positive samples). For each sample, four wells must be sensitised with the positive serum and four wells with the negative one.

ii) Dilute the liver extract to 1/5 and 1/30 (two replicates for each dilution) in ELISA buffer (see above), directly in the wells of the plate (e.g. add 45 µl of the buffer into all the wells of the plate, add 10 µl of the sample to the first two wells and then, after rocking, transfer 9 µl to the second wells). Treat the controls, both positive and negative, in the same way as the samples.

iii) After incubation and washing (see above), incubate with the HRPO conjugate.

iv) After a last series of washing, add the chromogenic substrate. Orthophenylene-diamine (OPD) must be used as peroxidase substrate for the final development of the reaction. Use 0.15 M citrate phosphate buffer, pH 5.0, with 0.5 mg/ml OPD and 0.02% H₂O₂. The reaction is stopped after 5 minutes by the addition of 50 µl of 1 M H₂SO₄.

v) Absorbance is read at 492 nm. Positive samples are those showing a difference in absorbance >0.3, between the wells coated with RHDV-positive serum and wells coated with the negative serum. Usually, at the dilution 1/30, positive samples taken from rabbits with the classical acute form of RHD give an absorbance value >0.8, while the absorbance value of the negative sample, at the dilution 1/5, ranges from 0.1 to 0.25.

For diagnosis of EBHSV, it is possible to use this RHDV-specific sandwich ELISA, but, due to the high antigenic difference existing between the two viruses, there is a risk of obtaining false-negative results. Therefore, the adoption of an EBHSV-specific sandwich ELISA technique using either a high-titre positive anti-EBHSV hare serum, or cross-reacting RHDV MAbs (5, 8), or specific EBHSV MAbs, instead of rabbit serum, is highly recommended (8).

d) Immunostaining

Tissue fixed in 10% buffered formalin and embedded in paraffin can be immunostained using an avidin–biotin complex (ABC) peroxidase method. The sections are first deparaffinised in xylene and alcohol, counter-stained with haematoxylin for 1 minute and rinsed in tap water. They are then put in a methanol bath containing 3% H₂O₂ and washed in PBS three times for 5 minutes each. To limit background interference due to nonspecific antibody binding, the samples are incubated with normal rabbit serum for 1 hour at room temperature prior to the addition of biotin. The slides are incubated overnight in a humid chamber at room temperature with biotinylated rabbit anti-RHDV serum or MAbs, are washed as before and incubated again for 30 minutes at 37°C with an ABC peroxidase. The slides are then washed three times. Amino-ethyl-carbazole is used as substrate. Finally, the slides are rinsed in tap water and mounted (25).

Intense nuclear staining and diffuse cytoplasmic staining of necrotic cells in the liver, mainly in the perportal areas, are characteristic and specific. Positive staining of macrophages and Kupffer’s cells is also observed, as well as hepatocellular reactions. Positive reactions can also be detected in the macrophages of the lungs, spleen and lymph nodes, and in renal mesangial cells (25).

Tissue cryosections fixed in methanol can be directly immunostained by incubation for 1 hour with fluorescein-conjugated rabbit anti-RHDV serum or MAbs. Specific fluorescence can be detected in the liver, spleen, and renal glomeruli.

e) Western blotting

When other tests such as HA or ELISA give doubtful results (low positivity) or the samples are suspected of containing s-RHDV particles, western blotting analysis is useful for determining the final diagnosis.

The samples are prepared as described previously, and are further treated in order to concentrate viral particles (tenfold) by ultracentrifugation (100,000 g for 90 minutes) through a 20% (w/w) sucrose cushion.
Both the supernatant and the pellet can be examined to detect, respectively, the RHDV 6S subunits (5) and the denatured VP60 structural protein of RHDV or its proteolytic fragments, which can range in size from 50 to 28 kDa. A positive and negative control sample should be used on each occasion.

Proteins could be detected with polyclonal antibodies or MAbs. If MAbs are used, they should recognise continuous epitopes. RHDV-specific MAbs recognising internal or buried epitopes could be used also to detect EBHSV. Rabbit anti-RHDV hyperimmune sera are less efficient than MAbs at recognising the same band patterns (6).

The proteins are denatured for 2 minutes at 100°C in the presence of 60 mM Tris, pH 6.8, 2% sodium dodecyl sulphate (SDS), 2% beta-mercaptoethanol, and 5% glycerol, separated on 10% SDS/PAGE (polyacrylamide gel electrophoresis), and then transferred by electoblotting to nitrocellulose or PVDF (polyvinylidene flouride) membranes, in 25 mM Tris, 192 mM glycine pH 8.3 and 20% (v/v) methanol at 1.5 Å for 60 minutes with cooling or at 0.15 Å overnight. After transfer the membranes are saturated for 30–60 minutes with 2% bovine serum albumin (BSA), dissolved in phosphate buffer, pH 7.4, and incubated for 2 hours at room temperature with the appropriate serum diluted in phosphate buffer, pH 7.4, and 1% BSA. The filters are washed thoroughly with PBS and incubated for 1 hour at room temperature with anti-species alkaline phosphatase-labelled immunoglobulins at a dilution predetermined by titration. Finally, the filters are again washed and the chromogenic substrate (5-bromo-4-chloro-3-indolylphosphate nitro blue tetrazolium) is added.

Positive test samples and the positive control will produce a pattern consistent with reaction to proteins of molecular weights of, respectively, 60 kDa (the single structural protein of RHDV) or 41–28 kDa (the fragments of the VP60 associated with the transition from RHDV to s-RHDV), when examining the pellet, and 6 kDa (the subunits) when examining the supernatant.

Western blot analysis can also be used to identify EBHSV. The test procedure is identical. The pattern of protein bands, detected using either an anti-EBHSV polyclonal serum or cross-reacting anti-RHDV MAbs, is similar. However, the percentage of samples showing viral degradation is higher and therefore several fragments of lower molecular weight, originating from the VP60 structural protein, are often observed.

f) Nucleic acid recognition methods

The application of the RT-PCR to the detection of RHDV-specific nucleic acid has been described by several authors (14, 17). This method is carried out on organ specimens, urine, faeces and sera using different oligonucleotide primers derived from the capsid region of the RHDV genome (N-terminal portion). cDNA obtained from the RT reaction is usually amplified as described by Baginski et al. (1). To reveal the PCR product, the amplified DNA reaction mixture is subjected to electrophoresis on agarose gel. If needed, specificity of the PCR product can be determined by sequencing or by transfer of the agarose gel on to a nylon filter that can be finally hybridised with a labelled internal probe, and examined by auto-radiography.

A similar RT-PCR method has been used to identify the nonpathogenic RCV (4). Several primers, specific for the RHDV RNA polymerase gene and complementary to the VP60 and ORF2 genes, are used and the amplified fragments are subjected to Southern blot analysis.

RT-PCR appears to be an extremely sensitive method for the detection of RHDV, and is 10⁴-fold more sensitive than ELISA (17). It is not strictly necessary for routine diagnosis, but it is more appropriate for studies on molecular epidemiology, to study the early stages of the infection and the pathogenesis of RHDV and to detect the virions in young animals (<40 days of age), in nonspecific hosts (other vertebrates) and vectors (mosquitoes and fleas).

An in-situ hybridisation technique using either sense or antisense DNA probes has been developed for investigating the presence of RHDV in tissue samples (13). This method is highly sensitive and can be used for early diagnosis of RHD as it gives positive results as soon as 6–8 hours post-infection. However, it is expensive and difficult to carry out, and thus it is mainly indicated for research studies.

2. Serological tests

Infection by RHDV can be diagnosed through detection of a specific antibody response. As the humoral response has great importance in protecting animals from RHD, determination of the specific antibody titre after vaccination or in convalescent animals is predictive of the ability of rabbits to resist RHDV infection. Some epidemiological data suggested the existence of an ‘apathogenic’ viral strain antigenically related to RHDV. In fact, the presence of so called ‘naturally acquired’ antibodies that confer full protection against virulent RHDV infection was shown in colonies where no previous outbreak had been reported or vaccination performed (24). The existence of nonpathogenic calcivirus provides a likely explanation for the early discrepancies found in
the course of serological surveys of the rabbit populations in European countries as well as in Australia and New Zealand.

Three basic techniques are applied for the serological diagnosis of RHDV: haemagglutination inhibition (HI) (19), indirect ELISA (I-ELISA) and C-ELISA (8). Each of these methods has advantages and disadvantages. With respect to the availability of reagents and the technical complexity of carrying out the test, HI is the most convenient method, followed by the I-ELISA and C-ELISA, respectively. On the other hand, both ELISAs are quicker and easier than HI, particularly when a large number of samples are tested. The specificity of the C-ELISA is markedly higher than those achieved with the other two methods (8). For improved serological interpretation and for correctly classifying the immunological status of rabbits, a combination of ELISA techniques that distinguish IgA, IgM and IgG antibody responses is also available. An alternative C-ELISA method has been described (10).

a) Haemagglutination inhibition

**Antigen:** The antigen is prepared using infected rabbit liver collected freshly at death. The liver is homogenised in 10% (w/v) PBS, pH 6.4, and clarified by two consecutive low speed centrifugations (500 g for 20 minutes and 6000 g for 30 minutes). The supernatant, drawn from the tube so as to avoid the superficial lipid layer, is filtered through a 0.22 µm pore size mesh, titrated by HA, and divided into aliquots, which are stored at −70°C.

**Serum samples:** Before testing, sera are inactivated by incubation at 56°C for 30 minutes. The sera are then treated with 25% kaolin (serum final dilution: 1/10) at 25°C for 20 minutes and centrifuged. This is followed by a second kaolin treatment, also at 25°C for 20 minutes, this time with 1/10 volume of approximately 50% packed human Group O RBCs. These are freshly collected, stored overnight in Alsever’s solution and washed in 0.85% PBS, pH 6.5. The sera are clarified by centrifugation.

- **Test procedure**
  i) Dispense 50 µl of serum into the first well of a round-bottom microtitre plate and make double dilutions into wells 2–8 using PBS with 0.05% BSA.
  ii) Add 25 µl of RHDV antigen containing 8 HA units to each well and incubate the plate at 25°C for 30–60 minutes.
  iii) Add 25 µl of human Group O RBCs at 2–3% concentration to each well and allow to settle at 25°C for 30–60 minutes.
  iv) Titrate the antigen with each test to ensure that 8 HA/25 µl were used, and include positive and negative serum controls.

The serum titre is the end-point dilution showing inhibition of HA. The positive threshold of serum titres is correlated to the titre of the negative control sera; it usually is in the range 1/20–1/80.

Because of the practical difficulty of obtaining, keeping and the risk from working with human Group O blood cells, and because of the difficulty in obtaining consistent results, this test is being superseded by the serological or antibody-detection ELISA.

b) Competitive enzyme-linked immunosorbent assay

**Antigen:** An international standard strain is not yet available; however, as only one serotype has been identified so far world-wide, reliable results can be obtained by different laboratories each using their own standard virus. Even the antibodies induced by the identified RHDV variants are recognised by the standard method described here. In addition, the test can also easily detect antibodies originating from infection of rabbits with the nonpathogenic RCV, due to its high genetic correlation with RHDV (6, 7).

The antigen can be prepared as described previously for HI (Section B.2.a.), taking care to store it at −20°C in the presence of glycerol at 50% (v/v) to prevent freezing. If necessary, the virus can be inactivated before the addition of glycerol, using 1.0% binary ethylenimine (BEI) at 33°C for 24 hours. Antigen must be pretitrated in ELISA and then used as the limiting reagent; i.e., the dilution that corresponds to 60–70% of the plateau height (absorbance value at 492 nm in the range 1.1–1.3).

**Anti-RHDV serum:** specific polyclonal sera with high anti-RHDV titre can be obtained in different ways. Two possible and currently used methods are as follows:

i) Rabbits are infected with a RHDV-positive 10% liver extract diluted 1/100 in PBS to obtain convalescent sera (21–25 days) containing a high level of anti-RHDV IgG. Due to the high mortality rate associated with RHDV, it is necessary to infect at least 10–15 seronegative rabbits or to infect rabbits that are only partially protected (e.g. 4–6 rabbits infected from 3 to 7 days post-vaccination). Rabbits that survive the infection must be bled 21–25 days post-infection to obtain the convalescent...
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sera. Alternatively, convalescent rabbits can be re-infected 3–4 months post-infection and bled 10–15 days later to obtain RHDV hyperimmune sera.

ii) RHDV is purified from the livers of experimentally infected rabbits that died from an acute form of the disease (between 28 and 40 hours post-infection), using one of the methods that has been published (5, 8, 9, 20, 22). Then the purified RHDV can be used to immunise sheep or goats according to classical protocols using oil adjuvants. The same procedure can also be used to inoculate rabbits if the purified virus is inactivated before inoculation.

Anti-RHDV MAbs may be used instead of rabbit polyclonal sera. Purification of rabbit IgG and conjugation to HRPO can be done following the standard protocols. The conjugated antibody is tinted in a sandwich ELISA in the presence and absence of RHDV antigen (negative rabbit liver). It is then used at the highest dilution showing maximum (plateau high) absorbance (if the serum had a good anti-RHDV titre, the value of the HRPO conjugate should range from 1/1000 to 1/3000).

Control sera: Negative serum is taken from rabbits fully susceptible to RHDV infection. Positive serum is either a convalescent serum diluted 1/100 in a negative serum or a serum taken from a vaccinated animal.

- Test procedure (example)
  i) The rabbit anti-RHDV serum diluted to a predetermined titre, e.g. 1/5000 in 0.1 M carbonate/bicarbonate buffer, pH 9.6, should be adsorbed to an ELISA microplate of high adsorption capability (e.g. Nunc Maxisorb Immunoplate) at 4°C overnight.
  ii) Wash the plate three times for 3–5 minutes each time, in PBS, pH 7.4, with 0.05% Tween 80 (PBST). When the plates are not immediately used, they can be stored, closed in a plastic bag, for at least 3 months at –20°C.
  iii) Distribute 25 µl/well PBST with 1% yeast extract (PBSTY) or 1% BSA (PBST-BSA) to all the wells needed on the plate (see below). Add 7 µl of the first serum sample to the first two wells (A1 and B1), 7 µl of the second serum to the second two wells (C1 and D1), and continue with the third (E1 and F1) and the fourth (G1 and H1) sera, thus completing the first column. If qualitative data (positive/negative) are needed, repeat the operation in the second column with sera samples from 5 to 8, and in the third column with sera samples from 9 to 12, and so on. If the titre of the serum needs to be determined, the serum must be diluted further. Agitate the plate and then use an eight-channel micropipette to transfer 7 µl from the wells in column 1 to the wells in column 2. This corresponds to a four-fold dilution of the sera. This last operation can be repeated once (titre 1/160), twice (titre 1/640), or four times (titre 1/10,240). Either in the case of testing sera for qualitative data (single dilution), or for getting the final titre (several dilutions), complete each plate leaving 12 wells free for the control sera. Add 7 µl of positive sera to wells G6 and H6, and 7 µl of negative sera to wells G9 and H9, then dilute them once and twice (1/40–1/160).
  iv) Add 25 µl/well antigen suspended in PBSTY to all the wells on the plate, at a dilution that is double the decided dilution, as described above in the antigen section (see the first part of this ELISA method description).
  v) Incubate the plate at 37°C on a rocking platform for 50 minutes.
  vi) Wash the plate as described in step ii.
  vii) Add 50 µl/well rabbit IgG anti-RHDV conjugated with HRPO at the decided dilution, as described above in the ‘anti-RHDV serum’ section (see the first part of this ELISA test description).
  viii) Incubate the plate at 37°C on a rocking platform for 50 minutes, and wash as described in step ii adding a fourth wash of 3 minutes duration.
  ix) Use 50 µl/well OPD as hydrogen donor under the following conditions: 0.5 mg/ml OPD in 0.1 M phosphate/citrate buffer, pH 5, and 0.02% H₂O₂. Stop the reaction after 5 minutes by addition of 50 µl/well 1 M H₂SO₄.
  x) Read the plate on a spectrophotometer using a 492 nm filter.

The serum titre corresponds to the dilution giving an absorbance value equal to 50% (±10) of the value of the negative serum at dilution 1/160 (reference value).

The serum is considered to be negative when the absorbance value of the first dilution (1/10) decreases by less than 20% of the reference value, while it is positive when the absorbance value decreases by 30% or more. When the absorbance value of the 1/10 dilution decreases by between 20% and 30% of the reference value, the sera is considered to be doubtful. A wide range of titres will be found, depending on the origin of the sample. Positive sera range from 1/640 to 1/10,240 in convalescent rabbits, from 1/80 to 1/640 in vaccinated rabbits and from 1/10 to 1/160 in ‘nonpathogenic’ infection. Knowing the origin of the sample
allows a choice to be made between testing one or more dilutions. Testing only the first dilution gives a positive or negative result. The titre is established by testing all dilutions, up to the sixth one.

Due to the significant antigenic differences existing between RHDV and EBHSV (8, 27), the serological techniques described above, which use RHDV as antigen, are not recommended for the serological diagnosis of EBHS. However, a direct ELISA method could be employed for the detection of positive and negative EBHSV hare sera; in fact, the adsorption of RHDV on to the solid phase of an ELISA microplate exposes cross-reactive antigenic determinants. Alternatively, a specific C-ELISA for EBHSV can be arranged in a similar way, using specific reagent (antigen and antisera) prepared as described above for RHDV.

c) Isotype enzyme-linked immunosorbent assays (isoELISAs)

These ELISAs enable the detection and titration of isotypes IgA, IgM and IgG (7). The isotype titres are critical for the interpretation of field serology in four main areas: cross-reactive antibodies, natural resilience of young rabbits, maternal antibodies, antibodies in previously infected rabbits (12).

To detect RHDV-specific IgG, one RHDV-specific MAb is adsorbed to the Maxisorp plate at a concentration of 2 µg/ml by the method described above for the polyclonal serum in the C-ELISA (see above Section B.2.b, test procedure step i). Virus is added to the plates at a concentration double that used in the C-ELISA and after incubation and washing, sera are added and serially diluted four-fold starting from 1/40. An MAb anti-rabbit IgG HRP conjugate is used to detect IgG bound to the virus. The final step for the isoELISAs for IgG, IgM and IgA is the addition of OPD and H₂SO₄ as for the C-ELISA. To detect IgM and IgA isotypes the phases of the ELISA reaction are inverted in order to avoid competition with IgG, which is usually the predominant isotype. MAb anti-rabbit IgM or anti-rabbit IgA is adsorbed to the wells and then the sera are diluted as described above. Incubation with the antigen follows and then HRP-conjugated MAb is used to detect the RHDV bound to the plate. Sera are considered to be positive if the OD₅₂₉ (optical density) value at the 1/40 dilution is more than 0.2 OD units (two standard deviations) above the value of the negative serum used as a control. The titre of each serum is taken as the last dilution giving a positive value. Because isoELISA tests do not follow identical methodology, equivalent titres do not imply that isotypes are present in the same amounts.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

In all the countries where RHD is endemic, indirect control of the disease is achieved by vaccination using the appropriate type of vaccine – one that is prepared from clarified liver suspension of experimentally infected rabbits, and that is subsequently inactivated and adjuvanted. The methods of inactivation (formaldehyde, beta-propiolactone or other substances) and the adjuvants used (incomplete mineral oil or aluminium hydroxide), can vary according to the protocol used by the different manufacturers.

In the last ten years several studies were carried out on the expression of RHDV capsid protein in Escherichia coli, in vaccinia virus, and in attenuated myxomavirus (MV). Moreover, it has been shown by various authors that a recombinant capsid protein, VP60, expressed in the baculovirus/Sf9 cell expression system, self-assembled into virus-like particles (VLPs) that are structurally and antigenically identical to RHD virions. While the fusion protein expressed in E. coli is highly insoluble and of low immunogenicity, active immunisation can be achieved with VLPs obtained in the baculovirus system by using recombinant vaccinia, MV and canarypox, administered either intramuscularly or orally. In particular rabbits vaccinated with recombinant MV expressing the RHDV capsid protein were protected against lethal RHDV and MV challenges. The resulting recombinant virus was also capable of spreading horizontally and promoting protection of contact animals, thus providing the opportunity to immunise a wild rabbit population (26). Similarly, the immunogenicity of VLPs administered by the oral route as an alternative to parenteral immunisation offers an economical and practical way to administer a vaccine for mass immunisation of wild animals.

More recently, the VP60 structural protein has been expressed in transgenic plants, either with a new plum pox virus (PPV)-based vector (PPV-NK), or in transgenic potato plants under the control of a cauliflower mosaic virus 35S promoter or a modified 35S promoter. In both cases the immunisation of rabbits with extracts of Nicotiana clevelandii plants infected with the PPV-NK VP60 chimera and with leaf extracts from potatoes carrying this modified 35S promoter, respectively, induced an efficient immune response that protected animals against a lethal challenge with RHDV.

However, at the present time, recombinant vaccines are not yet registered and commercially available.

In France, a vaccine (Dercunimix®, Merial) has recently been registered and commercialised that is a combination of a traditional inactivated liver-derived RHD vaccine and a live attenuated Myxovirus vaccine, and which can be administered by the intradermal route.

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The usual programme is to administer the inactivated vaccine twice with an interval of at least 2 weeks. Normally, a 1-ml dose is inoculated subcutaneously in the neck region. In those units with no history of disease, where the anamnesis for RHD is negative, it is advisable to vaccinate only the breeding stock; the first injection should be given at 2–3 months of age. Annual revaccination is strongly recommended to ensure a good level of protection, although experimental data indicate that protection usually lasts for a long time (over 1 year). The vaccination of meat animals is not necessary if disease has not occurred on the farm. Following an outbreak of RHD, even if strict hygiene and sanitary measures are adopted, including cleaning and disinfection, safe disposal of carcasses and an interval before restocking, it is strongly recommended to vaccinate meat animals at the age of 40 days, because the incidence of re-infection is very high. Only after several production cycles is it advisable to stop vaccination of meat animals. In order to verify the persistence of infective RHD inside the unit, a variable number of rabbits, starting with a small sentinel group, should not be vaccinated.

Vaccinated animals quickly produce strong immunity against RHDV infection, therefore vaccination is considered to be effective in protecting non-exposed rabbits and its primary use is in rabbitries after an outbreak of the disease has been diagnosed; once RHD has been confirmed in some sick or dead rabbits, the remaining healthy animals are immediately vaccinated.

The administration of immune serum is also effective in producing a rapid, but short-lived, protection against RHDV infection.

Vaccine should be stored at 2–8°C and it should not be frozen, or exposed to bright light or high temperatures.

1. Seed management

a) Characteristics of the seed

The source of seed virus for the production of inactivated tissue vaccines is infected liver homogenates obtained by serial passages in rabbits that have been inoculated with a partially purified RHD viral suspension. The latter is obtained by centrifuging the 1/5 liver suspension (w/v) in PBS at 10,000 g for 20 minutes at 4°C. The resulting supernatant is treated with 8% (v/v) polyethylene glycol (PEG 6000) overnight at 4°C. The pellet is resuspended at a dilution of 1/10 in PBS, and subsequently centrifuged at 10,000 g for 20 minutes at 4°C. The supernatant is ultracentrifuged at 80,000 g for 2 hours at 4°C through a 20% cushion of sucrose. The pellet is resuspended in PBS (1/100 of the starting volume). This viral suspension is then characterised by negative-stain EM examination, determination of reactivity in ELISA, and capability of HA at room temperature with slow elution (HA titre against RBCs of human Group O higher than 1/1280). Seed virus is titrated before use and should contain at least 10^5 LD50. It should be stored frozen (−70°C) or freeze-dried.

b) Method of culture

At present, RHDV replication can be obtained exclusively in susceptible animals. The rabbits used for inoculation are selected from colonies shown to be susceptible to the disease by periodic serological testing. The animals (at least 4 months old) must be kept in strict quarantine on arrival, in a separate area and reared under satisfactory health conditions (see Laboratory animal facilities in Chapter I.1.6.). Seed virus propagation and production of vaccine batches relies on the same protocol of experimental infection, involving intramuscular injection of a dose of at least 100 LD50.

c) Validation as a vaccine

The seed virus used for vaccine production must be shown to be free from other viruses, bacteria, mycoplasma and fungi. Seed virus is controlled by direct inoculation into susceptible rabbits followed by evaluation of the clinical signs in the course of the experimental infection. Suitable seed virus should cause the death of 70–80% of the rabbits within 24–72 hours post-inoculation, with the internal organ lesions characteristic of RHD. To validate the test, gross and histopathological examination of all rabbits should be performed to exclude intercurrent diseases.

2. Method of manufacture

Following inoculation of susceptible rabbits, the liver and spleen of those rabbits that die between 24 and 72 hours post-inoculation are collected. The organs are minced in 1/10 (w/v) sterile PBS, pH 7.2–7.4, and the mixture is homogenised for 10 minutes in a blender in a refrigerated environment. The mixture is then treated with 2% chloroform (18 hours at 4°C), followed by centrifugation at 6000 g for 1 hour at 4°C. The supernatant is collected by high pressure continuous pumping and is subsequently inactivated. The viral suspension is assayed by HA test and ELISA (see Section C.3.) and, once the number of HA units from the initial titration is known, more sterile PBS is added in sufficient volume to provide, after inactivation and adsorption on adjuvant, a concentration of 640–1280 HA units/dose in the commercial product. Various agents have proved effective at
abolishing viral infectivity. The most frequently used are formaldehyde and beta-propiolactone, which can be used at different concentrations and temperatures, for variable periods of time and also in combination. During inactivation, it is advisable to continuously agitate the fluid. Aluminium hydroxide, Freund's incomplete adjuvant or another oil emulsion is then incorporated into the vaccine as adjuvant. A preservative, thiomersal (merthiolate), is finally added at a dilution of 1/10,000 (v/v) before distribution into bottles.

3. In-process control

**Antigen content:** The RHD titre is determined before inactivation by calculating the HA titre, which should be higher than 1/1280, and the ELISA reactivity. Both values are again determined after inactivation and adsorption on adjuvant. Negative-staining EM confirms the identity of RHD.

**Sterility:** The organs are tested for the presence of viable bacteria, viruses, fungi or mycoplasma according to the protocol used for testing master seed virus. PBS solution and aluminium hydroxide gel are sterilised by autoclaving; oil emulsion is sterilised by heating at 160°C for 1 hour.

**Inactivation:** Before incorporation of the adjuvant, the inactivating agent and the inactivation process must be shown to inactivate the vaccine virus under the conditions of manufacture. Thus, a test is carried out on each batch of the bulk harvest as well as on the final product. Five rabbits are inoculated with a 2-ml dose of the suspension and five unvaccinated rabbits are kept as controls. After 10 days, adequate inactivation and absence of undesirable side-effects are demonstrated by the absence of clinical signs and by similar weight increments in the two groups. At the end of the trials, the animals are slaughtered and liver extracts are tested by HA, ELISA and EM.

4. Batch control

Sterility, safety and potency tests should be carried out on each batch of final vaccine; tests for duration of immunity should be carried out once using a typical batch of vaccine, and stability tests should be carried out on three batches.

a) **Sterility**

   Each batch of vaccine must be tested for the presence of viable bacteria, viruses, fungi or mycoplasma according to the same protocol recommended for testing master seed virus.

b) **Safety**

   Ten rabbits should be inoculated by the recommended routes with three times the vaccinal dose. The rabbits are observed for 3 weeks. No abnormal local or systemic reaction should develop.

c) **Potency**

   Ten seronegative adult rabbits that are at least 4 months old are vaccinated with one full dose of vaccine given by the recommended route. Two other groups of five animals each are vaccinated with 1/4 and 1/16 of the full dose, respectively. A fourth group of ten unvaccinated rabbits is maintained as controls. All animals are challenged at 4 weeks post-vaccination by intramuscular inoculation of a dose of RHDV containing at least 100 LD$_{50}$ or presenting a HA titre higher than 1/2560. No vaccinated rabbits should show signs of infection, while the mortality rate among control animals should be higher than 70%. The antibody response of each vaccinated animal is then determined with reference to titrated standard antisera; the mean antibody level should not be significantly less than the level recorded in the protection test performed using as vaccine the inactivated seed virus.

d) **Duration of immunity**

   The data reported in the literature indicate a long-term duration of immunity induced by a single vaccination (up to 15 months). However, it is advisable to carry out the following test: 20 rabbits vaccinated once are divided into four groups and are serologically tested at monthly intervals over a period of 1 year. Each group is inoculated with virulent RHDV at 3, 6, 9 months or 1 year post-vaccination (see Section C.4.c.). Challenge infection should produce increasing seroconversion, which is directly related to the time that has elapsed since vaccination. The absence of clinical signs and of mortality proves that the RHDV has not multiplied.

e) **Stability**

   Evidence should be provided to show that the vaccine passes the batch potency test at 3 months beyond the suggested shelf life.
f) **Preservatives**
   A suitable preservative is normally required for vaccine in multidose containers (see Section C.2.). Its persistence throughout shelf life should be checked.

**g) Precautions (hazards)**
   When oil-emulsion vaccines are prepared, vaccinators should be warned against the risk and consequences of accidental self-injection, which must be treated urgently as a ‘grease-gun’ injury.

5. **Tests on the final product**
   The tests for safety, potency and sterility of the final product must be performed after bottling and packaging. Thus, it is important that these two last manufacturing steps be performed following standardised good manufacturing procedures. The tests are conducted by removing samples from a statistically determined number of randomly taken multidose containers (20 or 100 doses) of vaccine.

a) **Safety**
   See Section C.4.b.

b) **Potency**
   See Section C.4.c.

c) **Sterility**
   See Section C.4.a.

**REFERENCES**


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NB: There is an OIE Reference Laboratory for Rabbit haemorrhagic 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).
INTRODUCTORY NOTE ON BEE DISEASES

Bees are insects that are closely related to ants and wasps. There are many thousands of species of bee, most of which are not social insects, living solitary lives. The honey bee, Apis species, lives as a colony, which is a family of social insects. There are many species, subspecies, races and subraces of honey bees that are adapted to their environment.

Two species are important for bee keeping – the western honey bee Apis mellifera, and the eastern honey bee A. cerana. The Africanised bee, which is found in South and Central America and some states of the United States of America, is a cross between two subspecies of the western honey bee, the European bees and the South African bee. Apis cerana is important in South and South-East Asia. The colonies are small and docile, but the honey yields are low. In a suitable climate, the western honey bee, A. mellifera, is sometimes preferred for its greater honey production.

It is thought that all bees are susceptible to the known diseases of bees, but different races may have varying susceptibility. For example, A. cerana is less susceptible to varroosis. When sampling a colony of bees for diagnosis of diseases, live bees must first be killed with diethyl ether or in a deep freezer (−20°C) overnight. Bees may also be killed by submersion in 70% ethyl alcohol, e.g., when collected for diagnosis of acariosis (Acarapis). Larval and pupal smears must be made when testing for brood diseases or a piece of comb containing brood showing visible signs of disease may be sent to the laboratory.

* * *
CHAPTER 2.9.1.

ACARIOSIS OF BEES

SUMMARY

Acariosis or acarine disease is a disease of the adult honey bee Apis mellifera L. and other Apis species. It is caused by the Tarsonemid mite, known as the tracheal mite, Acarapis woodi (Rennie). The mite is approximately 150 µm in size, and it is an internal parasite of the respiratory system, living and reproducing mainly in the large prothoracic trachea of the bee. Sometimes they are also found in the head, thoracic and abdominal air sacs. The mites feed on the haemolymph of their host.

The pathogenic effects found in infected bees depend on the number of parasites within the trachea and are attributable both to mechanical injuries and to physiological disorders consequent to the obstruction of the air ducts, lesions in the tracheal walls, and the depletion of haemolymph. As the parasite population increases, the tracheal walls, normally white and translucent, become opaque and discoloured with blotchy black areas, probably due to melanin crusts.

The mortality rate may range from moderate to high. Early manifestations of infection normally go unnoticed, and only when infection is heavy does it become apparent. This is usually in the early spring. The infection spreads by direct contact. Generally, only newly hatched bees under 10 days old are susceptible. Reproduction occurs within the tracheae of adult bees, where female mites may lay 8–20 eggs. There are 2–4 times as many females as males. Development takes 11–12 days for males and 14–15 days for females.

Identification of the agent: The parasites are demonstrated only by laboratory methods and under the microscope. The mites need to be observed inside the tracheae or removed from them to be observed microscopically. Several techniques are available for demonstrating the mites, such as dissection, grinding and staining.

The thoraces of suspect bees are dissected to expose the trachea. Each trachea is examined under a dissecting microscope (×18–20), where the mites will be seen through the transparent wall as small oval bodies.

Alternatively, larger samples of suspect bees can be ground or homogenised in water, followed by coarse filtration of the suspension, and centrifugation. The deposit is treated with undiluted lactic acid for 10 minutes. This is then mounted for microscopic examination.

The parasites may be stained by histological techniques so that they can be observed within the bee trachea. The tracheae are separated out, cleared with 8% potassium hydroxide, and stained with 1% methylene blue. This is the best method for large numbers of samples.

Serological tests: Serological tests are not available.

Requirements for vaccines and diagnostic biologicals: There are no biological products available. Menthol crystals or oil patties made with vegetable oil (not animal fat) and white granulated sugar will keep mite levels under control.

A. INTRODUCTION

Acariosis is a disease of the adult honey bee Apis mellifera L. and other Apis species, caused by the microscopic Tarsonemid mite Acarapis woodi (Rennie). The mite is approximately 150 µm in size, and it is an
internal parasite of the respiratory system (Figure 1). These tracheal mites enter, live, and reproduce mainly in the large prothoracic tracheae of all bees, feeding on the haemolymph of their host (Figure 2). Sometimes they are also found in the head, thoracic and abdominal air sacs (5).

**Fig. 1.** *Acarapis woodi* Rennie. Top: Adult male, Centre: Adult Female, Bottom: Egg.

**Fig. 2.** Main thoracic tracheae of honey bee where *Acarapis* is commonly found; light infestations are near the spiracle opening.
Chapter 2.9.1. — Acariosis of bees

The pathogenic effects on individual bees depend on the numbers of parasites within the tracheae, and are attributable both to mechanical injuries and to physiological disorders consequent to the obstruction of the air ducts, lesions in the tracheal walls, and to the depletion of haemolymph. As the parasite population increases, the tracheal walls, which are normally whitish and translucent, become opaque and discoloured with blotchy black areas, probably due to melanin crusts (4).

The mortality rate may range from moderate to high. Early signs of infection normally go unnoticed, except for a slow dwindling in the colony size. Only when infection is heavy does it become apparent. This is generally in the early spring after the winter clustering period when the mites have bred and multiplied undisturbed into the longer-living winter bees. This applies mainly to the Northern Hemisphere where there are seasonal variations in the reproduction of bees.

Infection spreads from one bee to another by direct contact. Generally, only newly hatched bees under 10 days old, are susceptible. Attempts to rear A. woodi on artificial and synthetic diets have been unsuccessful, while culturing them on immature stages of the honey bee itself has been only partially successful (6). The life span of the mites in dead bees is approximately 1 week. Reproduction occurs within the tracheae of adult bees, where female mites may lay 8–20 eggs. There are 2–4 times as many females as males; development takes 11–12 days for males and 14–15 days for females.

There are no reliable clinical signs for the diagnosis of acarosis as the signs of infection are not specific and the bees behave in much the same way as do bees affected by other diseases or disorders. They crawl around in the front of the hive and climb blades of grass, unable to fly. Dysentery may be present.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Acarosis can be detected only in the laboratory using microscopic examination or an enzyme-linked immunosorbent assay (ELISA). There is no reliable method for detection of very low levels of infection. The number of bees sampled determines the detection threshold of the method. It has been shown that a 2% rate of infection can be detected by sampling 50 bees, while a 1% rate of infection is detected using 100 bees (confidence limit is 80% for a colony of average size in spring). Because of the high level of manual work involved, it is suitable to examine 50 bees. Sequential sampling data are given in ref. 15. The best time to take bee samples is in the early spring or late autumn (Northern hemisphere), when Acarapis populations are high. Visualisation of mites is easier in older bees, which have more mites. Samples of queens, drones or workers can be used, but Acarapis prefer drones.

a) Dissection (7)

A sample of 50 bees is collected at random from the suspected colony. These are mainly bees crawling and unable to fly, found within about 3 metres of the front of the hive. This is preferable to random collection from within the colony. The bees may be living, dying, or dead. Live bees must first be killed with ethyl alcohol or in a deep freezer (~20°C); bees must not have been dead for over 2–3 days unless kept at 4°C for up to 4 weeks or –20°C for up to 2 months. They may be preserved indefinitely in a preservative such as Oudemann solution: glacial acetic acid (80 ml); glycerol (50 ml); 70% ethanol (870 ml).

• Test procedure
  i) Lay and secure bees on their backs or hold with thumb and first finger.
  ii) Remove the heads and forelegs using a small forceps and remove the collar surrounding the neck opening to expose the tracheae (Figure 3). Check the tracheae nearest to the spiracle (as mites enter through the spiracle) to see light infestations. Heavy infestations are easily visible as shadows or dark objects in clear to dark brown tracheae. Old and heavy infestations will make the tracheae brown to black.
  iii) Cut through the thorax in front of the middle pair of legs and the base of the forewings with a sharp razorblade. These thin disks can be further treated to clear muscle tissue.
  iv) Macerate either by gentle heating in an 8% solution of potassium hydroxide for approximately 20 minutes or by leaving them to stand overnight without heating.
  v) Examine the first pair of tracheae, which are covered by muscle tissue, under a dissecting microscope at a magnification of ×18–20, or transfer the tracheae to another slide, add glycerin or water and observe at higher magnification.
  vi) Mites are easily seen through the transparent wall as small, oval bodies.
This is the simplest and most reliable technique for the laboratory diagnosis of acariosis, allowing the detection of early infections and enabling the infection rate to be established. Even light infections can be detected by the use of a dissecting microscope with this technique. Only in very exceptional instances will it be necessary to employ higher magnifications in order to make a diagnosis. However, this is a demanding technique, especially when a large number of acariosis diagnoses have to be made. If it is necessary only to distinguish between heavily infected and lightly or non-infected colonies, dissection can be stopped at step ii and the colour of the tracheae observed.

b) Grinding (3)

A sample of about 200 bees is collected at random from the suspect colony. The wings and legs of each bee are removed from the thorax, and the bodies are pooled in a 100 ml container that has been one-quarter filled with water. This suspension is homogenised three times, each time for several seconds, in a homogeniser at 10,000 rpm with the addition of more water. The resulting suspension is strained through a sieve (mesh 0.8 mm) and the sieve is rinsed with water to a final volume of approximately 50 ml. The filtrate is centrifuged at 1500 g for 5 minutes and the supernatant fluid is discarded. A few drops of undiluted lactic acid solution are added to the debris of the deposit, which will contain the mites. This is left for 10 minutes to allow the muscle fibres to dissolve, and is then mounted under a cover-slip for microscopic examination. This technique is quicker than dissection, but may be less accurate. External mites A. externus, A. vagans and A. dorsalis, all of which are morphologically similar to A. woodi, are often found on the thorax of healthy bees and can very easily be mistaken for A. woodi (Table 1). It seems, however, that they do not cause any serious threat to bees or beekeeping. This method should therefore only be chosen if all that is required is a rough estimation of the degree of infection in a region. It is not suitable for determining a first outbreak.

<table>
<thead>
<tr>
<th>Table 1. Differential diagnosis of Acarapis species (13)</th>
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<tbody>
<tr>
<td>Character</td>
</tr>
<tr>
<td>Notch of the coxal plate</td>
</tr>
<tr>
<td>Space between stigmata</td>
</tr>
<tr>
<td>Length of tarsal limb (IV leg pair)</td>
</tr>
</tbody>
</table>

c) Staining (9)

The mites and trachea can be stained specifically, rendering them easily visible by microscopy.

- Test procedure
  i) Remove the head and forelegs.
  ii) Make a transverse cut through the membranous areas behind the forelegs.
  iii) Make a second transverse cut in front of the middle pair of legs at the base of the forewings.
  iv) To clear the sections (1–1.5 mm thick), place them in an 8% solution of potassium hydroxide.
  v) Stir gently and heat near to boiling point for approximately 10 minutes until the soft internal tissues are dissolved and cleared, leaving the chitinous tissues intact.
vi) Retrieve sections by filtration and wash with tap water.

vii) Stain and mount the sections.

viii) Examine for mites by low-power microscopy.

Permanent mounts are prepared by the usual histological techniques.

Cationic stains are the most suitable and specific as they stain the mites intensely but the tracheae only weakly. A solution of 1% aqueous methylene blue is the most suitable, prepared by dissolving the methylene blue first and then adding sodium chloride to make a 0.85% NaCl solution.

- Test procedure
  i) Stain in 1% aqueous methylene blue.
  ii) Differentiate sections in distilled water for 2–5 minutes.
  iii) Rinse the sections in 70% alcohol.

When kept in 95% ethanol, the mites will retain the stain for 6 hours (1). It is essential with this technique to macerate the tissues effectively in the potassium hydroxide solution. Using this method, it is possible to process a large number of samples rapidly and conveniently.

d) Enzyme-linked immunosorbent assay

An ELISA for trachea mites has been developed (8, 11, 12). This test may produce false-positive results, and is therefore only recommended for survey examinations. Another method is the visualisation of guanine, a nitrogenous waste product of mites (10).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available. Menthol crystals (50 g for a two story colony) control mites if left in the colony for 28 days, providing the ambient temperature is at least 18°C. The optimum temperature range for the vapours to work is 27–29°C. Small cakes made with vegetable shortening (e.g. margarine, not animal fat) and white granulated sugar will keep mite levels to 10%. The cake (about 100 g in weight) should be placed on the top bars of the frames in the brood nest in the autumn and early spring (14). Formic acid may be used to treat infected colonies.

Some races of bees, such as Buckfast bees (2) and some hygienic strains, are less susceptible to attack by Acarapis.

ACKNOWLEDGEMENTS

Illustrations by Diana Sammataro and are reproduced with her permission.

REFERENCES


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NB: There are OIE Reference Laboratories for Bee diseases (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

American foulbrood affects the larval stage of the honey bee Apis mellifera and other Apis spp., and occurs throughout the world. Paenibacillus larvae subsp. larvae (White), the causative organism, is a bacterium that can produce over one billion spores in each infected larva. The spores are extremely resistant to heat and chemical agents, and only the spores are capable of inducing the disease.

Identification of the agent: Combs of infected colonies have a mottled appearance due to a mixture of healthy capped brood, uncapped cells containing the remains of diseased larvae, and empty cells. This is not characteristic only of American foulbrood. Cell cappings of a diseased larva appear moist and darkened, becoming concave and possibly punctured as infection progresses. The larval or pupal colour changes to creamy brown and then to a dark brown with a ropy appearance when drawn out. A distinctive odour develops in the advanced stage. The diseased brood eventually dries out to form characteristic brittle scales that adhere tightly to the lower sides of the cell. The formation of a pupal tongue is one of the most characteristic, but rarely seen, signs of the disease and precedes the formation of the scales.

The method of choice for diagnosis of American foulbrood depends on whether clinical signs of the disease are present or not. In case of clinical illness, a range of simple confirmational laboratory techniques are available. Some of them require the isolation of the pathogenic agent by subculturing. The presence of heat-resistant spores, growth characteristics of the bacterium, colony morphology, combined with the following simple laboratory tests are considered to be conclusive: Gram staining, catalase test and nitrate reductase test (facultative). Thorough identification of the isolated bacteria can be done by biochemical profiling or polymerase chain reaction (PCR). The latter also permits direct examination of the larval remains without the previous long cultivation step. Antibody-based techniques are useful when no cross-reactivity with other bacilli has been demonstrated, for instance against Paenibacillus alvei, often found in late phase European foulbrood.

When clinical signs are absent or information on the appearance of the brood combs are missing (examination of honey bee products) a thorough identification of the pathogenic agent is recommended. This can be done by biochemical profiling (on suspicious isolated colonies) or by PCR (directly on the samples or after cultivation). Only experienced persons can rely on growth characteristics, colony morphology, and the above-mentioned simple confirmational laboratory techniques alone.

Serological tests: There are no serological tests available.

Requirements for vaccines and diagnostic biologicals: There are no biological products available.

A. INTRODUCTION

American foulbrood is a disease of the larval stage of the honey bee Apis mellifera and other Apis spp., and occurs throughout the world where such bees are kept. Paenibacillus larvae subsp. larvae (White), the causative organism, is a bacterium that can produce over one billion spores in each infected larva. The bacterium is a round-ended, straight and sometimes curved rod, which varies greatly in size (0.5 µm wide by 1.5–6 µm long), occurring singly and in chains and filaments; some strains are motile. The sporangia are often sparse in vitro, and the ellipsoidal, central to subterminal spores, which may swell the sporangia, are often found free (15). The
spores are extremely heat stable and resistant to chemical agents. Only spores are capable of inducing the disease.

The infection can be transmitted to a larva by nurse bees or by spores remaining at the base of a brood cell. Although the larval stages of worker bees, drones and queens are susceptible to infection, infected queens and drone larvae are rarely seen under natural conditions. The susceptibility of larvae to American foulbrood disease decreases with increasing age (32); larvae cannot be infected later than 53 hours after the egg has hatched. The mean infective dose ($\text{ID}_{50}$= spore dose at which 50% of the larvae are killed) needed to initiate infection, though very variable, is 8.49 spores in 24–48-hour old bee larvae (13). Exchanging combs containing the remains of diseased larvae is the most common way of spreading the disease from colony to colony. In addition, feeding or robbing of spore-laden honey, artificial swarms and the introduction of queens from infected colonies can also spread the disease. The early detection of American foulbrood helps to prevent further spread.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

A healthy larva has a glistening, pearly white appearance. It first develops at the base of the cell in the shape of the letter ‘C’ and subsequently grows upright to fill the cell. Infected larvae die in this erect position. In severely infected colonies, the combs appear to be mottled due to a pattern of healthy capped brood, uncapped cells containing the remains of diseased larvae, and empty cells. The capping of a cell that contains a diseased larva appears moist and darkened and becomes concave and punctured as the infection progresses. Also, the larva or pupa changes colour, first to a creamy and eventually to a dark brown. The larvae become glutinous in consistency and can be drawn out as threads when a probe is inserted into the larval remains and removed from the cell. A distinctive odour develops at this stage, resembling that of animal glue. Finally, after 1 month or more, the remains of the diseased brood dry out to form typical hard, dark scales that are brittle and adhere strongly to the lower sides of the cell (Figure 1). If death occurs in the pupal stage, the formation of the pupal tongue, a protrusion from the pupal head that traverses the top of the brood cell, is one of the most characteristic signs of the disease, although it is rarely seen (Figure 2c). The tongue may persist also on the dried scale.

The method of choice for diagnosis of American foulbrood depends on whether clinical signs of the disease are present or not. In case of clinical illness, a range of simple confirmational laboratory techniques are available. Some of them require the isolation of the pathogenic agent by subculturing. The presence of heat-resistant spores, growth characteristics of the bacterium, colony morphology, combined with the following simple laboratory tests are considered to be conclusive: Gram staining, catalase test and nitrate reductase test (facultative). Thorough identification of the isolated bacteria can be done by biochemical profiling or polymerase chain reaction (PCR). The latter also permits direct examination of the larval remains without the previous long cultivation step. Other methods for direct examination of larval remains are described: the modified hanging-drop technique, the Holst milk test and different antibody-based techniques. The modified hanging-drop technique, based on the Brownian movements of $P. l. larvae$ spores and their morphology, is poorly specific. The same is
true for the Holst milk test, based on a high level of proteolytic activity during sporulation of *P. l. larvae*. Both tests should not be used alone. Antibody-based techniques are useful when no cross-reactivity with other bacilli has been demonstrated, for instance against *Paenibacillus alvei*, often found in late phase European foulbrood.

When clinical signs are absent or information on the appearance of the brood combs is missing (examination of honey bee products) a thorough identification of the pathogenic agent is recommended. This can be done by biochemical profiling (on suspicious isolated colonies) or by PCR (directly on the samples or after cultivation). Only experienced persons can rely on growth characteristics, colony morphology, and the above-mentioned simple confirmational laboratory techniques alone.

**Fig. 2. Clinical American foulbrood (a–c) and Gram staining (d): (a) Combs have mottled appearance. (b) A matchstick draws out the brown, semi-fluid larval remains in a ropy thread. (c) The formation of a pupal tongue is a very characteristic sign, but rarely seen. (d) Microscopic examination of isolated colonies reveals Gram-positive rods, occurring singly and in chains.**

#### a) Sample preparation

When the typical signs of disease are observed at the apiary it is recommended to send to the laboratory a piece of brood comb of about 20 cm², containing as much of the dead or discoloured brood as possible. Little or no honey should be present in the sample. The sample can be loosely wrapped in paper, and wrappings, such as plastic bags, aluminium foil, waxed paper, tin or glass, should be avoided as these materials allow samples to become mouldy, making an accurate diagnosis almost impossible. The sample can be dispatched in a heavy cardboard or wooden box. If a portion of comb cannot be sent, the probe used to examine cell contents must have enough material on it for any test. This too can be wrapped in paper or put into an appropriate tube. However, such a small sampling size can only be considered when the sampler has sufficient expertise or is well trained to identify the diseased areas on the comb.

Sometimes, the larval remains are difficult to locate because of the condition of the comb. Scale material can be conveniently located by using ultra-violet or near ultra-violet light. Exposure between 310 and 400 nm will cause any scale material to fluoresce. Some discretion must be used when using this technique as both honey and pollen will also fluoresce.

When macroscopic examination at the apiary by an experienced sampler reveals that the brood combs have a healthy appearance, honey samples may be sent for laboratory analysis. Samples of food supplies collected from sealed cells close to the brood nest can be taken with a spoon and transferred into a plastic bag or tube (31). Harvested honey ready for sale can be taken as well, although this does not enable the identification of diseased colonies if there are spores present in the sample. Samples size should be 30–50 g.
b) Culture techniques

To culture *P. l. larvae* from larval remains, spore suspensions are prepared by mixing diseased material in 5–10 ml of sterile water, physiological solution (phosphate buffered saline or 0.9% NaCl) or liquid medium (same composition as the solid media listed below, but without agar) in a test tube. All culture media should be subjected to quality control and must support the growth of *P. l. larvae* from small inocula. The reference strain should also be cultured in parallel with the suspect samples to ensure that the tests are working correctly.

Honey samples to be examined for spores are heated to 45–50°C and shaken to distribute any spores that may be present. Dilution with an equal volume (25 ml) of water permits easier handling. The diluted honey is transferred into 44 mm width dialysis tubing that has been tied at one end. The open end is tied after filling. The tubes are submerged in running water for 18 hours or in a water bath with 3–4 water changes over the same time period. After dialysis, the contents are centrifuged at 2000 g for 20 minutes. The supernatant liquid is discarded leaving approximately 1 ml (or less) of residue in each sample. The deposit is then resuspended in 9 ml of water (29).

Honey can also be prepared for culturing without the dialysis step, however this requires longer (30 minutes) and faster centrifugation (3000 g). Likewise, the volume in which the deposit finally is resuspended can be kept much smaller (200 µl) in order to improve the sensitivity of the test (6). Whatever the method of choice will be, when the outcome of honey analyses is done in a quantitative way and threshold values are set, the methodology that was used to establish these values should always be strictly followed.

Both the sample preparations derived from brood comb samples and honey samples can be processed in the same way from this point on. The suspension is heated at 80°C for 10 minutes to kill nonsporulating bacteria. A sterile cotton swab is used to transfer a portion of the suspension on to the surface of Petri dishes containing solid medium, which are then incubated for 2–4 days at 34–37°C. For a quantitative evaluation, it is recommended to spread a fixed volume of the suspension on solid agar with a sterile scraper rather than using cotton swabs. Inoculated plates are best incubated in an atmosphere of 5–10% CO₂ in air, although aerobic incubation will do as well.

Different solid media can be used including brain–heart infusion agar supplemented with thiamine HCl (29), J-medium (19), MYPGP (7), Michael’s medium (5) and Columbia agar containing 5% horse blood (15). The latter may become discoloured or partially haemolysed when *P. l. larvae* grows on it.

Samples from clinically diseased larvae will result in confluent grown plates after 2–4 days, leading to a subculturing step in order to have isolated colonies. On Columbia blood agar, colonies are small (< 1 mm in diameter), regular, glossy, butyrous, and greyish or discoloured with blood pigments (15). On Michael’s medium, the colonies are whitish, opaque, flattened, with irregular edges and usually with a diameter of 1–3 mm (5). Inexperienced technicians are advised to run *P. l. larvae* reference strains in parallel, for instance LMG 9820 (other designation: ATCC 9545). A proven positive brood or honey sample might serve as a positive control for the entire examination.

Difficulties can occur when the spore suspensions contain other spore-forming bacteria, which may completely overgrow the culture. If so, it is recommended to supplement the solid medium with nalidixic acid (18) and/or pipemidic acid (2). Stock solutions are prepared by dissolving 0.3 g nalidixic acid or 0.4 g pipemidic acid in 2 ml 1 N NaOH and diluting to 100 ml with 0.01 M phosphate buffer or water, sterilised by filtration and stored under refrigeration. These stock solutions are added to molten agar medium to give a final concentration of 6–9 µg/ml nalidixic acid and 10–20 µg/ml pipemidic acid.

Spores similar to *P. l. larvae* have also been recovered from bees wax by chloroform extraction (21) and from pollen by an aqueous filtration (10).

c) Simple confirmational tests

*Paenibacillus l. larvae* is a Gram-positive rod with some characteristics that make it distinguishable from many other bacilli that contaminate bees and bee products. Indeed, the bacterium is catalase negative (14) and reduces nitrate to nitrite (23). In consequence, a number of simple laboratory tests can confirm American foulbrood if clinical signs are observed and when cultivation of heat-treated samples yields colonies with the characterised growth velocity (slow) and colony morphology (see above) containing Gram-positive rods.

*Catalase test*: a drop of 3% hydrogen peroxide is placed on an actively growing culture on solid medium. Most aerobic bacteria break down the peroxide to water and oxygen, producing a bubbly foam, but *P. l. larvae* is almost always negative for this reaction (14). When Columbia blood agar is used for cultivation, the test cannot be done on the solid medium as the presence of horse blood will cause a false-positive
reaction. In this case, colonies should be transferred to a clean microscope slide for the execution of the test.

Nitrate reductase test: the bacterium can be grown on a medium such as brain–heart infusion agar containing potassium nitrate (1–2 mg/litres of medium). When growth has occurred, the addition of a drop of sulphanilic acid-alpha-naphthyl reagent produces a red colour if any nitrate has been reduced to nitrite. Nitrate reductase negative *P. l. larvae* strains have also been described (16, 20).

d) Biochemical profile

When the analyst cannot rely on the presence of clinical signs (for instance, examination of honey bee products) or when the disease is still in its subclinical phase, a more profound identification of the pathogenic agent is recommended. Biochemical profiling of suspicious isolated colonies, in addition to their basic characteristics (heat-resistance, growth velocity, colony morphology and bacterium morphology) can be considered to be conclusive. The biochemical profiling includes – besides the above-mentioned catalase and nitrate reductase tests – the production of acid from carbohydrates, the hydrolysis of starch and casein, the use of citrate and the liquefaction of gelatin.

Production of acid from carbohydrates (11): bacteria are grown in J-broth (same composition as J-medium, but without agar) in which 0.5% of the test substrate, separately sterilised in aqueous solution, is substituted for the glucose. The carbohydrates used are L (+)-arabinose, D (+)-glucose, D (+)-xylose and D (+)-trehalose. The cultures are tested at 14 days by aseptically removing 1 ml or less to a spot plate, mixing the sample with a drop of 0.04% alcoholic bromocresol purple, and observing the colour of the indicator.

*Paenibacillus l. larvae* produces acid aerobically from glucose and trehalose. No acid is produced from arabinose and xylose (1). Differentiation between *P. l. larvae* and *P. l. pulvificiens* – the latter associated with the rare disease named ‘powdery scale’ – can be done based on acid production from mannitol and salicin (15). This seems to be one of the few characteristics that allow differentiation at the subspecies level. In addition, *P. l. pulvificiens* also grows at 20°C (*P. l. larvae* does not) and some strains produce yellow/orange-pigmented colonies (15).

Hydrolysis of starch (11): 1 g of potato starch is suspended in 10 ml of cold distilled water, mixed with 100 ml of J-medium without glucose, autoclaved, cooled to 45°C, and then thoroughly mixed and poured into five Petri dishes. After 3 days’ storage at room temperature (to allow the excess moisture to evaporate), duplicate plates are streaked with each culture. The plates are flooded with Gram’s iodine after 5 and 10 days’ incubation. After 15–30 minutes, the unchanged starch becomes white and opaque. A clear zone underneath (after the growth was scraped off) and around the growth, measures the hydrolysis of the starch. *Paenibacillus l. larvae* strains do not hydrolyse starch.

Hydrolysis of casein (5): the medium that is used for this test is composed of a solution A (10 g skim milk powder, 90 ml distilled water) and a solution B (3 g agar, 97 ml distilled water) that are sterilised separately (121°C for 20 minutes), brought to 45°C in a double boiler and then mixed. The medium thus prepared (25 ml) is poured into Petri dishes, which are inoculated with two to three drops of a young (3–4 days) culture in semisolid J-medium. After 14 and 21 days’ incubation, a small amount of the culture is mixed on a spot plate with phenol red indicator. An alkaline reaction signifies use. *Paenibacillus l. larvae* does not use casein.

Use of citrate: this is tested using semisolid J-medium without glucose but supplemented with 2 g of sodium citrate (11). The medium, sterilised in test tubes, is inoculated with two to three drops of a young (3–4 days) culture in semisolid J-medium. After 14 and 21 days’ incubation, a small amount of the culture is mixed on a spot plate with phenol red indicator. An alkaline reaction signifies use. *Paenibacillus l. larvae* does not use citrate.

Growth in nutrient broth (11): bacteria are inoculated into a tube of nutrient broth (3 g beef extract, 5 g peptone, 1000 ml distilled water) and incubated either until growth occurs or for 14 days. If the culture grows, a loopful is transferred to another tube of nutrient broth. This procedure is repeated serially either for ten successive serial transfers or until growth fails. Only cultures that survive ten serial transfers are considered able to grow in nutrient broth. *Paenibacillus l. larvae* is unable to withstand serial transfer in nutrient broth (1). On the contrary, *P. l. pulvificiens* can grow on this routine medium (15).

Liquefaction of gelatin (11): cultures in tubes of plain gelatin (120 g gelatin, 1000 ml distilled water, pH 7.0) incubated at 28°C are tested for liquefaction at 3- to 4-day intervals for 4 weeks. Before testing, cultures are placed at 20°C for approximately 4 hours to allow the unchanged gelatin to harden. *Paenibacillus l. larvae* causes gelatin to liquefy (1).

The use of commercial kits, such as API 50 CHB (5) and BBL CRYSTAL (8), for the biochemical characterisation of *P. l. larvae* can be taken into consideration. However, as it was proven that these kits
produce different results for some of the biochemical reactions, a profile for \( P. l. larvae \) has to be drawn up for each system independently (8).

e) **Polymerase chain reaction**

The PCR is a genetic fingerprint technique that permits the identification of suspicious bacterial isolates and the detection of \( P. l. larvae \) in clinically and subclinically diseased larvae and honey bee products. Sample treatment depends on the application of the test.

One colony of a suspicious colony is suspended in 50 µl of distilled water and heated to 95°C for 15 minutes (12). Following centrifugation at 5000 \( g \) for 5 minutes, 1 µl of the supernatant is used as template DNA in a PCR 50 µl mixture containing 2 mM MgCl₂, 50 pmol of a forward and a reverse primer (primer sequences are given below), a 25–200 mM concentration of each deoxynucleoside triphosphate, and 1–1.25 U of \( Taq \) polymerase. Amplification of a specific DNA fragment occurs in a thermocycler under the following PCR conditions: a 95°C step (1–15 minutes); 30 cycles of 93°C (1 minute), 55°C (30 seconds) and 72°C (1 minute); and a final cycle of 72°C (5 minutes). The molecular weights of the PCR products are determined by electrophoresis in a 0.8% agarose gel and staining with ethidium bromide.

The remains of two diseased honey bee larvae are suspended in 1 ml of sterile distilled water and mixed thoroughly; 100 µl of this suspension is diluted with 900 µl distilled water. This dilution is vortexed and 100 µl of it is used to extract DNA by heating and centrifugation (see above) (9). The PCR method remains the same for the different applications.

The above-mentioned preparation method for template DNA based on heating and centrifugation can only be used when vegetative stages of the bacteria are present. Indeed, spore suspensions are centrifuged at 6000 \( g \) and 4°C for 30 minutes. The pellet is then subjected to microwave treatment for 5 minutes at maximum power to break the spores, and the released DNA is suspended in 30 µl of 10 mM Tris/HCl, pH 8.0, containing 1 mM ethylene diamine tetra-acetic acid (EDTA). When spores are to be detected from honey, DNA was serially diluted with sterile distilled water to eliminate PCR inhibition caused by honey (28). Another DNA extraction method, based on lysozyme and proteinase K treatment, has been described (4).

Good results can also be obtained by incubating a pelleted spore suspension (for instance, from a honey sample or subclinically infected larvae) in MYPGP broth at 37°C for 2–24 hours. Thereafter, the suspension is centrifuged at 14,500 \( g \) for 5 minutes, washed with sterile distilled water and resuspended in 200 µl of sterile distilled water. This short incubation step causes spores to hatch, making them sensitive for DNA preparation by heat treatment again (see above) (22).

Several primer combinations based on the 16S rRNA gene are proven to be species specific. Differentiation between \( P. l. larvae \) and \( P. l. pulvifaciens \) is possible with the primer set provided by Piccini et al., when the number of cycles is reduced from 30 to 25 (28). The sequences of the primers are:

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Direction</th>
<th>Sequence</th>
<th>PCR-product size</th>
<th>Specificity level</th>
</tr>
</thead>
<tbody>
<tr>
<td>(12)</td>
<td>forward</td>
<td>5'-AAG-TCG-AGC-GGA-CCT-TGT-GTT-TC-3' 5'-TCT-ATC-TCA-AAA-CCG-GTC-AGA-GG-3'</td>
<td>973 bp</td>
<td>species</td>
</tr>
<tr>
<td>(9)</td>
<td>reverse</td>
<td>5'-CTT-GTG-CTT-TCT-CTG-GAA-GAC-GCC-A-3' 5'-TCT-TAG-AGT-GCC-CAC-CTC-TGC-G-3'</td>
<td>1106 bp</td>
<td>species</td>
</tr>
<tr>
<td>(28)</td>
<td>reverse</td>
<td>5'-CGA-GCG-GAC-CTT-GTG-TTT-CC-G-3' 5'-TCA-GTT-ATA-GGC-CAG-AAA-GC-3'</td>
<td>700 bp</td>
<td>subspecies</td>
</tr>
</tbody>
</table>

Identification of the two subspecies is also possible by further digest of a PCR-amplified 16S rDNA fragment with the endonuclease HaellI (3). For the latter, the primers to be used have a much lower specificity and amplify the 16S rRNA genes from Bacillus, Paenibacillus, Brevibacillus and Virgibacillus species.

f) **Modified hanging-drop technique**

The modified hanging-drop technique (21) can be done directly on larval remains. However, due to its low specificity this technique is not conclusive.

Suspect material is mixed with water, and a drop of this suspension is placed on a cover-slip, dried and fixed by heat, and stained with carbol fuchsin or a suitable spore stain for 30 seconds. Any excess stain is washed off with water. While the preparation is still wet, the cover-slip is inverted on to a slide on which
there is a very thin layer of immersion oil. Excess water will emerge. The slide is gently blotted dry and
examined by high-power microscopy. By examining fields where pockets of water have formed in the oil, the
spores of *P. l. larvae* will be seen exhibiting Brownian movement. Spores from other bacilli often remain
fixed; in addition this technique allows the microscopic examination of the characteristic morphology of the
foulbrood spores. If the infection is under 10 days old, long vegetative forms of the bacterium are present
and some newly formed spores may be seen (24).

g) The Holst milk test

The Holst Milk test (17) uses the fact that a high level of proteolytic enzymes is produced by sporulating
*P. l. larvae*. The test is performed by suspending a suspect scale, or a smear of a diseased larva, in a tube
containing 1–4 ml of 1% powdered skim-milk in water. The tube is then incubated at 37°C. If *P. l. larvae* is
present, the suspension will clear in 10–20 minutes.

h) Antibody-based techniques

Different antibody-based techniques have been developed for the diagnosis of American foulbrood. Most of
them rely on polyclonal rabbit serum developed against pure cultures of *P. l. larvae*. In an immunodiffusion
test, the antibodies interact with the bacterial antigen during a double diffusion process, leaving precipitation
marks behind (27). In the fluorescent antibody technique these antibodies are conjugated with a
fluorochrome dye. The resulting fluorescent antibody reacts with a bacterial smear on a slide. Any excess
antiserum is washed off and the smear is examined by fluorescence microscopy. *Paenibacillus l. larvae*
stains specifically as brightly fluorescing bacteria on a dark background (26, 30, 33). Antibody-based
techniques are useful when no cross-reactivity with other bacilli has been demonstrated, for instance
against *Paenibacillus alvei*, often found in late phase European foulbrood.

An enzyme-linked immunosorbent assay using a monoclonal antibody specific to *P. l. larvae* exists (25).

2. Serological tests

No serological tests are available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No vaccines or diagnostic biological products are available.

ACKNOWLEDGEMENT

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respectively Ruth Waite and Frans J. Jacobs.

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**NB:** There are OIE Reference Laboratories for Bee diseases (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](http://www.oie.int)).
CHAPTER 2.9.3.
EUROPEAN FOULBROOD

SUMMARY

The causal organism of European foulbrood of honey bees is the bacterium Melissococcus pluton. The identification of its presence by the observation of signs of disease in the field is unreliable. The most usual and obvious sign is the death of larvae shortly before they are due to be sealed in their cells, but this may be for reasons other than European foulbrood. Most infected colonies display few visible signs, which themselves often quickly abate spontaneously before the end of each active season. Infection remains enzootic within individual colonies because of mechanical contamination of the honeycombs by the durable organism. Recurrences of disease can therefore be expected in subsequent years.

Identification of the agent: Examination, by high-power microscopy, of suitable preparations of larval remains for the presence of numerous lanceolate cocci is adequate for most practical purposes, especially when it is done by experienced individuals.

The only certain means of making a diagnosis is by isolating and identifying the causative organism. This can be differentiated quite readily from all other bacteria associated with bees by its fastidious cultural requirements.

The isolated bacterium can be identified and differentiated by means of simple tube agglutination tests.

Serological tests: No tests for detecting antibodies in bees are available.

Requirements for vaccines and diagnostic biologicals: There are no biological products available.

A. INTRODUCTION

Bee larvae usually die of European foulbrood 1–2 days before being sealed in their cells, or sometimes shortly afterwards, and always before transformation to pupae. The disease is caused by Melissococcus pluton and occurs mostly during the period when colonies are growing quickly. Most sick larvae become displaced from their coiled position in the bottom of their cells before they die. Many are quickly detected and removed by nurse bees, leaving empty cells scattered randomly among the remaining brood.

Infected larvae that escape detection by adult bees and then die, first become flaccid and turn a light yellow colour that becomes increasingly brown, and at the same time they dissolve into a semi-liquid mass. They then become dry and form a dark brown scale that can easily be removed from the cells. Severely affected brood may have a very stale or sour odour, sometimes acidic, like vinegar, but often there is no smell.

Signs of disease usually disappear spontaneously from infected colonies before the end of the active season, but are likely to return in subsequent years (3, 10).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Microscopy

Freshly dead larvae are best for diagnosis. Before any decomposition occurs, diseased larvae can be smeared on a microscope slide or pulled apart by pinching the cuticle about the centre of the body with two
pairs of forceps, which are then pulled apart. The mid-gut contents are left exposed on the slide, still within the gelatinous, transparent peritrophic membrane. This is partially or almost completely filled with bacteria, which are easily seen as opaque chalk-white clumps. The contents of the mid-guts of healthy larvae, which are less easily dissected, have a golden-brown colour. Apparently healthy larvae may contain a mixture of bacteria and pollen. The mid-guts of healthy larvae that contain much light-coloured pollen may resemble those that are filled with bacteria.

For a bacteriological investigation, a loopful of a dilute aqueous suspension of the mid-gut contents is transferred to a clean microscope slide and mixed with a loopful of 5% aqueous nigrosin. This is spread over one or two square centimetres, dried gently over a flame, and examined directly by high-power microscopy. The presence of numerous lanceolate cocci, about 0.5 x 1.0 μm in size, occurring either singly or in clusters, and arranged end to end in pairs or short chains, is almost certainly diagnostic of European foulbrood. Some very slender square-ended rod-like bacteria are also usually present (Figure 1). The cocci are Gram positive and the rods are Gram negative. Similar preparations made from aqueous suspensions of whole dead or decomposing larvae are likely to present a confusing array of bacteria in which M. pluton will be difficult to distinguish.

b) Culture methods

Melissococcus pluton (type strain NCDO 2443) is the most abundant bacterium during the early stages of an infection (4, 5). Melissococcus pluton can be cultivated on a medium (expressed in g/litre or ml/litre) comprising: yeast extract or certain peptones, 10 (4); cysteine or cystine, 0.2–2.0; glucose or fructose, 10; soluble starch, 10; 1 M KH₂PO₄, 100 at pH 6.6; and agar, 2. The medium is preferably autoclaved in 100 ml lots in screw-capped bottles at 116°C for 20 minutes and poured into Petri plates immediately before use. These plates are streaked with dilute aqueous suspensions of diseased larvae, or ideally, of diseased larval mid-guts. The latter can be prepared beforehand by allowing them to dry on a slide, which may then be kept, for years if necessary, at 4°C or –20°C. All culture media should be subjected to quality control and must support the growth of M. pluton from small inocula. The reference strain should also be cultured in parallel with the suspect samples to ensure that the tests are working correctly. The preparation and storage of dried smears also eliminates most secondary organisms after a few weeks without affecting the viability of M. pluton. This organism is isolated most efficiently by inoculating decimal dilutions of the aqueous suspension into agar that has been maintained molten at 45°C and which is then poured into plates. The plates must be incubated anaerobically, such as in McIntosh and Fildes jars in an atmosphere of approximately 5–10% carbon dioxide (CO₂) at 35°C. Small white opaque colonies of M. pluton usually appear within 4 days. This bacterium is somewhat pleomorphic in vitro, often appearing in rod-like forms. The final pH of the medium may reach 5.5. Decreasingly fastidious strains become selected in vitro. Simplified or modified forms of the medium then support multiplication, especially of a serologically distinct M. pluton group from Brazil (1) that will multiply on chemically defined media (2). CO₂ remains essential. Incubated slopes should be sealed when bacterial growth is apparent and may then be kept at 4°C for up to 6 months. Alternatively, the cultures can be suspended in a medium of 10% sucrose, 5% yeast extract and 0.1 M KH₂PO₄, pH 6.6, and then lyophilised.

A number of other bacteria are often associated with and may be confused with M. pluton. Bacterium eurydice inhabits the alimentary tract of adult bees and occurs commonly in the gut of healthy larvae in small numbers. It is more numerous in larvae infected with M. pluton. The incidence of B. eurydice in healthy bees is very low in winter and early spring, but it increases in summer. It forms thin square-ended rods, which can grow either singly or in chains. When grown in certain media, it sometimes resembles streptococci and has been confused with M. pluton. However, its cultural characteristics closely resemble
those of *Corynebacterium pyogenes* (10), and it multiplies poorly in the form of thin rods, under the conditions necessary for the cultivation of *M. pluton*.

*Enterococcus (=* *Streptococcus*) *faecalis* closely resembles *M. pluton* morphologically and has often been confused with it, although they are both culturally and serologically distinct. Unlike *M. pluton*, it does not remain viable for long when dried, or persist as mechanical contamination within bee colonies. It is probably brought into the hive by foraging adult bees, and is responsible for the sour smell sometimes encountered with European foulbrood.

*Enterococcus faecalis* grows well *in vitro* under the conditions suitable for *M. pluton*, but it may be readily differentiated by its ability to grow aerobically. It forms small transparent colonies within 24 hours and is a facultative anaerobe. It multiplies on a variety of the more common media with or without carbohydrates or CO₂. The final pH in the presence of glucose is 4.0. *Enterococcus faecalis* rarely exceeds the number of *M. pluton* in bee larvae, and can usually be diluted out. When it is not diluted out it produces sufficient acid to prevent the *in-vitro* multiplication of *M. pluton*.

*Enterococcus faecalis* does not multiply in bee larvae in the absence of *M. pluton*, so its presence in large numbers can be taken as presumptive evidence of European foulbrood.

*Paenibacillus (= *Bacillus*) *alvei* is generally more common than *E. faecalis* in bee colonies affected with European foulbrood, but it is not invariably associated with the disease and so cannot act as a reliable indicator of it. In bee colonies, it multiplies only in the decomposing remains of larvae, and then its spores often predominate over all other bacteria, even to their apparent exclusion. *Paenibacillus alvei* forms very resistant spores and becomes well established in bee colonies with enzootic European foulbrood. It causes a characteristic stale odour. *Paenibacillus alvei* multiplies poorly under the conditions necessary for the *in-vitro* growth of *M. pluton*. It produces a spreading growth of transparent colonies, some of which are motile and move in arcs over the surface of the agar. Cultures have the characteristic stale odour that is associated with European foulbrood when the bacillus is present. Spores are formed rapidly.

c) Immunological methods

For the identification of *M. pluton*, antisera can be prepared in rabbits against washed cultures of *M. pluton* either by intravenous injections (6) or by a single intramuscular injection of 1 ml of antigen suspension mixed with an equal volume of Freund’s incomplete adjuvant.

Assays are made by agglutination tests in tubes containing suspensions of bacteria equivalent to 0.25 mg dry weight/ml. End-points are noted after tubes have been incubated for 4 hours at 37°C.

d) Nucleic acid recognition methods

Detection of *M. pluton* by polymerase chain reaction has been reported (7, 9). Specific primers to regions of the 16S rRNA gene have been identified, but the technique is not yet widely used. DNA profiles of *M. pluton* have been described and DNA restriction endonuclease (*AluI*) analyses could be used to reveal genotypic diversity among geographically diverse isolates of *M. pluton* (8).

2. Serological tests

No tests for detecting antibodies in bees are available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available.

ACKNOWLEDGMENT

Illustrations by Karl Weiss, extracted from Bienen-Pathologie, 1984. Reproduced with the kind permission of the author and Ehrenwirth-Verlag, Munich (Germany).

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CHAPTER 2.9.4.

NOSEMOSIS OF BEES

SUMMARY

The microsporidium Nosema apis (Zander) is a parasite of the adult honey bee that invades the epithelial cells of the ventriculus. Infections are acquired by the uptake of spores during feeding or grooming. The disease occurs throughout the world, but testing of bees can help to prevent the spread of infection to unaffected bee colonies.

The parasite invades the posterior region of the ventriculus, giving rise to large numbers of spores within a short period of time. The parasite is ubiquitous and occurs in greatest numbers in the spring when there is an increase in the brood. The disease is transmitted among bees via the ingestion of contaminated comb material and water, and by trophallaxis; honey stores and crushed infected bees may also play a role in disease transmission. Spores are expelled with the faeces where they may retain their viability for more than 1 year. Spores may also remain infective after immersion in honey and in the cadavers of infected bees; however they may lose viability after 3 days when submerged in honey at hive temperature.

The relative importance of faeces, honey and cadavers as reservoirs of infective spores is not fully understood. However, it seems likely that faecal contamination of wax, especially in combs used for brood rearing, or other hive interior surfaces, provides sufficient inoculum for Nosema apis to be successfully transmitted to the next generation of bees. The spores are inactivated by acetic acid or by heating to 60°C for 15 minutes. To be effective, these treatments, which inactivate spores on hive surfaces, need to be combined with feeding colonies with the antibiotic fumagillin to suppress infections in live bees.

Identification of the agent: In some acute cases, brown faecal marks are seen on the comb, with sick or dead bees in the vicinity of the hive. However, the majority of colonies show no obvious signs of infection, even when the disease is sufficient to cause significant losses in honey production and pollination efficiency. During winter, there may be an increase in bee mortality. In affected bees, the ventriculus, which is normally brown, is white and very fragile. Microscopic examinations of homogenates of the abdominal contents of affected bees will reveal the oval spores of Nosema, which are approximately 5–7 µm with a dark edge. Their internal contents cannot be distinguished when fresh spores are viewed using bright-field or phase-contrast microscopy. After staining with Giemsa’s stain, Nosema spores have a distinctive appearance, with a thick unstained wall and a blue-stained featureless interior. The nuclei within the spores are not visible. This method can help to distinguish N. apis from other microbes found in bees.

The appearance of Nosema spores can be confused with yeast cells, fungal spores, fat and calciferous bodies or cysts of Malpighamoeba mellifica. The latter are similar in size to Nosema spores, being 6–7 µm in diameter, but are completely spherical instead of oval.

Positive identifications can be made only by observation of typical spores in the ventriculus or faeces. Very mild infections may not be demonstrable. The extent of infection is determined by counting the spores on a microscope grid and calculating the average number of spores per area and estimating from that the number of spores per bee.

Serological tests: There are no applicable serological tests.

Requirements for vaccines and diagnostic biologicals: There are no biological products available.
**A. INTRODUCTION**

The microsporidium *Nosema apis* (Zander) is a protozoan parasite exclusive to the epithelial cells of the ventriculus of adult bees and the disease occurs throughout the world (15). Infection occurs by the ingestion of spores in the feed (5, 13), via trophallaxis (19) or perhaps after grooming of the body hairs (6, 10, 17).

The polar tube of the spore is everted and penetrates the peritrophic matrix of the intestine, particularly in the posterior region of the ventriculus. The sporoplasm passes down the tube and enters the cytoplasm of the epithelial cells, where it reproduces. Autoinfections can occur at the same time as new infections. After a short interval, spores develop in large quantities. Infected bees are unable to fly and have been shown to be infected with up to 500 million spores.

The parasite is ubiquitous and multiplies at a specific rate throughout the year, with maximum numbers occurring during spring, coinciding with the increase in the brood (17, 20). In winter, spores are rarely to be found, or are only found in heavily infected bees.

Any inherent natural defence by a bee colony against a heavy infection with the parasite depends on the colony size as well as on the prevailing weather conditions during the early part of the autumn of the previous year (18). If these conditions are unfavourable, the overall life expectancy of the colony is reduced. This may lead to the premature death of bees during winter or early spring. In a typical case of a colony being depleted because of a *Nosema* infection, the queen can be observed surrounded by a few bees, confusedly attending to brood that is already sealed.

In faecal droppings, spores may retain their viability for more than 1 year (3). Spores may also remain viable for up to 4 months after immersion in honey (21) and for up to 4.5 years in the cadavers of infected bees (12). The spores may lose viability after only 3 days when submerged in honey at hive temperature (14). It is likely that faecal contamination of wax, especially in combs used for brood rearing, or other hive interior surfaces, provides sufficient inoculum for *N. apis* to be successfully transmitted to the next generation of bees. The relative importance of faeces, honey and cadavers as reservoirs of infective spores is not fully understood and it seems that temperature may have a marked effect on the rates at which spores lose viability, regardless of their medium (14).

Spores may be killed by heating hive equipment or tools to a temperature of at least 60°C for 15 minutes. Combs may be sterilised by heating to 49°C for 24 hours (8). Fumes from a solution of at least 60% acetic acid will inactivate any spores within a few hours, depending on the concentration; higher concentrations are even more effective and will kill spores within a few minutes (2, 9). Such procedures come under the jurisdiction of national control authorities with protocols that vary from country to country. Disinfection can be carried out, for example, by putting acetic acid solution into bowls or onto sponges that can soak up the liquid. Following disinfection after an outbreak, all combs should be well ventilated for at least 14 days prior to use. Suppression of *Nosema* disease can also be achieved by feeding an antibiotic, fumagillin (also known as Fumidil B), in sugar syrup to the colony. It is thought to work by preventing the parasite from multiplying in adult bees that have ingested the antibiotic. The most effective control of *Nosema* disease is achieved by combining the sterilisation of equipment using heat or acetic acid with fumagillin treatment (8).

**B. DIAGNOSTIC TECHNIQUES**

1. Identification of the agent

In acute forms of infection, especially in early spring, brown faecal marks may be noted on the comb (4). At the entrance to the hive, sick and dead bees may be seen, although other causes, such as pesticide poisoning, should be eliminated first if this is the case. During winter, *Nosema*-infected colonies may become severely depleted of bees or die out altogether. The majority of *Nosema*-infected colonies will appear normal, with no obvious signs of disease even when the disease is sufficient to cause significant losses in honey production and pollination efficiency (1, 11). During winter, there may be an increase in bee mortality. A proper diagnosis can be made only by microscopic examination of the adult bee ventriculus. To diagnose a *Nosema* infection, the posterior pair of abdominal segments is removed with a forceps to reveal the ventriculus, complete with the malpighian tubules, the small intestine and rectum. The ventriculus is normally brown but, following a *Nosema* infection, becomes white and very fragile. However, this appearance is given by other causes of intestinal disturbance, for example feeding on indigestible food stores, such as syrup containing actively growing yeast. For a reliable diagnosis, a number of bees in a sample should be examined.

a) Microscopy

It is necessary to attempt to distinguish between a *Nosema* infection and an infection caused by *Malpighamoeba mellificae* (16). There is quite often an indication of dysentery in a *Nosema* infection. In an *M. mellificae* infection, there may be a diarrhoea, often of a sulphur-yellow colour and with a distinct odour.
Characteristics of *M. mellificae* cysts are described later. Secondary mixed infections may occur (17). A simple, nonquantitative method for detecting *Nosema* infection is as follows: sampled bees should be obtained from the hive entrance in order to avoid sampling individuals under the age of 8 days, which would lead to 'false negatives' because no spores from the protozoan in question would be determined. At least 60 bees should be collected in order to detect 5% of diseased bees with 95% confidence (10). Before sending to the laboratory, the bees should be fixed in 4% formol in order to prevent them from decomposing and to improve their reception and organisation in the laboratory. The abdomens of the bees to be examined are separated and ground up in 2–3 ml of water. Three drops of the suspension are placed on a slide under a cover-slip and examined microscopically at ×400 magnification, under bright-field or phase-contrast optics. This is a slight simplification of Cantwell's original method (7). The spores are about 5–7 µm long and 3–4 µm wide. They are completely oval with a dark edge. Their contents, consisting of nucleus, sporoplasm and polar tube, cannot be seen. Dyes are usually not necessary.

*Nosema* spores must be differentiated from yeast cells, fungal spores, fat and calciferous bodies, and from *M. mellificae* cysts, which are spherical and approximately 6–7 µm in diameter.

When air-dried, ethanol-fixed smears of infected tissue are stained with Giemsa's stain (10% in 0.02 M phosphate buffer) for 45 minutes. *Nosema apis* spores will have a distinctive appearance, with thick unstained walls and an indistinct blue interior, without visible nuclei. Insect cells, fungal spores and other protozoa stained in this way will generally have thinner walls, blue/purple cytoplasm and magenta-coloured nuclei.

In order to obtain accurate, reliable and meaningful quantification of levels of *Nosema* infections in honey bees, a standardised procedure must be used. A suitable protocol is as follows:

A sample of older worker honey bees is taken, from which the abdomens of ten individuals are macerated in 5 ml of water using a mortar and pestle. When tissue pieces have become quite fine, the suspension is filtered through two layers of muslin (thin loosely woven cotton fabric) in a funnel leading to a graduated centrifuge tube. A second 5 ml of water is used to rinse the pestle, swirl around the inside of the mortar and pour through the subsample in the funnel. Water levels are equalised in the tubes and the suspensions are centrifuged for 6 minutes at 800 g. The supernatants are decanted and the tubes are refilled to the 10 ml level. Using disposable pipettes and a rubber bulb, the pellets are resuspended by repeated uptake and forcible ejection through the pipette tips. When the solution appears to be homogenous, a sample is taken to fill the calibrated volume under the cover-slip of a haemocytometer (blood cell counting chamber). After a few minutes the spores will have settled to the bottom of the chamber. *Nosema* spores appear transparent but with a very distinct dark edge and are 5–7 µm long and 3–4 µm wide. They are best seen using a magnification of ×400 and bright-field or phase-contrast optics. The number of spores in each square is counted. Where a spore lies over the edge of a square, count only those spores that straddle the left and upper edges of the square, not the right and bottom edges. One *Nosema* spore, observed in the area that covers the entire etched grid (4000 small squares), is equal to an average of 4 million spores per bee. If no spores are seen, the result should be designated 'not detected', but that does not mean that the bees are not infected. Regulatory agencies will decide on the level of infection useful for their purposes.

A laboratory method for the simultaneous detection of *Nosema* spores and *M. mellificae* cysts consists of the individual examination of the colonies using 30–60 bees per colony. A suspension of the abdomens of dead bees is prepared by grinding with 5–10 ml water; the volume of water depending on the number and condition of the bees. The suspension must be filtered to remove debris that would interfere with the examination, first through a 100 µm and then a 40 µm filter. Parts of the malpighian tubules pass through the 100 µm filter, but are collected on the 40 µm filter. They are placed on a slide or bacterial counting chamber and examined at ×400 magnification. Only a few tubules are filled with cysts after an *M. mellificae* infection. The normal structure of malpighian tubules is not visible in this case. Only cysts inside the malpighian tubules can be taken as a positive result, because *M. mellificae* cysts are often confused with fungal spores and yeast cells.

b) Culture

There are no cultural methods for growing these organisms.

2. Serological tests

There are no serological tests available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No biological products are available.
ACKNOWLEDGEMENT

The authors would like to thank Dr F. Gnädinger for his valuable advice.

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**NB**: There are OIE Reference Laboratories for Bee diseases (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 2.9.5.

VARROOSIS

SUMMARY

The mite Varroa destructor (formerly Varroa jacobsoni) is a parasite of adult bees and their brood. It penetrates the intersegmental skin between the abdominal sclera of adult bees to ingest haemolymph. It can sometimes be found between the head and thorax. The number of parasites steadily increases with increasing brood activity and the growth of the bee population, especially late in the season when clinical signs of infestation can first be recognised. The life span of the mite depends on temperature and humidity but, in practice, it can be said to last from some days to a few months.

Identification of the agent: The clinical signs of varroosis can only be recognised at a late stage of infestation, so that diagnosis entails the examination of the hive debris. The debris produced during the summer is especially useful for diagnosis. The earliest and most precise diagnosis can be made only after the application of a medication that forces the mites to drop off the bees or kills them directly. Larger amounts of debris can be examined using a flotation procedure. Bees are washed in petroleum spirit, alcohol or detergent solution. However, this method is less accurate due to the unequal distribution of mites and the usually small sample sizes.

Serological tests: No serological tests are applicable.

Requirements for vaccines and diagnostic biologicals: No biological products are available.

A. INTRODUCTION

The Varroa mites are parasites of adult bees and their brood. Four species have been recorded: Varroa jacobsoni, V. destructor, V. underwoodi and V. rinderi. Until recently Varroa mites that affect Apis mellifera worldwide were assumed to be V. jacobsoni. However it has been shown that these mites are V. destructor (Figure 1). They are responsible for the condition of varroosis or varroatosis (1, 2). The mite inserts itself between the abdominal sclera in adult bees (10) where it penetrates the intersegmental membranes in order to ingest haemolymph. Sometimes it can also be found between the head and thorax. For reproduction, the female enters the cells with the bee brood shortly before the cells are sealed. They prefer drone brood to worker brood. After the brood cell is sealed, the mite lays up to seven eggs in intervals of about 1–2 days. These hatch into nymphs, but only two to three reach the adult stage.

Fig. 1. Varroa on pupa and adult bee. Left: pupa with four Varroa female mites. Right: worker bee with two female mites.
Chapter 2.9.5. — Varroosis

The number of mites usually increases slowly at the beginning of the season. Clinical signs may be seen at any time during the active season, although usually maximum numbers are reached late in the season (Figure 2), when the first clinical signs of infestation can be recognised. The course of this parasitism is usually lethal, except in some areas, such as tropical Latin America (6, 12). The life span of mites on larval or adult bees depends on temperature and humidity. Under practical conditions, the life span may vary from some days to a few months.

**Fig. 2.** Graph of populations of bees and mites over 1 year in a temperate Northern Hemisphere climate: brood numbers (solid line); mite numbers (broken line).

In heavily infested bee colonies, clinical signs of varroosis can often first be seen in the latter part of the season when the brood is reduced (12). Heavy infestations are usually reached 3–4 years after the primary invasion, but can occur within weeks if infested by bees from nearby colonies that are collapsing.

Essentially, the brood is damaged by the parasitic mites. Bees and their offspring that have been infected during the brood phase by only one parasitic mite show various ill effects, such as a shortened life span, changes in behaviour and an increased disease susceptibility (8). The parasitism is critical if more than one mite enters the brood cell for reproduction. Only in the lethal stage immediately before the collapse of the colonies do clinical signs, such as shrunken wings and shortened abdomen, appear (Figure 3). This is due to an increased susceptibility to deformed wing and acute paralysis virus, as well as to the infection of wounds and loss of haemolymph (3, 4). If the brood dies shortly before or after sealing, clinical signs of European foulbrood appear without the presence of the specific agent *Melissococcus pluton*. If the brood survives, the emerging bees show various behavioural changes and their life span is considerably shortened (7, 11).

**Fig. 3.** Effect of Varroa on bee morphology. Left: normal bee appearance. Right: bee heavily attacked by mites. This newly emerged bee has a deformed wing and reduced abdominal volume.

**B. DIAGNOSTIC TECHNIQUES**

1. Identification of the agent

The female mite is a dark reddish/brown colour and has a flat, oval-shaped body approximately 1.1 mm × 1.5 mm. It is the only common parasite of honey bees that can be seen with the naked eye (13).
Chapter 2.9.5. – Varroosis

a) Debris examination

An easy method of diagnosis of varroosis is by the examination of the debris generated by bees themselves. An insert covered with a screen mesh is placed on the floor of the hive. Unless this insert is covered with such a gauze, or smeared with grease, the bees will dispose of the mites outside the hive.

The debris produced within a few days in the late season usually contains little other than visible mites (9, 11). The debris collected in winter, however, must be examined in the laboratory. An insert is placed in the hive as before, but an effective medication is used to cause the mites to fall off the bees, so that after a given time, a number of mites may be observed on the floor insert. Some countries demand the diagnostic application of certain medications for proving the absence of mites.

Large amounts of debris can be examined in the laboratory using a flotation procedure (5).

- **Test procedure**
  i) Dry the debris for 24 hours.
  ii) Flood the debris with industrial alcohol.
  iii) Stir continuously for around 1 minute or, if debris contains wax or propolis particles, stir for 10–20 minutes.
  iv) Identify and observe the mites that float to the surface.

b) Brood examination

For the second method, drone brood is examined, if available, otherwise worker brood is examined.

When a large number of samples are examined, a rough determination of the degree of infection can be obtained.

- **Test procedure**
  i) Remove the cappings of the brood cells with a knife.
  ii) Wash the brood cells directly into a sieve system with warm water from a hand-held shower.
  iii) Collect the mites in the lower fine sieve (mesh width 1 mm) while the brood is gathered in the upper coarse sieve (mesh width 2–3 mm).
  iv) Place the contents of the sieve on a bright plate, where the mites can be easily identified and counted.

When a smaller number of samples are being studied, the individual cells are examined using an appropriate source of light. After removing the cappings and the bee brood, infected cells can be identified by the presence of small white spots – the faeces of the mite – found on the cell wall. The mites themselves should be sought for confirmation, by examining the bottom of the cell and the bee brood for attached mites.

c) Bee examination

In a third method, approximately 200–250 bees are removed from unsealed brood combs. Samples should be taken from both sides of at least three uncapped brood combs. To determine an apiary’s percentage of infestation, it is necessary to collect and analyse individual samples from at least 10% of the beehives, and to determine later the average infestation rate based on these individual results.

- **Test procedure**
  i) Kill the bees in a special container by submersion in alcohol.
  ii) Stir the container for 10 minutes.
  iii) Separate the bees from the mites by means of a sieve with a mesh size of approximately 2–3 mm.

Under some circumstances, the *Varroa* mite may be confused with the bee louse, *Braula coeca* (Figure 4). The latter is round, not oval, and being an insect, has only three pairs of legs. A number of different species of mite may be associated with *Varroa* mites on bees, but these are easily distinguished. In addition, other parasitic mites, such as those of the *Tropilaelaps* spp., are known to cause similar damage to bee colonies as the *Varroa* mites.

2. Serological tests

No serological tests are available for routine laboratory diagnosis.
**Chapter 2.9.5. – Varroosis**

**Fig. 4.** Diagram of Varroa destructor (formerly Varroa jacobsoni Oudemans) (female).

<table>
<thead>
<tr>
<th>a</th>
<th>Dorsal aspect</th>
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<tbody>
<tr>
<td>b</td>
<td>Anterior aspect</td>
</tr>
<tr>
<td>c</td>
<td>Ventral aspect</td>
</tr>
<tr>
<td>d</td>
<td>The bee louse (Braula coeca, female)</td>
</tr>
</tbody>
</table>

Note the flat shell-like back and four pairs of legs.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products and vaccines available. Formic acid, oxalic acid, lactic acid and thymol can be used to control Varroa mites (http://www.apis.admin.ch/english/Themes/Varroa.htm). Some hygienic strains are less susceptible to Varroa parasites.

- Acknowledgement

Illustrations by Karl Weiss, extracted from Bienen-Pathologie, 1984. Reproduced with the kind permission of the author and Ehrenwirth-Verlag, Munich (Germany).

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CHAPTER 2.9.6.

TROPILAEELAPS INFESTATION OF HONEY BEES
(Tropilaelaps clareae, T. koenigerum)

SUMMARY

The mites Tropilaelaps clareae Delfinado and Baker and T. koenigerum Delfinado-Baker and Baker (Mesostigmata: Laelapidae), are parasites of honey bee brood. Feeding on bee larvae and pupae causes brood malformation, death of bees and subsequent colony decline or absconding. Development requires about 1 week, and the mites are dispersed on bees.

Identification of the agent: An infestation by Tropilaelaps can be recognised either visually on bees or by examining hive debris. Irregular brood pattern, dead or malformed immatures, bees with malformed wings that crawl at the hive’s entrance, and especially the presence of fast-running, large, red-brown, elongated mites on the combs, are diagnostic for the presence of T. clareae. An early diagnosis can be made after opening brood cells and finding immature and adult mites therein. The hive (colony) may be treated with various chemicals that cause the mites to drop off combs and bees. Sticky boards on the bottom of the colony can be used to examine hive debris and mites.

Serological tests: No serological tests are applicable.

Requirements for vaccines and diagnostic biologicals: No biological products are available.

A. INTRODUCTION

The mites Tropilaelaps clareae occurs in Asia, from Iran in the north-west to Papua New Guinea in the south-east (10), whereas T. koenigerum is known only from Sri Lanka and Nepal (5). Tropilaelaps clareae can easily be distributed by the honey bee Apis mellifera. There has been a single report of T. clareae from Kenya, Africa (10), but this record has not been subsequently substantiated (11). The natural host of both species of Tropilaelaps is the giant Asian bee Apis dorsata, but T. clareae has been collected from A. cerana, A. florea, A. mellifera and A. laboriosa, whereas T. koenigerum occurs on A. laboriosa and A. dorsata (5). Tropilaelaps clareae can easily be distributed by the honey bee Apis mellifera.

1. Life cycle

The colonising Tropilaelaps female (or females; as many as a dozen may occur within individual a single cells) places from one to four eggs on mature bee larvae shortly before the brood cell is capped. The drone brood is preferred by Tropilaelaps and may be almost 100% parasitised (2). The mite progeny, usually one male and several females feed on and seriously damage the bee brood. Development of the mite requires about 1 week. The adults, including the foundress female, emerge with the adult bee and search for new hosts.

The short life-cycle, as well as a very brief stay on adult bees, explains why populations of T. clareae increase faster than those of Varroa mites. When both T. clareae and Varroa destructor infest the same colony, the former may out-compete the Varroa mite (2, 15). It has been reported that when both mite species are in the same cell, the reproduction of both mites declines (14).

Phoretic survival on bees is quite short (only 1–2 days) because Tropilaelaps cannot pierce the integument of adult bees. The phoretic time for Tropilaelaps spp. is important in understanding the life cycle, and recent research suggests the period can be as long as 5–10 days (17, 18). Gravid female mites will die within 2 days unless they deposit their eggs (19).

Infestation by Tropilaelaps causes the death of many bee larvae (up to 50%), resulting in an irregular brood pattern and of which the cadavers that may partially protrude from the cells. Many malformed bees occur, with
distorted abdomens, stubby wings and deformed or missing legs. Some of the affected bees crawl at the hive’s entrance (1). In addition, perforated cappings are seen, the result of sanitation activities by the worker bees, which evict the infested bee pupae or young adults. Some infested colonies abscond, carrying the mites to a new location.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The first sign of an infestation by *T. clareae* is often the occurrence of large (almost 1 mm in length), red-brown, elongated mites on the combs or on adult bees (Fig. 1). *Tropilaelaps koenigerum* is slightly smaller, only about 0.7 mm in length (4). Both species of *Tropilaelaps* can easily be recognised and separated from the *Varroa* mite using a ×10 magnifying glass. The body of the *Varroa* mite is wider than it is long and it moves slowly, whereas the body of *Tropilaelaps* is elongated, with a heavily sclerotised holoventral or similar shield, and it is a fast-running mite.

![Fig. 1. Tropilaelaps clarea. Photo by J. Waddell.](image)

a) Mite collection

Methods to collect mites include an ether or sugar roll (15). Collect approximately 100–200 bees in a wide-mouthed jar with lid. Scrape the bees into the jar or use a modified vacuum to suck them in. Knock the bees to the bottom of the jar with a sharp blow; there should be about a 1–2 inch (2.54–5.08 cm) layer of bees on the bottom. Remove the lid and spray a 2-second burst with ether starter fluid. Alternatively, use enough 70% alcohol or soapy water to cover the bees; or add around 25 g (1 oz) powdered sugar (or flour). If using ether replace the lid and agitate or roll the jar for about 10 seconds; mites should stick to walls. If using soap or alcohol, agitate and then strain out the bees with a coarse hardware cloth or mesh strainer; mites will be in the liquid. If using sugar or other powder, put screening material (such as hardware cloth) on top of the jar and shake the mites on to white paper to count; repeat every 2 minutes. For a more accurate count, finish with an alcohol or soapy water wash to collect all the mites.

b) Colony and brood examination

When monitoring honey bee colonies for the presence of *Tropilaelaps* (or *Varroa*), an examination of both drone and worker brood may provide an early indication of infestation. Mites can be observed inside capped bee brood by using a honey scratcher (with fork-like tines) to pull up capped pupae. The mites are clearly visible. The younger mite stages are whitish and may be almost motionless while feeding on their hosts’ bodies, as their mouthparts and front legs are fixed to the cuticle of the bee host (15). The extent of parasitisation can be estimated by opening a predetermined number of brood cells; infestation rates are then calculated as per cent of capped brood containing live mites (3).

c) Sticky board examination

A precise diagnosis can be made using a sticky board covered with a mesh, such as fly screen, that prevents the bees from removing the dislodged mites. The mesh must be large enough for mites to pass through. Make a sticky board with poster board, cardboard or other white, stiff paper coated with Vaseline or other sticky substance (8, 12, 16), or use a sheet of sticky shelf paper. Cut the paper to fit the bottom board of a hive. Cut a piece of hardware cloth or screen to fit on top of the sticky board. To keep the bees from cleaning off the board, fold under the outside edges of the screen to raise it off the board, and staple or tape
Chapter 2.9.6. — Tropilaelaps infestation of honey bees (Tropilaelaps clareae, T. koenigerum)

in place. Leave the board in the colony for up to 3 days, collecting and examining the debris for mites. For faster mite diagnosis, smoke each colony adding 25 g (1oz) pipe tobacco in the smoker. Puff the bees 6–10 times, close up the hive for 10–20 minutes. Pull out the sticky board after 10 minutes and count the mites. Acaricides are sometimes used to knock mites off bees and will appear on the sticky boards.

2. Serological tests

No serological tests are available for diagnosis.

3. Treatment

In countries with infestations of T. clareae, fluvalinate in slow-release formulations controls T. clareae (9, 13), as do monthly dustings with sulphur (1) and treatments with formic acid (6). The inability of this mite to feed on adult bees, or to survive outside sealed brood for more than a few days, such as caging the queen for a few weeks, is being used as a non-chemical control method (19, 20).

Many of the same acaricides used for Varroa will kill Tropilaelaps. Strips of plastic-impregnated fluvalinate (Apistan™) or cimiazol (Apitol) trickled on to bees will kill mites. Alternatively, tobacco smoke in the smoker will cause mites to drop off bees. Strips of filter paper, available in some countries as Folbex strips, are prepared by soaking in an aqueous solution of 15% potassium nitrate to which two drops of amitraz (usually 12.5%) are added (9). After the paper dries, the strip is ignited and inserted into the hive. The smoke causes many mites to drop off. Another method is to use plates or pads soaked with 20 ml of 65% formic acid (very caustic and will burn hands and face). The pads are placed in the colonies, near the top (7). These last methods are not recommended, as they can harm both bees and humans.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available.

REFERENCES

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


* *

**NB:** There are OIE Reference Laboratories for Bee diseases (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 2.10

DISEASES NOT COVERED BY LIST A AND B

CHAPTER 2.10.1.

CYSTICERCOSIS

SUMMARY

Cysticercosis of farmed and wild animals is caused by the larval stages (metacestodes) of cestodes (tapeworms), the adult stages of which occur in the intestine of humans and dogs or wild Canidae. Bovine cysticercosis (primarily in muscle) and porcine cysticercosis (primarily in muscle and the central nervous system) are caused by the metacestodes (cysticerci) of the human cestodes Taenia saginata and T. solium, respectively. Cysticerci of T. solium also develop in the central nervous system and musculature of humans. Cysticerci of T. saginata asiatica occur in the liver of pigs. Cysticercosis and coenurosis of sheep and goats (in the muscles, brain, liver and peritoneal cavity) are caused by T. ovis, T. multiceps and T. hydatigena, adults of which occur in the intestines of dogs and wild canids.

Most adult and larval tapeworm infections cause little or no disease. Exceptions are severe, potentially fatal human neurocysticercosis (NCC) caused by T. solium, and occasionally neurocoenurosis caused by T. multiceps in humans. These parasites are also occasional causes of muscle or ocular signs in humans. 'Gid' caused by T. multiceps in ruminants can require slaughter of the animal. Acute T. hydatigena coenurosis in sheep and goats is rare. Cysticercosis causes economic loss through condemnation of infected meat and offal.

Identification of the agent: Adult Taenia tapeworms are dorsoventrally flattened, segmented and large, reaching from 20 to 50 cm (species in dogs) to several metres (species in humans). Anteriorly, the scolex (head) has four muscular suckers and may have a rostellum, often armed with two rows of hooks, the length and number of these being relatively characteristic of a species. A neck follows the scolex, and this is followed by immature and then by mature reproductive segments, and finally gravid segments filled with eggs. Segment structure, although unreliable, can aid diagnosis. Taenia species cannot be differentiated by egg structure. Metacestodes consist of a fluid-filled bladder with one or more invaginated protoscoleces. These ‘bladderworms’ are each contained within a cyst wall at the parasite–host interface. This structure comprises the cysticercus or coenurus.

Adult Taenia are recognised at post mortem or by passage of segments or eggs. Metacestodes are grossly visible at post mortem and meat inspection, but light infections are often missed. NCC can be diagnosed by imaging techniques.

Immunological tests: Adult Taenia infections can be recognised by detection of Taenia coproantigen in faeces using an antigen-capture enzyme-linked immunosorbent assay, but the test does not differentiate species and is not commercially available. Use of species-specific probes remains experimental.

Serological tests: Tests for antibodies in serum are not used currently for the diagnosis of cysticercosis diagnosis is by meat inspection.

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1 The first four diseases on this list are included in some individual species sections of List B, but these chapters cover several species and thus give a broader description.
Requirements for vaccines and diagnostic biologicals: Vaccine antigens have been identified for the metacestode, but not for the adult stages of T. ovis, T. saginata and T. solium. A T. ovis vaccine is registered in New Zealand, but is not commercially available.

A. INTRODUCTION

The metacestodes (or larval cestodes) of Taenia spp. tapeworms of humans and the Canidae are the cause of cysticercosis in various farmed and wild animals and in humans. Adult tapeworms are found in the small intestine of carnivore definitive hosts – humans, dogs, and wild canids. Taenia saginata of humans causes bovine cysticercosis, which occurs virtually world-wide, but has a particularly high prevalence in Africa, is found in Caucasian and South/Central Asian and eastern Mediterranean countries; the infection occurs in several countries in Europe. Taenia solium of humans causes porcine cysticercosis and human neurocysticercosis (NCC). It is found principally in Central and South America, sub-Saharan Africa, parts of the Commonwealth of Independent States (CIS), the People’s Republic of China and non-Islamic countries of Asia where there are free-ranging, scavenging pigs. The cysticerci of T. saginata asiatica of humans in South-East Asia occur in the liver of pigs. Dogs and wild canids are the definitive hosts of metacestodes of sheep, goats and other ruminants, which occur throughout most of the world, although T. multiceps has disappeared from the United States of America (USA) and New Zealand. Taenia ovis occurs in the muscles of sheep, T. multiceps in the brain (occasionally in the muscles) of sheep, goats, sometimes other ruminants and rarely humans, and T. hydatigena is found in the peritoneal cavity and on the liver of ruminants. Diagnosis is usually based on the host and the location of the metacestode when identified at meat inspection. Adults are acquired by the ingestion of metacestodes in meat and offal that has not been adequately cooked or frozen to kill the parasite.

Gravid segments that are shed by the adult tapeworms migrate spontaneously from the anus to fall to the ground and release eggs on the ground, or the segments and free eggs are passed in the faeces. Taenia solium segments, however, are often passed in chains. Eggs are immediately infective when passed. Animals acquire infection from ingestion of segments and eggs contaminating herbage or in faeces. Humans may be infected with T. solium by eggs on vegetables, etc., that have been contaminated by faeces or dirty hands, by faeco–oral transmission or through retro-peristalsis and hatching of eggs internally. Diagnosis continues to be mainly based on the morphology of the adult tapeworm and the presence of eggs or segments in the faeces of infected definitive hosts.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Taenia saginata (the beef tapeworm): The adult is large, 4–8 metres long and can survive many years, usually singly, in the small intestine of humans. The scolex (or head) has no rostellum or hooks. Useful diagnostic techniques are presented in Table 1 (23, 24). Gravid segments usually leave the host singly and often migrate spontaneously from the anus.

The eggs are typical ‘taeniid’ eggs that cannot be differentiated from other Taenia or Echinococcus spp. eggs. Taenid eggs measure about 30–45 μm in diameter; contain an oncosphere (or hexacanth embryo) bearing three pairs of hooks; have a thick, brown, radially striated embryophore or ‘shell’ composed of blocks; and there is an outer, oval, membranous coat, the true egg shell, that is lost from faecal eggs.

Metacestodes (or cysticerci) of T. saginata usually occur in the striated muscles of cattle (beef measles), but also buffalo, reindeer and deer. They are oval, about 0.5–1 cm long, translucent and contain a single white scolex that is morphologically similar to the scolex of the future adult tapeworm. They are contained in a thin, host-produced fibrous capsule. Cysts occasionally are found in the liver, lung, kidney and fat.

Taenia solium (the pork tapeworm) is smaller than T. saginata being up to 3–5 metres. The scolex has an armed rostellum bearing two rows of hooks; the number and size of hooks can aid differentiation of Taenia spp. (Table 1). Gravid segments have 7–13 (<17) uterine branches and do not usually leave the host spontaneously, but passively in chains with the faeces.

Metacestodes occur in the muscles and central nervous system of pigs (pork measles), bear and dogs and in the muscles, subcutaneous tissues and central nervous system of humans. Cysts are grossly similar to those of T. saginata, but may be larger than the T. saginata cyst. They have a scolex bearing a rostellum and hooks similar to the adult. Occasionally, in the brain of humans, they develop as racemose cysts up to 2 cm or more across that lack a scolex.
Table 1. Useful features for identification of scoleces and segments of Taenia spp.

<table>
<thead>
<tr>
<th>Parasite species</th>
<th>Number of hooks</th>
<th>Length of hooks (µm)</th>
<th>Number of testes</th>
<th>Layers of testes</th>
<th>Cirrus sac extends to longitudinal vessels</th>
<th>Number of uterine branches</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Large hooks</td>
<td>Small hooks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>T. hydatigena</strong></td>
<td>28–36 (26–44)</td>
<td>191–218</td>
<td>118–143</td>
<td>600–700</td>
<td>1 Yes</td>
<td>6–10 that re-divide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(170–235)</td>
<td>(110–168)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(131–202)</td>
<td>(89–157)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>T. multiceps</strong></td>
<td>22–30 (20–34)</td>
<td>157–177</td>
<td>98–136</td>
<td>284–388</td>
<td>2 Yes</td>
<td>14–20 that re-divide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(120–190)</td>
<td>(73–160)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>T. saginata</strong></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>765–1200</td>
<td>1 No</td>
<td>14–32 that re-divide</td>
</tr>
<tr>
<td><strong>T. solium</strong></td>
<td>22–36</td>
<td>139–200</td>
<td>93–159</td>
<td>375–575</td>
<td>1 Yes</td>
<td>7–16 that re-divide</td>
</tr>
</tbody>
</table>

*Taenia saginata asiatica* (Asian/Taiwan *Taenia*): Closely related to but genetically distinguishable from *T. saginata* (2), the adult in humans has an ovary, vaginal sphincter muscle and cirrus sac like those of *T. saginata*, but *T. s. asiatica* has a rostellum and posterior protuberances on segments and 11–32 uterine buds. Segments are passed singly and often spontaneously. The metacestodes are small, about 2 mm, and have a rostellum and two rows of primitive hooks, those of the outer row being numerous and tiny. They occur mainly in the parenchyma and on the surface of the liver of domesticated and wild pigs; they may be found on the omentum and, rarely, on the lungs and colonic serosa. Occasionally they are found in cattle, goats, and monkeys.

*Taenia ovis*: Adults in the intestine of dogs and wild carnivores reach 1–2 metres in length and have an armed rostellum (Table 1). Metacestodes that occur in the musculature of sheep and less commonly goats reach 3.5–1.0 × 0.2–0.4 cm. Commonly, the cysticerci are degenerate with a green or cream, caseous or calcified centre. A similar parasite occurs in wild carnivores and dogs and the muscles of reindeer and deer in northern areas.

*Taenia hydatigena*: Adults are 1–5 metres long, are found in the intestine of dogs and wild carnivores, and have an armed rostellum (Table 1). Metacestodes are large, from 1 cm up to 6–7 cm, and the scolex has a long neck. They are found attached to the omentum, mesentery and occasionally on the liver surface, particularly of sheep, but also of other domesticated and wild ruminants and pigs. A wolf and reindeer/deer cycle exists in northern latitudes, in which the metacestodes are found in the liver of the intermediate host; dogs are also infected as definitive hosts.

*Taenia multiceps*: Adults are 40–100 cm long in the intestine of carnivores and have an armed rostellum (Table 1). The metacestodes are coenuri – large, white fluid-filled cysts that have up to several hundred scoleces invaginated on the wall in clusters. These grow to 5 cm or so in size in the brain of sheep, the brain and intermuscular tissues of goats, and also the brain of cattle, wild ruminants and occasionally humans. In neural
tissue the cysts are not encapsulated. The cysts induce neurological signs that in sheep are called ‘gid’, ‘sturdy’, etc.

a) Diagnosis of adult parasites in humans or dogs

All parasite or faecal material from humans with possible T. solium infections must be handled with suitable safety precautions to prevent accidental infection with the eggs. Taenia multiceps and Echinococcus spp. also infect humans and, as taeniid eggs in dogs cannot be differentiated to species or genus level, in areas where these are endemic, same safety precautions apply. In addition to Taenia spp., humans may be infected by Diphyllobothrium and Hymenolepis spp., while six other cestode genera are recorded occasionally in humans. These are described by Lloyd (13) and all can be differentiated from Taenia spp. by egg/proglottid morphology. Recently however, T. taeniaeformis with morphologically indistinguishable taeniid eggs was recorded in a child. In canids, Echinococcus spp. eggs cannot be distinguished from Taenia eggs, but the presence of the former can be determined by tapeworm size and, more recently, Echinococcus species-specific antigen-capture enzyme-linked immunosorbent assay (AG-ELISA). Other worms, Dipylidium, Diplopylidium, Mesocestoides and Diphyllobothrium spp. have morphologically distinct eggs and proglottids (13, 19).

Adult cestodes can be expelled from humans using an anthelmintic followed by a saline purgative and are identified on the basis of scolex and proglottid morphology. In animals, arecoline purgation has been useful; again, the recovered tapeworms are identified morphologically. Arecoline is no longer available as an anthelmintic, but can be obtained from chemical supply companies. As it has side-effects, old, infirm and pregnant animals should be excluded from treatment. A dose of 4 mg/kg should result in purgation in under 30 minutes. Walking and abdominal massage of recalcitrant cases or enema for constipated dogs may avoid the use of a second dose (2 mg/kg), which should be given only sparingly. Fortunately, arecoline purgation is being replaced rapidly by AG-ELISA for Echinococcus spp. and perhaps in the future this will also be the case for Taenia spp.

Verster (23) and Loos-Frank (14) have given descriptions of parasitic diagnosis of all the Taenia spp. of humans and animals, their hosts and geographical distributions. Mayta et al. (15) and Loos-Frank (14) give methods for mounting, embedding, sectioning and staining the proglottids. The following staining technique is that of Loos-Frank (14). Worms, after relaxation in water, can be stained directly, although small worms should be fixed in ethanol for a few minutes. Alternatively, worms can be fixed and stored in 70% ethanol containing 10% lactic acid, the scolex and worm being stored separately. The rostellar hooks of scolecites or protoscolecites should be cut off and mounted en face in Berlese’s fluid (made by dissolving 15 g gum arabic in 20 ml distilled water and adding 10 ml glucose syrup and 5 ml acetic acid, the whole then being saturated with chloral hydrate, up to 100 g). The stain is lactic acid carmine: 0.3 g carmine is dissolved at boiling point in 42 ml lactic acid and 58 ml distilled water, 5 ml of 5% iron chloride solution (FeCl₂.4H₂O) is added after cooling and can be used again to refresh older solutions. Specimens are left to sink in the stain within a vial, and after several minutes are washed in 1-day-old tap water until blue. They are then fixed in 50–70% ethanol and dehydrated under the slight pressure of plastic foil. Salicylic acid methyl ester is used as clearant.

When segments break from the end of the worm, some eggs are expelled in the intestine and can be found in the faeces. Spontaneous migration of T. saginata or T. s. asiatica from the anus is likely to be noticed by the patient (>95%). When the segments migrate, the sticky eggs are deposited in the peri-anal area and might be detected by application and examination of sticky tape. These signs are far less likely for chains of T. solium (1). Segments of all three may be found on the faeces, but are passed intermittently. In dogs, approximately 50% of the segments migrate spontaneously from the anus. These segments, when they fall to the ground, will migrate, shedding eggs. The remainder of the segments are passed in the faeces, but commonly, the segments migrate and void the majority of their eggs in trails on the surface of the faeces and surrounding area. Even if a migrating segment sheds all its eggs, it can be identified as a cestode by the many concentric calcareous corpuscles contained within its tissues. Faeces, after mixing to reduce aggregation, can be examined for eggs. Various techniques are used throughout the world and include ethyl acetate extraction and flotation. For the latter, NaNO₃ or Sheather’s sugar solution (500 g sugar, 6.6 ml phenol, 360 ml water), with their higher specific gravities, are superior to saturated NaCl as flotation media for taeniid eggs. Flotation can be carried out in commercially marketed qualitative or quantitative flotation chambers or by centrifugal flotation that includes a modified Wisconsin technique (faeces, diluted in water, are sieved and centrifuged, the pellet is resuspended in sugar or Sheather’s solution and centrifuged at 300 g for 4 minutes). Eggs adhering to the cover-slip can then be detected. Faecal egg examination will be less sensitive for T. solium than the other species. Species cannot be determined by egg morphology, but DNA probes, the polymerase chain reaction (PCR), and PCR restriction fragment length polymorphism (RFLP) have proved useful for differentiation in the laboratory. These have been largely used experimentally to differentiate faecal eggs of T. solium, T. saginata and T. s. asiatica (2, 5–7, 9). While equally applicable to differentiation in dogs, the same examinations have not been done.
An AG-ELISA to detect Taenia coproantigen in faeces is no longer available commercially. Information on availability for epidemiological studies or collaborative use can be obtained from Professor P.S. Craig, OIE Reference Expert on Echinococcosis (see Table given in Part 3 of this Terrestrial Manual). This AG-ELISA was developed experimentally by Allan et al. (1) to detect coproantigen in dogs, and so, with appropriate controls, could be used to detect Taenia infection in this species. The technique, however, is only genus specific. The test is a solid-phase, microwell assay with wells coated with polyclonal, rabbit anti-Taenia-specific antibody (TSA). The following is the basic technique:

i) Faecal supernatants are recovered from fresh, frozen or formalinised (5% formalin at 4°C) faecal samples. The sample is vigorously shaken forming a slurry in an equal weight/volume of 0.15 M phosphate buffered saline (PBS) containing 0.3% Tween 30. The supernatant is recovered by centrifugation at 2000 g for 30 minutes.

ii) Soluble aqueous non-gravid proglottids from Taenia are obtained following emulsification in PBS and centrifugation.

iii) Hyperimmune rabbit antiserum is produced against the soluble proglottid extract and the IgG fraction is isolated by passage into and elution of the bound IgG from Protein A-Sepharose CL 4B (Pharmacia). Some of the IgG fraction is conjugated to peroxidase type VI (Sigma). The sera are stored in small quantities frozen at –20°C. Sera may need to be absorbed by packed normal dog faeces in a ratio 2/1 with mixing for 1 hour and recovered by centrifugation.

iv) Flat-bottomed microtitre plates (Dynatech) are coated with rabbit anti-Taenia IgG (protein content 5–25 µg/ml determined by UV spectrophotometry) using 100 µl/well, the antiserum are diluted in 0.05 M NaHCO3/Na2CO3 buffer, pH 9.6. Plates are incubated overnight at 4°C, the wells are washed three times with PBS/0.1% Tween, blocked with PBS/0.3% Tween for 1 hour and washed again. 100 µl of faecal supernatant containing 50% fetal calf serum is added and the plates are incubated for 1 hour and then washed three times. 100 µl of the peroxidase-conjugated anti-Taenia IgG (diluted 1/100 or 1/200) is added and the plates are incubated for 1 hour before washing three times. Substrate solution (100 µl of 5-amino-salicylic acid [Sigma] and 0.005% H2O2 in 0.1 M phosphate buffer containing 1 mM Na2EDTA [ethylene diamine tetra-acetic acid] at pH 8.0) is added for 25 minutes and the result is read at 450 nm. Cut-off values are the mean plus 3 standard deviations of values for normal dog faeces.

b) Diagnosis of metacestodes

In live animals, T. solium or T. saginata metacestodes might be palpable in the tongue but, both in the living animal and on post-mortem examination or meat inspection, tongue palpation is of diagnostic value only in pigs or cattle heavily infected with metacestodes; these will also be difficult to differentiate from large sarcocysts.

- Meat inspection – the main diagnostic procedure

Metacestodes are visible first as very small, about 1 mm, cysts, but detection of these requires thin slicing of organs in the laboratory. Many young cysts are surrounded by a layer or capsule of inflammatory cells (mononuclear cells and eosinophils being prominent histologically). These may later degenerate, but the parasites’ abilities to evade the immune response mean that later in infection, as the cyst matures, few inflammatory cells are present in its vicinity and the cysticercus in its intermuscular location is surrounded by a delicate fibrous tissue capsule.

In theory, cysts can be visualised or felt in tissues such as the tongue of heavily infected animals as early as 2 weeks after infection. Cysts are readily visible by 6 weeks and, when mature, are usually oval, about 10 × 5 mm or larger, with a delicate, fairly translucent, white parasite membrane and host capsule; pale fluid within the cyst and the scolex, visible as a white dot within the cyst, usually invaginates in the middle of a long wall.

At meat inspection many of the cysts detected, often 85%, are dead. The rate at which cysts age and die and so degenerate varies with the parasite species and also with the tissue within which the cyst is embedded. Death usually occurs within 9 months of infection of adult cattle with T. saginata. However, cysts may remain viable for several years if young calves are infected within a few days of birth. Cysts of T. hydatigena in the peritoneal cavity of sheep and those of T. solium also have been described as surviving in sheep and pigs for long periods. Taenia solium cysts survive for many years in the brain of humans, and frequently symptoms begin only as the cyst begins to degenerate. In general, cysts tend to die more rapidly in the predilection sites. It is suggested that this is due to greater blood circulation to these muscles. Conversely, the higher rate of activity in these muscles (which in itself accounts for the greater circulation) may damage the parasites, allowing leakage of fluid and perhaps disrupting the parasite’s ability to evade the immune response. Cysts at different stages of viability and degeneration can be found in the same host.
Degenerating cysts vary in appearance. The host’s fibrous tissue capsule thickens and becomes opaque, but initially the cyst within remains apparently normal. The fluid gradually becomes colloid and inflammatory cells infiltrate. The cyst cavity becomes filled with greenish (eosinophilic) and then yellow, caseous material and is very unaesthetic, usually being large in size and certainly more obvious in meat than the original viable cyst. Later the cyst may calcify. While PCR tests have been used largely for the differentiation of adult taeniids in humans, they could be usefully applied to unambiguously identify a metacestode.

After treatment of *T. saginata* and *T. solium* in cattle and pigs with drugs such as albendazole and oxfendazole, the cysts may lose their fluid and collapse. The resultant lesion is much smaller than lesions observed following natural death. However, cysts that have died before treatment of the animal will remain large and visible.

Where very young (without a scolex) or degenerate cysts need to be differentiated from other lesions, compression of the cyst, smears of the caseous contents and histological examination of haematoxylin and eosin (H&E) stained sections are used. Microscopic examination may reveal the calcareous corpuscles (concentric concretions of salts that are around 5–10 µm in size). These indicate a cestode origin of the tissue and differentiate, for example, an immature cyst from a simple cyst. The presence of hooks and their length together with knowledge of the host and tissue may aid in identification of cestode species. This would be useful in the finding of a new cestode in a host species or geographical area from which, historically, the parasite was absent.

Meat inspection procedures vary with the parasite and the host involved, i.e. zoonosis or not, the tissue involved, and regulations within a country. Examinations tend to be more extensive with the zoonotic infections *T. saginata* and *T. solium*.

In general, meat inspection procedures consist in:

i) Visual inspection of the carcass, its cut surfaces and the organs within it. This may reveal *T. saginata*, *T. solium* and *T. ovis* in the muscles, *T. hydatigena* on the liver or mesenteries and omentum, or *T. multiceps* in the brain.

ii) The external and internal masseters and the pterygoid muscles each must be examined and one or two incisions made into each, the cuts being parallel to the bone and right through the muscle.

iii) The freed tongue is examined visually and palpated.

iv) The pericardium and heart are examined visually. The heart usually is incised once lengthwise through the left ventricle and interventricular septum so exposing the interior and cut surfaces for examination. Incisions may go from the base to the apex and regulations also may require additional, perhaps four, deep incisions into the left ventricle. Alternately, the heart may be examined externally and then internally after cutting through the interventricular septum and eversion.

v) The muscles of the diaphragm, usually after removal of the peritoneum, are examined visually and may be incised.

vi) The oesophagus is examined visually.

vii) In African countries in particular, the triceps brachii muscle of cattle is incised deeply some 5 cm above the elbow. Additional cuts into it may be made. The gracilis muscle also may be incised parallel to the pubic symphisis. These cuts are usually also undertaken for *T. solium* in pigs. Such incisions into the legs are made in Africa as it is suspected that more parasites lodge in these muscles in working or range animals walking long distances due to the exercise and consequent increased blood flow to these muscles. Other countries may also require such incisions into the legs. However, as this devalues the meat, such incisions are made most commonly once one or more cysts have been found at the predilection sites so as to determine the extent of the infection.

Overall, the initial incision into any tissue is the most important, but additional incisions may be required by the regulations or are required if cysts are found on the initial incision(s).

Additional or fewer procedures may be required for specific parasites and the judgements on the carcass, viscera, offal and blood will vary dependent on *Taenia* species and regulations within a country. Judgement on infected carcasses will fall into three main categories: i) approve for human consumption; ii) partially condemn and pass the remainder of the carcass, but in the case of the zoonoses, *T. saginata* and *T. solium*, the carcass, meat and viscera must be treated; and iii) totally condemn heavily infected carcasses or emaciated diseased ones.

*Taenia saginata*: Calves under 6 weeks or <32 kg are not usually examined. Predilection sites are the heart, tongue, masseters and diaphragm, presumably because they receive the greatest circulation. Nonetheless, cysts may be found in any muscle of the body. If one carcass in a lot is found to be infected, all carcasses...
from the same lot can be held until laboratory confirmation is obtained. If *T. saginata* infection is confirmed, additional incisions are usually made in the carcasses in the lot; all suspicious lesions found in the rest of the lot are considered to be *T. saginata* without laboratory confirmation. Lesions of *T. saginata* may need to be differentiated from *Sarcocystis* sarcocysts and actinobacillosis lesions.

**Judgement:** If a carcass is considered to be heavily infected then the carcass, meat, offal and blood all are condemned. The description of a heavy infection varies, but generally it is the detection of cysts at two of the predilection sites plus two sites in the legs. In the case of a lesser infection, the infected parts and surrounding tissues are removed and condemned. The carcass and edible viscera then must be treated; treatment varies with country and facilities available and includes: i) freezing at lower than –10°C for >10 or 14 days, or lower than –7°C for 21 days; ii) boxes of boned meat are frozen at less than –10°C for >20 days; iii) heated to above 60°C throughout; iv) steamed at moderate pressure (0.49 kg/cm²); v) heated at 95–100°C for 30 minutes; or vi) pickled in salt solution for 21 days at 8–12°C. Blast freezing needs examination; generally a 30 kg box is reported to require 2 x 24-hour cycles at –30.9°C followed by 72 hours cold storage at –23.3°C for death of scoleces. Often no treated meat can be exported although in some countries it can be exported in canned form. Even if only dead cysts are found on meat inspection, carcass treatment is still justifiable as about 10% of lightly infected carcasses were found on dissection to have both dead and viable parasites within them.

*Taenia solium*: The predilection sites are as for *T. saginata* although there are reports of higher prevalence in shoulder and thigh. Commonly one or more cuts are required 2.5 cm above the elbow joint. This is said to detect some 19% of infected carcasses that would otherwise have been missed.

**Judgement:** In some countries, any lightly or heavily infected pigs and their viscera and blood are condemned. In areas where infection is common, lightly infected carcasses can be passed for cooking and pickling and occasionally freezing.

*Taenia hydatigena*: The parasite migrating in the liver leaves haemorrhagic tracks that then become green/brown with inflammation and later white due to fibrosis. For the records, these must be differentiated from those of liver flukes, if possible, by identification of the cysticerci or adult flukes. White spot from *Ascaris* infection is differentiated as the lesions appear as pale to white, small, isolated foci. Some cysts remain trapped below the liver capsule. These usually are small and degenerate early and then calcify into cauliflower-like lesions. *Taenia hydatigena* usually is superficial in a subserosal, while *Echinococcus granulosus* hydatid cysts are deeper in the parenchyma. If viable, the former has a long-necked single scolex, while the latter, if fertile, has many scoleces. Histology may be required for differentiation. H&E-stained sections will reveal the laminated membrane on even young hydatid cysts. Its presence or absence can be confirmed by periodic acid–Schiff staining when the highly glycosylated proteins in the laminated membrane stain red. *Taenia hydatigena* lesions in cattle and pigs can be similar to tuberculosis. However, the portal lymph nodes are not involved, the contents of parasite cysts are more easily shelled-out and remainders of hooks and calcareous corpuscles may be seen or Ziehl–Neelsen staining may reveal bacteria.

**Judgement:** Usually only a few cysts or tracks are present and these can be trimmed. Heavily infected livers and omentum are condemned. Rarely, acute infections are seen with large numbers of migrating parasites producing traumatic hepatitis, ascites, oedema, etc., and would result in secondary condemnation of the carcass.

*Taenia multiceps*: The parasites have a predilection site for the brain and spinal cord.

Early migrating parasites can cause reddish, later grey purulent tracks in the brain and in heavy infections the sheep may show meningoencephalitis. Clinical signs of the mature cyst causing pressure atrophy vary according to location in the brain and the sheep gradually may be unable to feed and will become emaciated. In heavy infections, parasites migrate and begin development in other tissues, but they die early. These produce small lesions, 1 mm or so in size, that first contain an encapsulated cyst, then eosinophilic, caseous material that later may calcify.

**Judgement:** Initially only the head is condemned or rare cysts in intermuscular or subcutaneous sites are trimmed. Later the animal may have been unable to feed with condemnation due to emaciation, etc.

*Taenia ovis*: The predilection sites are as for *T. saginata*. Cysts may be confused with large *Sarcocystis gigantea* sarcocysts.

**Judgement:** Commonly detection of up to 2–5 cysts results in trimming and the carcass is passed. This does not prevent the unaesthetic presence of live or degenerate parasites in other tissues. Ultrasound and X-rays are being tested for detection of these. In heavy infections the carcass is condemned.
Meat inspection procedures detect only about 50% of the animals that are actually infected. Light infections are easily missed on palpation and meat inspection – in one study, 78% of carcasses infected with >20 cysts were detected, while only 31% of those with fewer cysts were detected (24). Meat inspection efficacy will vary with the number and location of incisions. For example, in Zimbabwe, 58% of cattle were positive in the head only, 20% in the shoulder only and 8% in the heart only, although overall 81% were found to be infected if all three organs were included. Walther & Koske (24) in Kenya also found that the predilection sites were not necessarily infected in 57% of the cattle found positive on dissection. They also confirmed the importance of the shoulder incisions in detection of infection in Africa as 20% of the cattle found to be infected were positive in the shoulder only.

In humans, the most common presenting sign in *T. solium* NCC is seizures followed by headache, but a range of signs, such as vomiting, psychoses, etc., are seen depending on the number, location and viability or level of degeneration of the cysticerci (viable, transitional dying, calcification) (3, 12). In humans, clinical evaluation and either computerised tomography (CT) scan (best for calcified cysts) or magnetic resonance imaging (MRI) (detects cysts in both parenchymal and extraparenchymal locations and can follow the progression of the lesion) are used to detect the exact locations and viability of *T. solium* and *T. multiceps* metacestodes. These remain the most efficient means of diagnosis, but access to imaging facilities may not be available in endemic areas. Calcified cysts are detected by radiography.

## 2. Serological tests

The development of an automated sensitive and specific diagnostic test would greatly reduce the costs of damage to the carcass and also the costs of labour. Serological tests for animals have not reached the stage where commercialisation for individual diagnosis or large-scale detection of infected carcasses in slaughter houses is possible. All assays tested – AG-ELISA, antibody ELISA, enzyme linked immunoelectro transfer blot (EITB) and tongue inspection – show low sensitivity in rural pigs infected naturally with low levels of *T. solium* (18). This contrasts with their high sensitivity and specificity when applied to commercially reared pigs free from infection and such pigs experimentally infected with *T. solium* (17, 18). This finding is also true for *T. saginata* infections in cattle (16, 22). Thus, only a small percentage (13–22%) of cattle carrying fewer than 30–50 viable cysticerci is detected by AG-ELISA. Conversely, antibody has proven most useful for detecting cysts that are no longer viable. Nonetheless, AG-ELISAs do have a use in field-based epidemiological studies for indicating transmission. For example, the detection of viable infections in cattle or pigs could indicate point sources of infection, season of transmission and age of animals at risk.

The EITB assay for *T. solium* (used to detect antibody to lentil–lectin bound glycoproteins in cerebrospinal fluid or serum) is widely used in humans (20, 21) in many laboratories and also is available commercially (Immunetics, Cambridge, Massachusetts, USA). An AG-ELISA using polyclonal or monoclonal antibody (used to detect antigen in cerebrospinal fluid) has a specificity and sensitivity of up to 86% in selected patients (8). The specificity of these tests tends to be very high but sensitivity is lower, this is in part related to cyst number. The hierarchy of clinical symptoms, imaging studies and serological tests has been presented by del Brutto et al. (4).

## C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Immunochemoic identification of protective antigens and their production by recombinant DNA technology has been uniquely successful in the Taeniidae compared with other eukaryotes, and is described by Lightowlers & Gauci (11). Vaccination with the resultant products has been highly effective. Overall the success was advanced by the fact that a strong protective immunity occurs after natural infection, high levels of protective immunity are induced by antigens in oncosperal extracts, there is good cross-immunity between *Taenia* species, and immunity is largely antibody mediated as evidenced by passive and maternal transfer of immunity so that antibody could be used to probe for protective antigens. Initially the *T. ovis* vaccine was developed. The *T. solium* 45W antigen was isolated as a recombinant protein from *Escherichia coli*. The antigen being a glutathione-S-transferase fusion protein was highly efficacious, although beta-galactosidase fusion proteins were not. Potency control is by AG-ELISA and *in vivo* immunogenicity, and the vaccine has given protection for a period of at least 1 year in field trials. Two other antigens (To16K and To18K) have been isolated and cloned and each individual *T. ovis* protein is protective. Using the benefits of cross-reaction and probing with the *T. ovis* cDNA, TSA9 and TSA18, equivalent to To45W and To18, were identified in *T. saginata* DNA and were cloned from *T. saginata* oncosphere mRNA. In contrast to the *T. ovis* individual antigens, immunisation with both *T. saginata* antigens was required to produce high level protection. The *T. ovis* 45W and 18K cDNAs were also used to clone the equivalent TSO45 and TSO18 antigens of *T. solium*. Both the *T. ovis* and *T. saginata* vaccines have given >94% and 98% protection in cattle and sheep, respectively. The *T. ovis* vaccine was registered in 1994 by the New Zealand Animal Remedies Board. However, due to market changes in New Zealand, the vaccine is not available commercially. Costs of large-scale production of antigens, processing conditions and potential variants in expressed antigens have been outlined by Lightowlers & Gauci (11). Synthetic peptides from the sequences of these Taeniidae antigens induced antibody but not protection, indicating that the protective epitopes seemed conformational. Recently however, reasonable levels of protection were induced experimentally in piglets.
exposed to natural *T. solium* challenge using synthetic peptides based on protein sequences of the murine parasite *T. crassiceps* (10). It is possible that cost–benefit analyses concerning the use of the *T. saginata* vaccine could obviate its use in many countries, as cost of the vaccine is very important to the livestock industries. The importance of *T. solium* in humans increases the costs of the disease, but it remains to be seen whether the significance of the disease in endemic countries will be sufficient to push commercial production of the vaccine for use in livestock.

REFERENCES


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CHAPTER 2.10.2.
BUNYAVIRAL DISEASES OF ANIMALS
(excluding Rift Valley fever)

SUMMARY

Of the more than 537 known arboviruses, some 250 are contained in the largest family – the Bunyaviridae. One Bunyaviral disease of veterinary importance has already been described, namely Rift Valley fever, the causal agent of which is a member of the genus Phlebovirus (see Chapter 2.1.8.). Other genera of veterinary importance in the family are Nairobi sheep disease (NSD) virus, and the largest genus, Bunyavirus, which is subdivided into 18 antigenic groups. This genus contains only a few viruses that are significant pathogens of animals, among them Cache Valley virus (CVV) and Akabane virus. Although they have been placed in different antigenic groups, these two viruses have a tropism for fetal tissues and are responsible for congenital disease in domestic ruminants. Members of the Nairobi virus and Bunyavirus genera are single-stranded, enveloped RNA viruses, and have been more fully described in Chapter 2.1.8. Rift Valley fever.

Identification of the agent: CVV can be isolated from the blood of febrile or viraemic adult animals. Attempts at isolation from the fetus at birth are generally unsuccessful due to virus clearance by the fetal immune response. Cell lines derived from monkey or baby hamster kidney are employed for isolation of the virus or alternatively, intracerebral inoculation of infant mice may be used. Virus is identified by a complement fixation (CF) or a neutralisation test. Group- and virus-specific polymerase chain reaction (PCR) techniques have been developed for the Bunyaviruses. Akabane virus can be isolated from the blood of viraemic animals and occasionally from fetal material. Cell lines of monkey, baby hamster and mosquito are used. The virus produces deformities in the developing chicken embryo. Yolk sac inoculation, as well as intracerebral inoculation of suckling mice, are used. Virus is identified by CF or neutralisation tests. Group- and virus-specific PCR techniques have been developed for the Simbu group viruses. NSD virus is best isolated from plasma from febrile animals, mesenteric lymph nodes or spleen. Laboratory-reared sheep, 2–4-day-old unweaned mice inoculated intracerebrally, or cell cultures may be used for primary isolation. Sheep are the most sensitive animals for this, whereas a baby hamster kidney cell line and lamb or hamster kidney cell cultures are the most sensitive cells. Subinoculation of plasma from an experimentally infected sheep into cell cultures or mice is also recommended. Identification of the virus may be made by direct immunofluorescence of inoculated tissue cultures or of mouse brain smears. The agar gel immunodiffusion test can also be used to demonstrate the presence of NSD antigen in tissues. Infected tissue cultures or mouse brain suspensions may be used as sources of complement-fixing or enzyme-linked immunosorbent assay (ELISA) antigens.

Serological tests: For CVV and Akabane virus, haemagglutination inhibition, CF and serum neutralisation tests are used to detect antibodies. An ELISA, based on one used for Rift Valley fever, has also been described. For NSD the most suitable test is the indirect fluorescent antibody test. CF and indirect haemagglutination tests have also been used to confirm NSD outbreaks in the field. Virus neutralisation tests give equivocal results, a feature that also occurs with other members of the Nairobi virus group. ELISAs are now being evaluated for NSD. Infected spleen may be used as a source of antigen in immunodiffusion tests.

Requirements for vaccines and diagnostic biologicals: No vaccine is currently available for CVV. Vaccines for domestic use against Akabane virus have been produced in both Japan and Australia. For NSD, an experimental attenuated live virus vaccine has been investigated, and a killed tissue culture vaccine has been shown to be immunogenic.
A. INTRODUCTION

- **Cache Valley virus**
  
  Cache Valley virus (CVV) is an arthropod-borne ruminant virus endemic to North America. It is a member of the Bunyamwera serogroup of the genus Bunyavirus in the family Bunyaviridae. It is the most widely distributed Bunyavirus in North America. Antibody surveys have demonstrated the presence of CVV antibodies in domestic ruminants, deer and horses. Although most infections are subclinical and occurrence of disease is relatively rare, infection of the fetus leads to arthrogryposis, hydrancephaly, stillbirth, mummification and abortion. Infection with CVV or a closely related bunyavirus should be suspected if arthrogryposis and/or hydrancephaly occur in sheep flocks following a wet season with an abundance of vectors. Outbreaks are characterised by the appearance of abortions, weak lambs, and the birth of lambs with articular rigidity, skeletal muscle hypoplasia and vertebral column deformities including scoliosis and torticollis.

- **Akabane virus**
  
  Akabane virus is a member of the Simbu serogroup of the genus Bunyavirus in the family Bunyaviridae. It is an insect-transmitted ruminant virus endemic in many countries, particularly Japan and Australia where regular epizootics of disease occur. Akabane virus is also widely distributed in Africa, the Middle East and Asia, and antibodies have been found in cattle, sheep, goats, camels, horses and several species of African game. Infection in adult animals is subclinical but may lead to fetal losses, particularly in cattle, though sheep and goats are also vulnerable. The presence of Akabane virus should be suspected when outbreaks of abortion, stillbirth, congenital arthrogryposis and hydrancephaly occur in seasons of above-average rainfall with an abundance of insect vectors. Such outbreaks are characterised by abortions, stillbirths and the appearance of weak dumb calves with skeletal defects of the limbs and vertebral column, including torticollis and scoliosis.

- **Nairobi sheep disease**
  
  Nairobi sheep disease (NSD) is caused by a Nairovirus of the family Bunyaviridae; it is a tick-transmitted disease that is not contagious. NSD should be suspected when a mortality rate, which may range between 40% and 90%, occurs in a sheep or goat population, especially when this follows movement from free areas into enzootic areas. The disease is characterised by pyrexia (41.5°C), collapse, and diarrhoea. Abortion is also a feature. Animals that die in the early stages of the disease show congestion of most organs and tissues, with enlarged and oedematous lymph nodes; the spleen may also be enlarged. There are likely to be petechial and ecchymotic haemorrhages throughout the carcase on the serous surfaces of organs and especially the lymph nodes. Later in the course of disease, inflammation of the gastrointestinal tract may become evident. Infestation with ticks, notably *Rhipicephalus appendiculatus*, substantiates any suspicion as to the agent involved. There is a low total white cell count in the early febrile stages. Ganjam virus in India is believed to be a strain of NSD virus and causes a similar syndrome. NSD is an apparently rare zoonotic agent in the field, causing a mild influenza-like disease in humans, but at least seven laboratory aerosol infections by Ganjam virus have been recorded.

B. DIAGNOSTIC TECHNIQUES

- **Cache Valley virus**
  
  CVV is a teratogenic Bunyavirus of North America affecting mainly sheep. It is a member of the Bunyamwera serogroup of the Bunyavirus genus, family Bunyaviridae and is also the most common of the Bunyaviruses of North America (2). CVV was first isolated from a mosquito pool in Utah, United States of America (USA) in 1956 (16), but was only linked to disease during an epizootic of neonatal loss and malformed lambs in a sheep flock in Texas in 1987 (6). The virus has also been isolated from a horse and a clinically healthy cow.

  Serological surveys have shown a widespread prevalence of antibodies in domestic and wild ruminants and horses. Seroprevalence to CVV is high in deer, and the 1–3-day viraemia is sufficient to infect vectors allowing deer to act as amplifying hosts (1). Vectors include both *Culicoides* midges and mosquitoes of the *Aedes*, *Anopheles*, *Coquillettidia* and *Culiseta* groups.

  CVV infection of adult animals is largely subclinical, and experimentally infected ewes show only a transient febrile response, but with a detectable viraemia.

  CVV was the first North American Bunyavirus to be linked to fetal arthrogryposis and hydrancephaly, however other related viruses have been shown experimentally to have the same potential. Fetal infection with CVV is age dependent in its outcome. Malformations take place between 27 and 45 days’ gestation, with infection at 28–
36 days giving rise to central nervous system (CNS) and musculoskeletal defects, and infection at 37–42 days giving rise to musculoskeletal deformities only. Infection after 50 days’ gestation does not result in lesions and after 76 days the fetus is immunocompetent and antibodies are produced. Most CVV fetal deaths occur between 27 and 35 days’ gestation. The fetus is, however, susceptible at any age demonstrating the tropism of many bunyaviruses for fetal tissues (3).

Gross pathology of the musculoskeletal system includes arthrogryposis of one or more limbs, torticollis, scoliosis of the vertebral column and muscular hypoplasia. CNS lesions include hydrancephaly, hydrocephalus, porencephaly, microencephaly, cerebral and cerebellar hypoplasia and micromelia (3, 15). Dead embryos and stillborn or mummified lambs with no obvious defects are also found. Anasarca is seen, as is oligohydramnion. This reduction in amniotic fluid is thought to contribute to restriction of fetal movement and thus to the skeletal deformities seen. Limb defects are also due to neurodegenerative changes seen histopathologically as areas of necrosis and loss of paraventricular neuropil in the brain together with a reduction in the number of motor neurons. Skeletal muscle changes involve poorly developed myotubular myocytes (15).

- **Akabane virus**

Akabane virus is a teratogenic Bunyavirus widely distributed across the world but not in New World countries. It affects mainly cattle. It is a member of the Simbu serogroup of the Bunyavirus genus, family Bunyaviridae (18). Among two Japanese and seven Australian Simbu group viruses, Aino, Peaton, Douglas and Tinaroo are also thought to be potential pathogens. Akabane virus is however, the best studied and most pathogenic of the Simbu viruses and a major cause of arthrogryposis and hydranencephaly.

Akabane virus was first isolated in Japan in 1961, initially from a mosquito pool and then a pool of Culicoides midges. This was followed in 1972 by isolations from Culicoides in Australia and mosquito pool isolations in Africa. Akabane virus antibodies have been demonstrated in sera from cattle, sheep, goats, horses, buffalo and camels. Many indigenous game species in Africa south of the Sahara have Akabane virus neutralising antibodies. The range of Akabane virus includes the Middle East, Asia, Cyprus and Africa, but it is in Australia and Japan where regular epizootics of Akabane virus disease occur. Conditions favourable to such outbreaks are susceptible animals in early pregnancy and a sudden increase in vector populations, particularly when the virus has been absent from the area for a number of years.

Akabane virus infection in adult animals is usually subclinical, but encephalomyelitis has been recently associated with Adabane virus infection in adult cattle. Cattle seroconvert after a 3–4-day viraemia.

In endemic areas, antibody in the female animal prevents fetal infection, but Akabane virus is capable of establishing a long-term infection of the placenta in susceptible cattle and sheep. This takes place between 30 and 70 days’ gestation in the ewe and between 30 and 150 days’ gestation in the cow. Akabane virus has a predilection for brain, spinal cord and muscle cells where non-inflammatory necrosis interferes with morphogenesis.

Akabane virus infection has been studied experimentally in sheep and goats with the production of arthrogryposis/hydranencephaly, kyphosis, scoliosis, micro- and porencephaly, stillbirths and abortions (25). Natural infection of the ovine fetus has been described in Australia where perinatal lamb mortality and congenital microencephaly were most often seen.

Experimental Akabane virus studies have been carried out in pregnant cattle and it was shown that the type of abnormality is dependent on the gestational age of the fetus with hydroencephaly seen at 76–104 days and arthrogryposis at 103–174 days’ gestation (19). This time differential in appearance of abnormalities is clearly seen in bovine fetuses whereas in sheep with a shorter gestation period, brain and skeletal lesions appear concurrently in the same fetus. The sequence of events during an epizootic of Akabane virus-induced fetal loss are the birth of uncoordinated calves, followed by those with arthrogryposis and dysplastic muscle changes, and lastly those with hydrocephalus and other severe CNS lesions. These events may be preceded by stillbirths and abortions (26). Akabane virus is responsible for severe neural and muscular abnormalities and lesions are characterised by a nonpurulent encephalomyelitis, focal cerebral degenerative encephalomyelopathy porencephaly, microencephaly, hydrocephalus, loss of ventral horn motor neurons and axons, depletion of myelin in spinal cord motor tracts, necrosis and polymyositis in the myotubules with parenchymal degeneration of skeletal muscles. Spinal cord abnormalities include scoliosis, and kyphosis and arthrogryposis may affect almost any skeletal joint.

- **Nairobi sheep disease**

NSD is a disease of sheep and goats caused by a Nairovirus of the family Bunyaviridae (8). It is characterised by a mortality rate, which may range between 40% and 90%, and should always be suspected when animals have recently been moved from an area free from the disease into one where it is endemic. Outbreaks also follow incursions of ticks into previously free areas, particularly following heavy rains (9). The clinical signs are similar in
both sheep and goats, although there are differences in susceptibility among the various breeds and strains in their response to infection with NSD virus, some being more susceptible than others. Some indigenous breeds tend to be more susceptible whereas exotic breeds may recover after a prolonged illness. Cattle and game are refractory to infection with NSD virus (30). The incubation period for the disease varies from 2 to 5 days, when a temperature reaction of 41–42°C develops. There is hyperventilation accompanied by severe depression, anorexia and a disinclination to move. Animals stand with lowered head, and show a conjunctivitis and serosanguinous nasal discharge. Some of the superficial lymph nodes, such as the prescapular and/or precrural, become palpable. Diarrhoea usually develops within 36–56 hours of the onset of the febrile reaction. This is at first profuse, watery and fetid, later haemorrhagic and mucoid, and accompanied by colicky pains and tenesmus. Abortion is a common sequel to the infection. Examination of the predilection sites for the attachment of ticks, such as the ears, head and body, is likely to reveal the presence of the Ixodid tick *Rhipicephalus appendiculatus*.

Deaths can occur in peracute cases within 12 hours of the onset of the fever and at any time during the febrile reaction, while the animal is acutely ill. Further deaths then follow the fall in temperature for a further 3–7 days, associated with severe diarrhoea and dehydration.

The gross pathology of NSD can be misleading, for most deaths are likely to occur during the period of viraemia, when the only signs are likely to be lymphadenitis with petechial and ecchymotic haemorrhages on the serous surfaces of the alimentary tract, spleen, heart and other organs. None of these signs allows a specific diagnosis of NSD to be made or even suspected, for they are shared with many other febrile diseases of sheep in NSD-endemic areas. Diseases with which NSD may be confused include Rift Valley fever, peste des petits ruminants, rinderpest, salmonellosis and heartwater. Later in the course of the disease, a haemorrhagic gastroenteritis becomes more obvious, with haemorrhages on the mucosa of the abomasum, especially along the folds, in the region of the ileo-caecal valve, and most commonly in the colon and rectum. Zebra striping of the latter is often seen. The gall bladder is usually enlarged and haemorrhagic. Inflammatory lesions with haemorrhage may be seen in the female genital tract, if there has been abortion. However, in many animals dying from NSD, there may be none of these gastroenteric lesions, and a tentative diagnosis based on post-mortem signs can rarely be made. Common histopathological lesions are myocardial degeneration, nephritis and necrosis of the gall bladder.

The post-mortem signs in the early stages are the nonspecific changes associated with death at the viraemic stage of NSD, with congestion and petechial and ecchymotic haemorrhages on serous surfaces, on lymph nodes, the spleen and other organs such as the kidney, lungs and liver. Later, signs of haemorrhagic gastroenteritis become apparent, with ulceration of the abomasum, duodenum, caecum and colon. The virus is principally transmitted by the tick *Rhipicephalus appendiculatus*, and any infestation with such parasites should arouse suspicion of the presence of the disease. NSD virus may also be transmitted by other species of the genus *Rhipicephalus* and by the bont tick *Amblyomma variegatum*.

NSD is an apparently rare zoonotic agent in the field, causing mild influenza-like disease in humans. Laboratory infection has been associated with fever and joint pains (30).

1. **Identification of the agent**

- **Cache Valley virus**

At birth virus cannot be isolated from the fetus, but CVV has been isolated from mosquito pools and the blood of viraemic adult animals. This has been done on tissue culture using hamster and monkey kidney cell lines including baby hamster kidney (BHK), African green monkey kidney (Vero) and LLC-MK2. Virus can be isolated from a febrile animal using a 10% buffy coat suspension in minimal essential medium (MEM) and co-cultivation with Vero cells in MEM supplemented with 2% fetal bovine serum.

Virus isolation is also commonly done in newborn or weaned mice by intracerebral or intraperitoneal inoculation.

Many Bunyaviruses have been sequenced as they are medically important pathogens associated with encephalitis in humans in both North and South America. Polymerase chain reaction (PCR) technology has been applied to mosquito-pool surveillance, instead of traditional isolation in infant mice, and sensitivity is reported to be one positive mosquito in a pool of 100, which is undetectable by traditional plaque titration in cell culture. Group-specific and virus-specific primers have been designed, and using the reverse-transcription PCR (RT-PCR) the Bunyamwera (BUN) and California (CAL) serogroup viruses can be distinguished. Using a nested RT-PCR technique the CAL and most of the BUN serogroup viruses can be distinguished from other Bunyavirus genus members (20).
• **Akabane virus**

Diagnosis of infection is rarely made by virus isolation, but rather by histopathology and serology. Virus has, however, been isolated from viraemic sentinel animals using buffy coat suspensions from vector pools and occasionally from fetal material. RT-PCR has been described for the detection of Akabane virus and differentiation from Aino. This could contribute to the diagnosis, but the diversity of the Simbu serogroup will require validation to confirm specificity of the test as there is evidence of reassortment among the Bunyaviruses.

Suckling mice, 1–2 days old are used and inoculated intracerebrally with 0.01 ml of a clarified 10% suspension of the test material. Virus isolation in tissue culture is frequently done using Vero, BHK-21 and HmLu-1 cell lines. If C6/36 mosquito cells are used, cultures are left stationary for 7 days and material is repassaged on to a hamster or Vero cell line where cytopathic changes in the cultures become visible.

Antigen detection in formalin-fixed material by peroxidase staining of bovine and ovine fetal material has been described. Nucleic acid detection methods have also been developed for the differentiation of Aino and Akabane viruses using a nested RT-PCR technique.

• **Nairobi sheep disease virus**

NSD virus may be isolated from material collected from field cases by the use of laboratory animals or cell cultures (13). Safety precautions against aerosol infections should be taken when working with this agent. Uncoagulated blood, mesenteric lymph nodes and spleen tissue submitted on ice are the optimal samples to be collected from febrile or dead animals, respectively. The plasma can be used directly as inoculum, and the lymph nodes or spleen should be homogenised to make an approximate 10% (w/v) suspension in a transport medium. This medium can be Hanks’ medium with 0.5% lactalbumin hydrolysate or 0.75% bovine serum albumin, and containing penicillin (500 International Units/ml), streptomycin sulphate (500 µg/ml), and mycostatin (50 units/ml) or fungizone (2.5 µg/ml).

A recommended initial procedure is to inoculate an NSD-susceptible sheep held in isolation with 1–2 ml of the tissue suspensions, or of the plasma. Any pyrexia and clinical disease that develops permits a tentative diagnosis of NSD and, at the same time, furnishes excellent samples for virus isolation. This is especially valuable where the original field samples have been transported in hot climates where some loss of virus has inevitably occurred. Sheep are at least 100 times more sensitive than mice to NSD virus infection.

Infant mice, 2–4 days old, can be inoculated intracerebrally with 0.01 ml of a 1/10 dilution of plasma or of the tissue suspension. Two litters, each of 8–10 suckling mice, should be used for each sample and one blind passage is made routinely. The mice become debilitated and die within 5–9 days post-inoculation. Their brains should be harvested aseptically, pooled and diluted 1/100 for passage into further mice.

Cell cultures may be used in conjunction with mouse inoculation for the primary isolation of NSD virus, as they have shown levels of sensitivity similar to that of the intracerebral inoculation of unweaned mice. The BHK-21-C13 cell line is especially valuable; the Vero cell line (30) and primary and secondary lamb or hamster kidney cells have also been used. Most strains of NSD virus produce a cytopathic effect (CPE) on first passage in BHK cells; others produce a more obvious CPE only after subinoculation. The appearance of a CPE is not such a regular finding with lamb testis and kidney cells, although it is usually seen on the second passage in lamb kidney cells. Tube cultures should be used both with and without flying cover-slips, or if plastic bottles are used for isolation, microwell slide cultures should also be prepared. Approximately 0.2 ml should be inoculated and a period of 1–2 hours allowed for adsorption. The CPE becomes evident in roller cultures as foci of granular rounded cells after 24–48 hours in BHK cells, and in a further 24–48 hours in other cell types. The CPE is not specific for NSD virus, which is identified in cover-slip cultures by immunofluorescence or by staining with haematoxylin and eosin. The latter method reveals pleomorphic eosinophilic intracytoplasmic inclusions peculiarly of a spindle form; other inclusions are bipolar, or surround the nucleus.

The virus can be specifically identified by immunofluorescence staining, which may be positive as early as 24–48 hours post-inoculation when no CPE has yet become evident. Conjugates for direct immunofluorescence may be prepared from hyperimmune mouse ascitic fluids, and from immune rabbit or sheep antiserum by standard methods. Some cross-fluorescence may occur with other Nairoviruses at low dilutions of the conjugate, but these viruses are not normally associated with disease in sheep or goats.

The agar gel immunodiffusion test (AGID) can be a valuable primary diagnostic tool for the detection of NSD antigen in tissues. The test can be carried out in laboratories without tissue culture facilities and at field investigation laboratories. The spleen and mesenteric lymph nodes are the tissues of choice to be used in the test. Aliquots of 0.5–1 g should be homogenised with sterile sand in a pestle or a homogeniser to give 10–20% suspensions in phosphate buffered saline (PBS) or saline. The suspension should be centrifuged for 10–15 minutes at approximately 1000 g and the supernatant fluid is used in the test. This test can also be used for the identification of NSD virus antigen in mouse brain harvested from experimentally infected mice (see above).
Rabbit hyperimmune serum against NSD can be prepared by repeated inoculation of NSD-infected mouse brain. A mouse brain suspension at 2–5 % (w/v) is prepared as above and centrifuged at 3–5000 g for 15 minutes. Aliquots are then mixed with an equal volume of Freund’s complete adjuvant. Various inoculation regimes may be used but 1-ml volumes may be given subcutaneously and/or intramuscularly at 7-day intervals for 3–5 weeks, or at multiple inoculation sites in 0.1-ml volumes for a similar period. Serum should be collected 5–7 days after the last injection and stored in aliquots at −20°C.

Difco Noble or other suitable agar may be used in the test, using 0.85% sodium chloride at pH 7.2. Slides are prepared to give an agar layer approximately 2 mm in depth. Six wells should be placed hexagonally around a central well. The hyperimmune rabbit serum is placed in the central well and positive control antigen in wells 1 and 4. The tissue under test is placed in wells 2 and 5. Negative control tissue is placed in wells 3 and 6. Wells containing test tissue that give a precipitin line of continuity with the line formed between the positive antigen and the hyperimmune serum are considered to be positive.

Mouse brain suspensions or infective tissue culture fluids can be used as antigens for complement fixation (CF) tests for virus identification. Both have proved satisfactory after partial purification with fluorocarbon; the mouse brain can also be used in the form of a suspension in a borate buffer solution.

An enzyme-linked immunosorbent assay (ELISA) antigen for virus identification purposes can be prepared from an infected tissue culture in a bottle. The cells are removed using a pipette fitted with a rubber bulb when approximately 20% of the monolayer is showing CPEs. They are sedimented and washed three times in borate saline buffer, pH 9. The cells are then lysed and solubilised with SDS (sodium dodecyl sulphate) and 1% Triton X100, diluted approximately 1/5 in borate saline buffer and sonicated to provide an antigen for the ELISA. A control negative antigen is prepared in the same manner from uninfected cells. These are adsorbed directly on to ELISA plates and the test is carried out with NSD immune and normal serum with both antigens.

An RT-PCR has been described. Use of the PCR has detected Dugbe virus RNA in the organs and haemolymph of *Amblyoma vaiegatum* ticks. (Dugbe virus is a nonpathogenic member of the NSD serogroup and has been isolated from the blood of cattle in Africa.) The test is less sensitive than isolation in mice, but results may be expected within 48 hours instead of 8 days with a biological assay (27).

2. Serological tests

These include haemagglutination inhibition (HI), CF and virus neutralisation (VN) tests and ELISA.

- **Cache Valley virus**
  
  a) **Virus neutralisation test**

  VN tests for CVV used to be done by a plaque reduction neutralisation method but are now usually performed using inhibition of CPE on Vero cells in microtitre plates (4).

  - **Test procedure**
    
    1. Inactivate test sera at 56°C for 30 minutes in a water bath.
    2. Make serial twofold dilutions of the sera in MEM from 1/2 to 1/16 and incubate at 37°C for 60 minutes with an equal volume of 100 TCID$_{50}$ (tissue culture infective dose) per ml of virus. Standard controls are prepared in a similar manner.
    3. Discard the medium in a 96-well flat-bottomed cell-culture grade microtitre plate containing a preformed 24-hour Vero monolayer.
    4. Add the serum/virus mixtures to the plate, 50 µl per well, using three wells per dilution.
    5. Back titrate the virus used in the test, making three tenfold dilutions using 50 µl per well and four wells per dilution.
    6. Cover the plates and incubate for a further 60 minutes at 37°C.
    7. Add 50 µl MEM maintenance medium to each well.
    8. Incubate the plates at 37°C for 6 days in a humidified CO$_2$ incubator.
    9. Read the plates microscopically, evaluate the CPE and determine the 50% end points.
    10. The virus control should give a value of 100 TCID$_{50}$ and there should be no neutralisation by the negative control serum at the lowest dilution tested. The positive control should give a titre within an expected range of its predetermined mean.
Chapter 2.10.2. — Bunyaviral diseases of animals (excluding Rift Valley fever)

b) Enzyme-linked immunosorbent assay

An ELISA, modified and based on the one for Rift Valley fever described by Meegan et al. (23), has been used for CVV serological surveys. Modifications include a 1/400 dilution of mouse ascitic fluid to coat the plates, followed by a 1/25 dilution of a sucrose/acetone mouse brain antigen in a sandwich ELISA format. The diluent used is PBS with 0.5% Tween 20, 5% equine serum and 500 µg dextran sulphate per ml. A horseradish peroxidase conjugate detection system and an ABTS (2,2’-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) substrate are used (23).

c) Other tests

Not all members of the Bunyamwera group produce haemagglutinins, but a HI test has been described for CVV using a sucrose/acetone sucking mouse brain antigen and goose erythrocytes at pH 6.2. The test is said to lack sensitivity compared with a VN test, providing only 50% detection of antibodies. The CF test is little used because of extensive cross-reactivity within the Bunyamwera group.

• Akabane virus

a) Agar gel immunodiffusion test

The AGID test is broadly cross-reactive within the Simbu group, is less sensitive than the VN test, but has been used in the past to screen large numbers of serum samples. Infectious tissue culture fluid is concentrated using a dialysis sac and PEG 20,000 (polyethylene glycol). A 1% agarose in borate saline buffer, pH 8.6, is used in a standard seven-well (hexagonal) pattern. The test is incubated in a humid chamber and read after 48–72 hours, using suitable controls.

b) Haemagglutination inhibition test

The HI test is modified after Clarke & Casals 1958 (5) and better haemagglutination is achieved with an increased NaCl molarity. The test is also pH dependant. Sera are pretreated with kaolin or acetone and then heat inactivated at 56°C for 30 minutes. The test is performed using four units of sucrose/acetone-extracted mouse brain antigen, 0.3% red blood cells and borate buffer, pH 9 (17).

c) Virus neutralisation test

VN tests have been described using HmLu-1 cells in tube cultures or Vero and BHK cells in flat-bottomed 96-well microtitre plates (7). Two techniques have been described with a serum/virus incubation period of 1 hour or incubation overnight before the addition of the cells.

• Test procedure

i) Inactivate the test sera at 56°C for 30 minutes in a water bath.

ii) Prepare serial twofold dilutions of the sera in Eagles medium from 1/2 to 1/16 in a 96-well flat-bottomed microtitre plate using duplicate wells and 25 µl per well. Standard controls are prepared in a similar manner.

iii) Add 25 µl per well of virus in Eagles medium diluted to provide 200 TCID₅₀ per 50 µl.

iv) Cover and incubate at room temperature for 1 hour.

v) Include a back titration of virus in triplicate, making three tenfold dilutions using 25 µl per well.

vi) Add 100 µl per well Vero cells in Eagles medium with 2% serum at 5 x 10⁵ cells/ml.

vii) Incubate the plates at 34–37°C for 5 days in a humidified CO₂ incubator.

viii) Read the plates microscopically and calculate the titre as the reciprocal of the highest serum dilution completely inhibiting the CPE.

ix) The virus and serum controls should give the expected results.

Where overnight incubation is used, duplicate twofold serial dilutions of inactivated serum are mixed with 100 TCID₅₀ of virus using 100-µl volumes in each case. Following incubation for 1 hour at 37°C and overnight at 4°C, 50 µl BHK cells is added to the test. The plate is examined at 3 and 5 days’ incubation at 37°C and checked for CPE.

d) Enzyme-linked immunosorbent assay

Akabane virus ELISAs, using both IgG and IgM, have been described and found to be more specific and sensitive than the VN test. Coating antigen is 10⁶ TCID₅₀ per ml of virus grown on HmLu-1 cells diluted in a
0.05 M carbonate/bicarbonate buffer, pH 9.6. The wash medium is PBS containing Tween 20 and alkaline phosphatase. Rabbit anti-bovine IgG and IgM conjugates are used (28).

A similar ELISA using horseradish peroxidase rabbit anti-bovine IgG conjugate has also been described.

e) Complement fixation test

The CF test, not described here, is a group-specific test and is used mainly to study intergroup relationships among Simbu viruses.

- Nairobi sheep disease

a) Indirect fluorescent antibody test

The indirect fluorescent antibody test (FAT) is the most suitable test for use with members of the Nairovirus group. There are however, some cross-reactions, particularly with Dugbe virus and also with other members of the group, such as Congo–Crimean haemorrhagic fever virus (10). The NSD antibody titres by this method range from 1/640 to 1/10,240, and such titres are not obtained with immune sera to other members of the group (11).

The method has been used in epidemiological studies and to study the response to experimental vaccines. There do not appear to be any serological differences among the 40–50 isolates that have been examined. An I-34 strain1 was the virus usually used to prepare antigen, and this has been adapted to grow in BHK-21-C13 cells, after a series of passages.

The virus antigen in the cell substrate of choice may be grown in loose cover-slips, multiwell slides, Teflon-coated slides or microtitre plates for the test. A method using Teflon-coated slides is described.

- Preparation of antigen slides

i) Wash and sterilise Teflon-coated slides. This is done briefly with a hot detergent that is used for tissue culture glassware in the laboratory, then three rinses in tap water for 30 minutes, each followed by similar rinses in distilled/deionised water. Slides are then placed in 70% alcohol for 10 minutes, removed with a sterile forceps and wrapped in greaseproof paper. They will then be found to be sterile, but further sterilisation in a microwave for two cycles of 5 minutes each is recommended.

ii) Place these slides in sterile dishes using a sterile forceps; a square polystyrene type is better than the round variety.

iii) Mix a suspension of BHK cells containing approximately 25,000 cells/ml in BHK growth medium (usually Eagles for BHK cells), and add 1000 TCID₅₀ of NSD I-34 strain per ml. Mix by pipetting. Prepare some uninfected negative control slides.

iv) Add the infected cells in 50 µl volumes (for the 12-well size) or as appropriate to the size of the Teflon wells. Replace the cover on the dishes and put into a humidified CO₂ incubator or anaerobic container.

v) Leave overnight for the monolayer to form. Then remove the plates from the incubator to a laminar flow cabinet, and flood with maintenance medium using a pipette to cover the slides to a depth of 2–3 mm. Return to the incubator.

vi) Harvest the antigen slides just as foci of CPE become detectable. This will be in 36–56 hours (more accurate determination of the optimal harvesting time may be made by fixing and staining one slide after 24, 36 and 48 hours).

vii) The slides are washed three times in PBS and dried. They are then fixed with dry heat (minimum 80°C) or with ice-cold acetone for 10 minutes. The slides are wrapped and may be stored at 4°C for 2–3 months, or at −20°C for 1–2 years. Slides stored at −20°C must be brought to 4°C overnight before use.

Similar procedures may be followed to prepare antigen on flying cover-slips or multiwell culture slides. When using Nunc tissue culture multiwell plates, however, fixation should be with 75% acetone.

- Test procedure

i) Hydrate the slides by adding a drop of PBS to the wells with a Pasteur pipette. Number the slides according to the number of sera to be tested. Include in the series control positive and negative sera with infected and uninfected cell cultures.

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1 The I-34 strain was a virulent NSD isolate made in Kenya that was used extensively as a reference strain at the Kabete Laboratory – Kenya Agriculture Research Institute, P.O. Box 58137, Kabete, Nairobi, Kenya.
ii) Discard the PBS and add the serum dilutions 1/80–1/2560 in a predetermined manner to wells 1 to 6. It is preferable to duplicate each dilution on the same side.

iii) Place the slides in dishes and hold at 37°C in a humid incubator for 40 minutes.

iv) Wash the slides in racks in three changes of PBS, 5 minutes per wash.

v) Add the fluorescein-conjugated anti-species conjugate (usually anti-sheep or anti-goat) at a predetermined working dilution; one drop can be added to each well with a Pasteur or other pipette.

vi) Incubate as before for 30 minutes.

vii) Wash three times in PBS and dry the slides.

viii) Examine the slides by fluorescent microscopy. NSD virus antigen is found in the cell cytoplasm, and foci of bundles of fluorescing BHK cells will be seen. The antigen is seen mainly in fine fluorescent particles, but larger irregularly shaped antigen clumps occur, often surrounding the nucleus, or in spindle-like masses filling the cytoplasm to the pole of the cells. These particles will not be seen with negative sera or in the uninoculated control culture.

ix) Sera that show this fluorescence at dilutions of 1/640 or 1/1280 are indicative of recent infection with NSD (11).

b) Other tests

CF tests are complicated by the marked anticomplementary activity of many sheep sera.

Immunodiffusion tests have been used successfully with crude antigens prepared from infected sheep tissue, tissue culture fluids or mouse brain material. Hyperimmune sera can be prepared in sheep, mice or rabbits for use in the test, using infected spleen taken from sheep dying from NSD as the source of antigen for immunisation. Control negative test systems should be established using uninfected positive and negative sheep spleens (or other antigen sources), with known NSD-negative sera. Such reagents can be prepared in any laboratory and do not require the support of a virological laboratory. This test is useful although relatively insensitive.

An ELISA using a partially purified tissue culture antigen has been described for antibody testing and is suitable for use in serological surveys. The indirect FAT test should, however, be used to check doubtful results (24).

Monoclonal antibodies to the antigens of NSD virus strain I-34 have been developed and are being evaluated for their application as diagnostic reagents.

RNA probes have also been developed from the S (small) and M (medium) genome segments of Dugbe virus and have been used to demonstrate that the NSD serogroup of the genus Nairovirus is more closely related to the Crimean–Congo haemorrhagic fever serogroup than any of the remaining serogroups (22, 29). These probes also have the potential to be applied as diagnostic tools.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

- **Cache Valley virus**

Due to the sporadic nature of disease outbreaks, no vaccine has been developed.

- **Akabane virus**

Major epizootics of Akabane virus disease have only been reported from Japan and Australia, albeit at irregular intervals, but vaccination is seen to have merit in preventing fetal loss.

Vaccines for domestic use have been produced in Japan and Australia.

An inactivated vaccine is used in Japan for immunising cattle and goats. It is a formalin-inactivated intramuscular preparation with an aluminium phosphate gel adjuvant. Two 3-ml doses are given at a 4-week interval and yearly boosters are recommended. It is safe for use in pregnant animals. In field trials 88% of animals developed high VN antibodies after the first inoculation and there was a 100% response after the second dose (21). Similarly, in Australia an inactivated vaccine has been produced for intramuscular use giving two doses at a 4-week interval just before mating.
Akabane virus has also been attenuated in Japan as a potential vaccine candidate. Pregnant cattle and calves were inoculated subcutaneously, intramuscularly and intracerebrally; no leukopaenia, viraemia or pyrexia was observed and a good VN antibody response was produced. A live Akabane virus vaccine, safe in cattle, was tested in pregnant ewes. During the trials, some ewes became viraemic and virus was found in the organs of several fetuses. Although no fetal deformities were produced, the vaccine is deemed unsuitable for use in sheep.

- Nairobi sheep disease

Epidemiological investigations have shown that in a state of enzootic stability, no problems are encountered with NSD. The disease arises from animal movements from free areas into endemic areas and can be avoided when such areas have been defined. Ecological changes that permit spread of the vector tick will result in extensions of these areas.

Experimental vaccines have been prepared for such situations. One vaccine has consisted of virus attenuated by 35 passages in adult mice, but such vaccines can produce severe reactions in some breeds of sheep, and are not considered to be safe for general use. A similar vaccine was developed in Entebbe by further mouse brain passages, but this has not been further developed for use in the field in Uganda or elsewhere.

A tissue-culture-adapted strain of NSD virus has been grown to high titre in roller culture. When precipitated with methanol, inactivated, and administered with an adjuvant, this was found to give good protection following two inoculations given at an interval of 14 days. Neither of these vaccines is routinely produced, for there has been little demand for their use from the field (12, 14).

REFERENCES


CHAPTER 2.10.3.

SALMONELLOSIS

SUMMARY

Salmonellosis is an infectious disease of humans and animals caused by organisms of the two species of Salmonella (Salmonella enterica and S. bongori). Although primarily intestinal bacteria, salmonellae are widespread in the environment and commonly found in farm effluents, human sewage and in any material subject to faecal contamination. Salmonella organisms are aetiological agents of diarrhoeal and systemic infections in humans, most commonly as secondary contaminants of food originating from the environment, or as a consequence of septicaemia in food animals. Human salmonellosis is the most common and important zoonotic disease caused by Salmonella organisms. These organisms are also found in feedstuffs, causing infectious disease in animals, particularly poultry and pigs. Salmonellosis has been recognised in all countries, but appears to be most prevalent in areas of intensive animal husbandry, especially in pigs and calves and some types of poultry reared in confinement. Reptiles are also commonly asymptomatic carriers of Salmonella.

The disease can affect all species of domestic animals; young animals and pregnant and lactating animals are the most susceptible. Enteric disease is the commonest clinical manifestation, but a wide range of clinical signs, which include acute septicaemia, abortion, arthritis and respiratory disease, may be seen. Many animals, especially pigs and poultry, may also be infected but show no clinical illness. Such animals may be important in relation to the spread of infection between flocks and herds and as sources of food contamination and human infection.

Fowl typhoid and Pullorum disease, poultry diseases caused by Salmonella, are addressed in Chapter 2.7.5. of this Terrestrial Manual.

Identification of the agent: Diagnosis is based on the isolation of the organism either from tissues collected aseptically at necropsy or from faeces, rectal swabs or environmental samples, food products and feedstuffs. When infection of the reproductive organs, abortion or conceptus occurs, it is necessary to culture fetal stomach contents, placenta and vaginal swabs and, in the case of poultry, embryonated eggs.

Salmonellae may be isolated using a variety of techniques, which may include pre-enrichment to resuscitate sublethally damaged salmonellae, enrichment media that contain inhibitory substances to suppress competing organisms, and selective plating agars to differentiate salmonellae from other enterobacteria.

Various biochemical and serological tests can be applied to the pure culture to provide a definitive confirmation of an isolated strain. Salmonellae possess antigens designated somatic (O), flagellar (H) and virulent (Vi), which may be identified by specific typing sera, and the serovar may be determined by reference to the antigenic formulae in the Kauffman–White scheme. Many laboratories may need to send isolates to a reference laboratory to confirm the full serological identity and to determine the phage type and genotype of the strain, where applicable.

Serological tests: Serological tests should be conducted on a statistically representative sample of the population, but are of limited value if vaccination is used. In poultry, the whole blood test is used for rapid diagnosis of S. Pullorum/Gallinarum on the farm, being a relatively reliable diagnostic test under certain circumstances. In the laboratory, the tube agglutination test is the method of choice for export and diagnostic purposes for samples from all species of farm animals. Enzyme-linked immunosorbent assays are available for some serovars and may be used for serological diagnosis and surveillance, especially in poultry and pigs. Vaccination contributes to the limited diagnostic value of serological tests.

Requirements for vaccines and diagnostic biologicals: Many inactivated vaccines are used against salmonellosis and some live vaccines are available commercially. Due to the rather low
efficacy of the inactivated vaccines, oil or alhydrogel adjuvants are used to improve their immunogenic properties. Field efficacy data are often lacking, although laboratory testing may provide a useful indication. Innocuity tests are performed in laboratory animals and, in the case of inactivated vaccines, sterility tests using bacteriological enrichment media are carried out. Further reassurances, such as environmental impact and stability, are necessary for vaccines produced using genetic manipulation. Competitive exclusion is used to reduce Salmonella infections in poultry and other animal species.

A. INTRODUCTION

The classification of salmonellae has been controversial for many years. According to the latest nomenclature, which reflects recent advances in taxonomy (33), the genus *Salmonella* consists of only two species: *S. enterica* and *S. bongori* (21, 32). *Salmonella enterica* is divided into six subspecies, which are distinguishable by certain biochemical characteristics and some of which correspond to the previous subgenera. These subspecies are:

- Subspecies I = subspecies *enterica*
- Subspecies II = subspecies *salamae*
- Subspecies IIIa = subspecies *arizonae*
- Subspecies IIIb = subspecies *diarizonae*
- Subspecies IV = subspecies *houtenae*
- Subspecies VI = subspecies *indica*

For the serovars of *S. bongori*, the symbol V was retained to avoid confusion with the serovar name of *S. enterica* subsp. *enterica*. Strains of *Salmonella* are classified into serovars on the basis of extensive diversity of lipopolysaccharide (LPS) antigens (O) and flagellar protein antigens (H) in accordance with the Kauffmann–White scheme; currently approximately 2500 serovars are recognised (32, 33). This number is constantly being increased. The most common serovars that cause infections in humans and food animals belong to subspecies *enterica*. The serovars of the other subspecies are common in poikilothermic (cold-blooded) animals and in the environment, although some serovars of *S. arizonae* and *S. diarizonae* have been associated with disease in turkeys and sheep.

Names are retained only for subspecies *enterica* serovars. These names must no longer be italicised. The first letter is a capital letter. In clinical practice the subspecies name does not need to be indicated as only serovars of subspecies *enterica* bear a name, e.g. Typhimurium, London or Montevideo are serovars of subspecies *enterica*. The name *Salmonella* Typhimurium may be used for routine practice.

In this chapter, the abbreviated new conventions are followed, i.e. *S. Typhimurium* rather than the more complete nomenclature *S. enterica*, subsp. *enterica* serovar Typhimurium.

Salmonellosis is an infectious disease of humans and animals caused by organisms of the two species of *Salmonella* (*Salmonella enterica* and *S. bongori*). Although primarily intestinal bacteria, salmonellae are widespread in the environment and may commonly be found in farm effluents, human sewage and in any material subject to faecal contamination. Salmonellosis has been recognised in all countries, but appears to be most prevalent in areas of intensive animal husbandry, especially of poultry or pigs.

The disease can affect all species of domestic animals; young animals and pregnant animals are the most susceptible. Enteric disease, often presenting as a bloody or profuse watery diarrhoea with pyrexia, is the commonest clinical manifestation, but a wide range of clinical signs, which include acute septicaemia, abortion, arthritis, necrosis of extremities and respiratory disease, may be seen. The signs and lesions are not pathognomonic. Many animals, especially poultry and pigs, may also be infected but show no clinical illness (46). Such animals may be important in relation to the spread of infection between flocks and herds and as causes of human food poisoning. In the latter case, this can occur when these animals enter the food chain thus producing contaminated food products (45, 46).

The course of infection, the clinical signs, the post-mortem findings and epidemiological patterns vary according to the serovar and the animal species involved. Some serovars only affect certain hosts, e.g. *S. Gallinarum* in poultry or *S. Choleraesuis* in pigs, although most serovars may cause disease in a wide range of animal species (37). Many serovars (including some that are host adapted) have been shown to cause human food poisoning, and animal attendants, veterinarians and abattoir workers may be infected directly during the course of their work, as may laboratory personnel.
Disease is usually referred to as salmonellosis, although the term paratyphoid may be used, e.g. swine paratyphoid. In poultry, pullorum disease or bacillary white diarrhoea and fowl typhoid are often used to describe infections caused by *S. Pullorum* and *S. Gallinarum*, respectively (37). Fowl typhoid and Pullorum disease are covered in detail in Chapter 2.7.5. of this Terrestrial Manual.

For detailed epidemiological investigations, strain identification is necessary and such investigations have traditionally relied on biochemical and serological methods, phage typing of some serovars, and antibiograms. Genotypic analysis of the organism by molecular fingerprinting of DNA has been used to good effect in recent years. Plasmid profile analyses supply a quick and relatively easy method to fingerprint strains, and have been used in both human and veterinary medicine to study the spread of *Salmonella*. This technique has limitations as not all strains of *Salmonella* harbour plasmids, and plasmids may be readily acquired or be of similar size but genetically different. Alternative genetic techniques, such as pulsed-field gel electrophoresis and ribotyping, have been used to supplement plasmid profiling (25, 39). Genotyping is a rapidly expanding field and many new methods have been developed in recent years. It should be remembered that a single method may not work for all isolates and it may be necessary to evaluate a number of different techniques to find a method or combination of methods that is satisfactory and capable of differentiating clones of a particular serovar or phage type.

The isolation and subsequent identification of salmonellae depend not only on the quality of the sample but also on the culture medium and growth characteristics of the serovar, particularly those adapted to a host species. A comprehensive review of *Salmonella* infection in domestic animals has recently been published (46).

National schemes have been implemented in many countries to control *Salmonella* infections in animals in order to protect the consumer. In the European Union, the Zoonoses Directive 92/117/EEC requires the monitoring of breeding flocks of more than 250 birds and hatcheries for *S. Enteritidis* and *S. Typhimurium* (9). Culture of chicken delivery-box liners and dead or culled chickens is carried out on the day of arrival. At 4 weeks of age and 2 weeks prior to laying, pooled faeces of up to 60 samples, depending on the flock size, are cultured. Subsequently, adults are sampled every 2 weeks. At the hatchery, the meconium or dead-in-shells are cultured every 2 weeks. Serological monitoring is permitted if the serological tests can offer guarantees equivalent to the system of inspection at the hatchery and if vaccination has not been used. In Denmark serological monitoring for *Salmonella* is used for pig herds and commercial laying flocks. At the time of writing this chapter, the Zoonoses Directive is subject to revision so requirements for sampling may change.

*Salmonella* infections of food animals play an important role in public health and particularly in food safety, as food products of animal origin are considered to be the major source of human *Salmonella* infections (45). Special programmes have been implemented for surveillance of poultry, swine and cattle and include the surveillance of healthy animals that may be subclinical carriers of *Salmonella* organisms. Cross-contamination during food processing is also monitored as contamination by healthy food handlers can occur (46).

Feed contaminated with *Salmonella* is a most common source of animal infections. As feed contamination is often caused by *Salmonella* serovars of relevance to public health, feedstuffs, particularly meat-and-bone meal, should be investigated for the presence of salmonellae (46).

Samples of food and feed tested for *Salmonella* should be truly representative. Proper steps should be taken to prevent damage during transport or storage (17, 18). Because of the large variety of food and feed products there is no single sampling method appropriate to all products. Therefore different methods specific to the product should be used (10, 19).

The World Health Organization provides information on the development of appropriate measures for the prevention and control of food-borne diseases, including *Salmonella* infections, of humans. The most common vehicles of infection are eggs and egg products, poultry meat and meat from other food animals, and meat products. *Salmonella Enteritidis* and *S. Typhimurium* are the most widespread serovars in European countries, while *S. Typhimurium* is the dominant serovar in North America (45).

A *Salmonella* control policy for public health purposes should cover all stages from ‘the stable to the table’. It should include the mandatory reporting of all outbreaks of the disease (12), and animal feed testing (46). Feed monitoring also includes sampling of compound feed and feed materials, raw materials of animal origin, as well as sampling during feed processing. World-wide epidemiological investigations should be done to monitor *Salmonella* transmission and support *Salmonella* control policies.

Health controls at slaughter are essential, and special precautions should also be applied when slaughtering potentially infected herds. Decontamination measures should be implemented during processing.

Another essential element in the prophylaxis of human salmonellosis is consumer education, in particular awareness of safe handling and storage of food, kitchen hygiene and proper cooking to limit the risk of infection.
1. Identification of the agent

The frequency of sampling and the type of samples obtained will depend largely on the clinical findings, laboratory facilities, epidemiological data and the objectives.

Individual samples for bacteriological tests are collected as aseptically as possible and before any antibiotic treatment has commenced. Preferably samples are collected during the acute phase of the disease or as soon as possible after death. In the case of intensively housed poultry flocks, environmental samples, such as litter and dust or drag or boot swabs from floor surfaces (4), may be the most cost-effective way to identify infected flocks. Precautions should be taken to avoid cross-contamination of samples during transit and at the laboratory. Packages should be kept cool and accompanied by adequate information. For smaller animal species, it may be preferable to submit a representative number of sick or recently dead animals to the laboratory, if that is possible (44). Host-adapted serovars are more difficult to isolate from faeces so if these are suspected, infected tissues should be cultured where possible.

Particular attention should be given to the isolation of salmonellae from animals with subclinical infection, as these may only excrete bacteria intermittently and in low numbers. An increased sample size, increased number of samples representing more individuals, combined with pooling of samples and repeat sampling can provide an increased diagnostic sensitivity. In such situations bacteriological or serological methods should be used to identify infected flocks or herds rather than to identify infected individual animals.

- Culture

There are numerous methods for isolation of Salmonella in use world-wide (8, 13, 16, 23, 34, 44). Some of the more common methods are described below. All culture media prepared should be subjected to quality control and must support growth of the suspect organism from a small inoculum. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are working correctly.

a) Pre-enrichment media

The number of salmonellae in faeces from asymptomatic animals, environmental samples, animal feed and food is usually low, and it is necessary to use pre-enrichment media, such as buffered peptone water or universal pre-enrichment broth, to assist isolation. This may allow the small numbers of salmonellae, which may otherwise be killed by the toxic effect of enrichment media, to multiply, or it may help to resuscitate salmonellae that have been sublethally damaged, e.g. by freezing, heating, exposure to biocides or desiccation. Pre-enrichment may not be the best method for isolating less vigorous Salmonella strains, such as the host-adapted strains, from faeces because of overgrowth by competing organisms during nonselective pre-enrichment.

b) Enrichment media

Enrichment media are liquid or semi-solid agar media that contain additives that selectively permit salmonellae to grow while inhibiting the growth of other bacteria. Some, however, are also relatively toxic to certain serovars of Salmonella, e.g. selenite inhibits S. Choleraesuis, and brilliant green is toxic to many strains of S. Dublin. Elevated temperatures have also been used to increase the selectivity of enrichment medium, and a temperature of 43°C is used in some laboratories, although this may be inhibitory with some media, e.g. tetrathionate and Rappaport–Vassiliadis at 43°C inhibit temperature-sensitive strains, especially S. Dublin and 41.5°C is now recommended for incubation of Rappaport–Vassiliadis broth. Selective motility enrichment may also be used to increase the sensitivity of Salmonella isolation and semi-solid enrichment media, e.g. modified semi-solid Rappaport–Vassiliadis or Diagnostic Semi-Solid Salmonella medium (DIASALM), may provide greater sensitivity (42). The formulation of the medium, temperature and duration of incubation, and the volume of the samples used to inoculate the medium, may all serve to improve the isolation rate, and these variables should always be taken into account. Examples of selective enrichment medium are sodium tetrathionate, as in Muller–Kauffman broth, selenite F, selenite cysteine, brilliant green broth and Rappaport–Vassiliadis broths, or semi-solid Rappaport–Vassiliadis medium. Additions such as Ferrioxamine E may be added to selective media to enhance isolation of Salmonella from iron or nutrient-limited samples such as eggs, water or soil (34).

c) Selective plating media

These are solid, selective agars that permit differential growth to varying degrees. They inhibit growth of bacteria other than Salmonella and give information on some of the principal differential biochemical characteristics – usually nonlactose fermentation and hydrogen sulphide (H₂S) production. The results are
read after 24 and 48 hours of culture at 37°C. Salmonellae form characteristic colonies on such media that are usually distinguishable from the colonies of other bacteria on the plate, with the possible exceptions of Proteus, Pseudomonas and Citrobacter. Lactose-fermenting salmonellae may occasionally be isolated and H₂S production may be variable. Such atypical strains may be more effectively detected when semi-solid selective media are used. DIASALM medium is particularly useful in this respect as presumptive confirmation by slide agglutination testing using polyvalent O, H or specific antisera can be carried out on liquid from the growth zone in the plate. Salmonella Abortusovis is a slow-growing serovar and it is usual to incubate plates for up to 72 hours and to use the nonselective blood agar. Examples of selective media are brilliant green agar, xylose lysine deoxycholate agar, deoxycholate/citrate agar, Rambach agar, and bismuth sulphite agar, although many more will be found in the literature and media catalogues.

- **Identification of suspect colonies**

Suspect colonies are subcultured on to selective and nonselective agars to ensure that possible contaminants, such as Proteus spp., are absent. If there is an abundant pure growth, suspect colonies may be tested by slide agglutination with polyvalent Salmonella-typing sera (22, 32). In some cases, the suspect colony may not agglutinate and it is necessary to use biochemical tests to confirm the identity. These tests can be performed with peptone water sugars or commercial systems (such as the Analytical Profile Index [API] system), or composite media (such as triple sugar iron agar [TSI]), can be used to screen organisms (11).

The determination of the O factor(s) and the H antigen(s), and in special circumstances the Vi antigen, is performed by direct slide agglutination or tube agglutination using specific antisera. In the case of biphasic organisms, it is necessary to determine both phases by phase inversion – this involves passage through semi-solid agar containing antiserum to the known phase. Screening is facilitated by the availability of antisera directed against several factors, which can be pursued further by the use of monovalent typing sera. While most laboratories can identify the more common serotypes, it is necessary to use the facilities of a reference laboratory to confirm the identity of an isolate and possibly to obtain information on the phage type, if there is a scheme available, and genetic profile.

Additional biochemical tests may be necessary to identify some serotype variants, e.g. d-tartrate, which can be used to differentiate S. Paratyphi B var. Java from S. Paratyphi B. Isolates should also be tested for their sensitivity to a range of antimicrobial agents.

- **Immunological and nucleic acid recognition methods**

Numerous alternative Salmonella detection methods are in use and are commercially available (3, 5, 7, 27, 35, 36, 38, 40, 47). These include electrical conductance/impedence, immunomagnetic separation, enzyme-linked immunosorbent assay (ELISA), gene probes polymerase chain reaction methods, including nucleic acid sequence based amplification (NASBA) (15). Many of these methods have not been validated for faecal and environmental samples, however, and are more suited to analysis of human foodstuffs (31). The rapid methods are usually more expensive than conventional culture, but can be economically viable for screening materials where a low prevalence of contamination is expected or where materials such as feedstuffs are held pending a negative test. Currently none of the rapid methods has been shown to be suitable for direct detection of Salmonella so nonselective or selective enrichment stages are required (29). Typically this introduces more steps and operator time in the detection procedure. For DNA-based methods, inhibition of the PCR reaction by elements of the test sample matrix is problematic and requires suitable DNA extraction techniques (20). Rapid isolation methodologies may also be linked with sophisticated detection systems, such as biosensors (30). There are many variations and developments in rapid methods for Salmonella detection, but none has been shown to satisfactorily replace culture of veterinary samples in all circumstances. It is therefore not possible to provide details of all the methods in this chapter or to make recommendations, but the review articles cited above will provide further information. Internet searches are also a useful source of further information. Efforts are currently being made to standardise the use of certain rapid methods internationally (24), but there is a considerable amount of work still to be done.

- **Example test procedures for isolation of Salmonella from food, feedstuffs, faecal and environmental samples**

  i) Add a 10–25 g sample to ×10 volumes of buffered peptone water at ambient temperature. (NB: for many host-adapted serovars and some arizonae serovars, it is preferable to add the sample to selective enrichment medium, such as selenite cysteine broth, and to test tissue samples where possible [including direct plating]; see culture method for S. Pullorum/Gallinarum in Chapter 2.7.5. of this Terrestrial Manual.)

  ii) Incubate buffered peptone water for 16–20 hours at 37°C.

  iii) Inoculate 20 ml modified semi-solid Rappaport–Vassiliadis or DIASALM in a Petri dish with 0.2 ml incubated buffered peptone water broth.
iv) Inoculate 10 ml tetrathionate broth with 1 ml incubated buffered peptone water broth.

v) Incubate modified semi-solid Rappaport–Vassiliadis or DIASALM at 41.5°C and tetrathionate broths at 37°C (ensure that a reputable brand of tetrathionate suitable for use at 37°C is used).

vi) After 24 and 48 hours of selective enrichment, plate out modified semi-solid Rappaport–Vassiliadis or DIASALM by taking 1 µl loop of material from the edge of the turbid growth zone and streaking over one plate of Rambach agar or brilliant green agar and one plate of xylose lysine desoxycholate agar plus novobiocin.

vii) Plate out 10 µl of tetrathionate broth on Rambach agar or brilliant green agar and xylose lysine desoxycholate agar plus novobiocin.

viii) Incubate plates at 37°C for 24 hours.

ix) Check up to five suspect colonies (red/pink with reddening of the media on brilliant green agar, crimson with pale borders or orange/colourless on Rambach agar, red with black centre on xylose lysine desoxycholate agar) using poly 'O' and poly 'H' (phase 1 and phase 2) antiserum or composite biochemical media.

x) Subculture strongly suspect colonies that do not agglutinate with poly H antisera on to nonselective media then repeat testing. If a strong poly 'O' and poly 'H' agglutination can be obtained, this is sufficient for presumptive confirmation. Such isolates can then be serogrouped. If agglutination results are unclear then carry out further biochemical testing using composite media, such as TSI or use ONPG (o-nitrophenyl-beta-d-galactopyranoside) and urea or commercial biochemical tests such as API ID 32 E.

2. Serological tests

- Serological identification of infected animals, flocks and herds

A number of serological tests have been developed for the diagnosis of Salmonella infections in animals. In poultry, the whole blood test, which uses a stained antigen, and the serum agglutination test (SAT) have been used successfully for over 50 years for the identification of flocks infected with S. Pullorum/Gallinarum. Because S. Enteritidis possesses the same group D somatic antigen as S. Pullorum/Gallinarum, the whole blood test and related tests can be used for the diagnosis of infection but the sensitivity is low. In recent years, other tests, such as the ELISA (2, 41) have been developed for the diagnosis of S. Enteritidis and S. Typhimurium infections in poultry and for other serovars in farm animals. The ELISA has been used effectively to identify serologically S. Dublin carrier cattle and can be applied to bulk milk for screening dairy herds. The mix-ELISA is used in Denmark on serum or tissue fluid released by freezing then thawing muscle samples to detect Salmonella infections in pigs (26). A similar test is used to detect antibodies to S. Enteritidis and S. Typhimurium in egg yolk from commercial laying flocks (12).

Some ELISAs are now in routine use and a number are available commercially. The purpose of this section is to consider the serological tests that have been fully evaluated and are in routine use for the diagnosis of salmonellosis in animals. Other tests that are still in the development stage will therefore not be considered. New tests for Salmonella diagnosis are developed frequently so an Internet search is often the best means of obtaining current information.

- Factors affecting serological diagnosis

1. Serological methods should be used to identify infected flocks/herds rather than to identify infected individual animals, although repeated herd tests can be used as an aid to selective culling of chronic carrier animals. Serological tests are normally designed to detect a limited range of Salmonella serovars or serogroups.

2. It is well recognised that some animals with a positive serological response may no longer be infected with Salmonella organisms. Likewise, animals that are actively excreting salmonellae may be serologically negative. Similar considerations may also apply to bacteriological culture methods, and negative faecal culture results may not necessarily indicate that an animal is not infected. However, neither of these situations should be considered to be a major problem if enough tests are carried out. Animals that are serologically positive may have ceased to excrete salmonellae although circulating immunoglobulin concentrations may remain high. Other animals on the farm may still be infected. Serologically negative animals may result from a recent infection causing excretion before immunoglobulin production is maximal, or infection with less invasive serotypes. Animals that have been infected recently would, in all probability, eventually be detected serologically by an appropriate monitoring programme throughout the life of the flock/herd.
3. Newborn animals are immunologically immature and do not respond serologically to the somatic LPS antigen until 2–3 weeks of age. They do, however, produce a serological response to the flagellar protein antigens. Cattle may be unresponsive until about 10–12 weeks of age.

Chickens may also acquire anti-Salmonella antibodies passively from their parents via the yolk sac; this may indicate an infected parent flock. Mammals can acquire maternally derived antibodies via the colostrum.

4. Following Salmonella infections, immunoglobulin concentrations remain elevated for 2–3 months. An IgM response indicates recent infection.

5. Immunisation has been used for many years to control Salmonella infections in farm animals, and if diagnostic serology is to be used, it is necessary to differentiate the vaccine response from that of actual infection. Many live vaccines given orally do not provide a significant serum antibody response.

6. The effect of antibiotic therapy on the serological response remains unclear. Some workers found reduced titres following therapy whereas others found no effect. Serology, however, may be a more useful diagnostic technique for salmonellosis than culture if antimicrobial therapy has been used.

7. Approximately 2500 different Salmonella serovars exist. Depending on the antigen and test used, serological cross-reactions between different serovars may occur, e.g. S. Typhimurium and S. Enteritidis.

8. In poultry, egg yolk may be tested for immunoglobulins to Salmonella, and eggs may provide a method to screen flocks. This approach is used for monitoring commercial laying flocks in Denmark. In cattle, milk may be tested for anti-Salmonella antibodies to screen dairy herds.

9. The use of filter-paper discs for serum collection obviates the necessity to separate serum. The discs also provide long-term storage and reduce transport costs to the laboratory. The sensitivity of the test may be slightly reduced compared with tests carried out on fresh serum.

a) The whole blood test

The whole blood test provides a rapid test for fowl typhoid and pullorum disease that can be used on the farm. The sensitivity of the whole blood test is low and in inexperienced hands false-positive and false-negative results may be recorded.

For a detailed description of the whole blood test, see Chapter 2.7.5. Fowl typhoid and Pullorum disease.

b) Rapid slide agglutination test

Serum (0.02 ml) is mixed with polyvalent crystal-violet-stained antigen (0.02 ml). The tile is rocked gently for 2 minutes, after which the test is read. The test components are stored at 4°C and must have reached room temperature before being used.

Test sera should be free from contamination and haemolysis. It may be helpful to centrifuge serum samples that have been stored for any period of time.

If nonspecific false-positive reactions are suspected, positive/suspicious sera may be retested after heat-inactivation at 56°C for 30 minutes.

c) Serum agglutination test

The SAT is relatively insensitive, and many older animals have low levels of agglutinins in their sera caused by enterobacteria other than Salmonella. Single samples are of little value except as a herd test or a preliminary screening test. Paired samples are needed as the minimum requirement for confirmation of active infection. The test is relatively inexpensive; the antigens can be readily prepared and expensive equipment is not necessary. The SAT can be adapted to the microtitre format and can be readily used to determine somatic and flagellar titres. It is advisable to have standard sera for quality control of SAT antigen preparation(s).

- Preparation of somatic antigen
  i) Plate out the Salmonella culture from the appropriate stock culture on to a blood agar base (BAB) plate, or other suitable medium, for single colony growth. Incubate overnight at 37°C (±2°C).
  ii) Select a smooth colony and carry out a slide agglutination test to ensure that the required somatic antigen is present.
iii) Using a sterile loop, inoculate a nutrient agar slope in a universal container from the selected colony.

iv) Incubate the culture for 8–12 hours at 37°C (±2°C).

v) Using a Pasteur pipette, wash off the culture, preferably inside a safety cabinet, with approximately 2 ml of absolute alcohol, and transfer into a sterile universal container.

vi) Leave the antigen for 4–6 hours at room temperature to enable the alcohol to kill the bacteria and detach flagellae.

vii) Spin the universal container in a bench-top centrifuge for 5 minutes at 1000 g. Pour off the liquid and add enough phenol saline to make the antigen up to an opacity equivalent to Brown’s tube No. 2 (approximately 10^8 colony-forming units/ml) or other appropriate standard.

viii) Carry out standard titration with known serum to ensure that the antigen is positive for the required factor.

ix) Store in a refrigerator at 4°C until required.

- Preparation of flagellar antigens
  i) Plate out the appropriate *Salmonella* stock culture on to a BAB plate, or other appropriate medium. Incubate overnight at 37°C (±2°C).
  ii) Passage in semi-solid agar (about 0.3%) in a Craigie’s tube, or other suitable container, to induce optimum expression of the appropriate flagellar antigen. If the serovar is biphasic, H antiserum corresponding to the phase to be suppressed is added to the agar.
  iii) Use slide agglutination to check that the *Salmonella* is in the required phase. If this is correct, inoculate a loop of culture into 20 ml of nutrient broth. Incubate for 12–18 hours at 37°C (±2°C) for optimum growth. (If the phase is incorrect, repassage through semi-solid agar.)
  iv) Pipette 250 µl of 40% formaldehyde into the antigen suspension (use gloves and preferably work in a safety cabinet), and leave overnight.
  v) Test the antigen by SAT using the appropriate typing serum.

- Test procedure
  i) It is easiest to screen the sera at a dilution of 1/20; 0.25 ml of antigen is added to 0.25 ml of serum prediluted to 1/10 in normal saline.
  ii) The tests are incubated in a water bath at 50°C for 24 hours in the case of somatic antigens and for 4 hours for the flagellar antigens. The dilution and time of incubation will vary depending on the antisera that is used.
  iii) Sera that give a positive reaction are then diluted from 1/20 to 1/320 and retested with the appropriate antigen.

**d) Enzyme-linked immunosorbent assays for *Salmonella Enteritidis***

Two main basic systems are available for detection of IgG (IgY) specific for *S. Enteritidis*: the indirect ELISA (2) and the competitive ‘sandwich type’ ELISA (41).

The indirect ELISA involves the use of a detecting antigen coated on to the wells of a microtitre plate. After the application of a blocking reagent to reduce nonspecific binding, test samples are applied to the wells. Specifically bound antibody in the sample is detected by an antibody/enzyme conjugate. A variety of antigens, including LPS, flagella, SEF14 fimbriae, outer membrane proteins and cruder whole cell antigen preparations have been used.

The competitive sandwich ELISA employs a specific reagent – a monoclonal antibody (MAb) or polyclonal antibody – for coating antigen to wells. This is then followed by a pure or crude antigen preparation. Test samples are applied followed by conjugated antibody, which will not bind to the antigen if the sample contained specific antibodies. The assay time can be shortened by adding both test sample and conjugate together. MAbs have been prepared for LPS, flagella and SEF14 for *S. Enteritidis*.

There are advantages and disadvantages to both systems. The indirect assay is simpler and reagents are available for all *Salmonella* serotypes of chickens, turkeys, ducks and mammalian hosts. The competitive ELISA can be applied to all animal species and in general shows higher specificity. However, reagents are not available commercially for all serotypes. There are also some affinity problems and it may be less sensitive than the indirect assays. In the field, both systems have produced false-positive reactions and in
some cases screening with an indirect LPS ELISA may be followed by confirmation with a flagellar competitive ELISA.

Both types of assay may be used with serum, egg yolk or reconstituted dried blood eluted from filter paper discs. A mix-ELISA (or meat-juice ELISA), is used in Denmark to detect *Salmonella* infections in pigs (28). This ELISA contains the ‘O’ LPS antigens 1, 4, 5, 6, 7 and 12, from *S. Typhimurium* and *S. Choleraesuis*, which enables it to detect serologically 95% of the *Salmonella* serogroups found in Danish pigs. Serum is used to screen breeding and multiplying herds, whereas for pigs in the abattoir, the assay is performed on the tissue fluid (‘meat-juice’) that is liberated when a frozen 10 g muscle sample is thawed.

With some ELISAs differentiation can be made between infections produced by *Salmonella* serotypes from different serogroups. Some cross-reaction occurs between groups B and D and other invasive serovars. There is, however, usually a greater antibody response when LPS from the homologous serotype is used in the ELISA. The optimal method for choosing a ‘cut-off’ absorbance value, above which sera are designated as having come from an *S.-Entertidis*-infected flock, without producing an unacceptable level of false-positive tests, has not yet been decided on and agreed upon internationally.

ELISAs are readily adapted to automation and hence to large-scale testing programmes. A major problem is that expensive equipment is necessary and many of the reagents are also expensive. Several commercial ELISA kits for *S. Enteritidis*, *S. Typhimurium* and Group B/C mix-ELISAs are available. Ideally these should be validated by international ring trials before adoption for surveillance purposes.

An example of a validated ELISA is the one developed at the OIE Reference Laboratory at VLA Weybridge (see Table in Part 3 of this Terrestrial Manual for address). The requirements are given below.

- **Equipment**
  Falcon microtest III PVC plates; appropriate pipettes and measuring cylinders; ultrawash microtest plate washer; ELISA plate reader; test filter of 405–410 nm and reference filter of 630 nm.

- **Antigen**
  i) Phenol-extracted *S. Enteritidis* LPS is available commercially (Sigma Cat. No. L6011). This is reconstituted in 1 ml deionised water and stored at −20°C in 100-µl aliquots in phosphate buffered saline (PBS), pH 7.2, at a concentration of 2.5 mg/ml. For use, the antigen should be thawed in coating buffer at the appropriate concentration.
  ii) The LPS antigen can also be prepared by the technique of Westphal & Luderitz (43) and standardised as to its carbohydrate concentration by the method of Gerhardt (14), and adjusted to 1000 µg/ml.

- **Serum and conjugate diluent**
  Add bovine serum albumin (BSA) (2 g) and Tween 20 (0.05 ml) to PBS (100 ml). (Alternatively, powdered milk [1 g] can replace the BSA.) Store at 4°C and make fresh solutions every week.

  **Coating buffer:** add sodium carbonate (1.59 g) and sodium bicarbonate (2.93 g) to deionised water (1 litre) and adjust to pH 9.6. (Alternatively, dissolve one tablet of 0.05 M Sigma carbonate/bicarbonate buffer [Cat. No. C-3041] in deionised water [100 ml].) Store at 4°C and renew every 2 weeks.

  **Substrate buffer:** make a 10% (v/v) solution of diethanolamine in deionised water. The diethanolamine should be prewarmed to 37°C before dispensing, and the pH of the solution should be adjusted to pH 9.8 with 1 M hydrochloric acid. Store at 4°C and renew every 2 weeks.

  **Enzyme conjugate:** goat anti-chicken immunoglobulin conjugated to alkaline phosphatase (e.g. ICN Immunobiologicals, alternative supply Sigma: A9171) or other species anti-chicken globulin. Store at 4°C diluted in diluent at the appropriate concentration and renew every week.

  **Enzyme substrate:** dissolve one tablet of *p*-nitrophenyl phosphate disodium (5 mg) in substrate buffer (5 ml) no earlier than 30 minutes before dispensing, and store in the dark.

- **Standards**
  i) Positive control antiserum prepared by intramuscular inoculation of four 1-week-old specific pathogen free (SPF) chickens with an inoculum containing 10⁶ *S. Enteritidis*. The serum is subsequently obtained 3–4 weeks later when antibody titres are maximal.
  ii) Negative control serum A from four 1-week-old SPF birds.
  iii) Negative control serum B from 58 1-week-old breeders known to be free from *Salmonella* infections. Pool the sera and store in 100 µl volumes at −20°C.
C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Many inactivated vaccines are used against salmonellosis caused by different serovars in various animal species, including a combined S. Enteritidis and S. Typhimurium vaccine for use in poultry. Inactivation is usually achieved by either heating or the use of formalin and an adjuvant, such as alhydrogel, is usually used. Live vaccines have also been used in a number of countries; these include the semi-rough strains, such as 9R for fowl typhoid and HWS51 for S. Dublin infections (26). Other attenuated vaccines include auxotrophic mutants, which are used to prevent Salmonella infections in farm animals in Germany and for S. Enteritidis in the United Kingdom. Mutant vaccines attenuated rationally by molecular biological gene-deletion techniques have been developed for poultry and other species; these include aroA mutants and strains with mutations in the genes encoding adenylate cyclase (cya) and the cyclic adenosine monophosphate receptor protein (crp) (6), which is available in the United States of America. In Europe genetically modified organisms are not normally permitted for use as vaccines.

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

For killed or living vaccines, the bacterial strains should be an organism as closely related to currently circulating field strains as possible. It should be carefully chosen from cases of severe clinical disease, and virulence and antigen production should be assessed. It is best to evaluate a panel of potential strains in this way before testing the final selection. The final vaccinal strain should be identified by historical records and characterised by stable phenotypic and/or genetic markers. Living vaccinal strains should be marked by stable characters allowing distinction from wild strains. Markers, such as resistance to antimicrobials, for example rifampicin, may be used. Attenuation of virulence should be stable and preferably obtained by two independent defined mutations.

b) Method of culture

The seed culture is propagated and maintained using suitable media, of which many have been described (in textbooks) for growth of Salmonella. The media used must not contain serum or animal tissues. Culture may be on solid medium, in Roux flasks, or in liquid medium, in which case large-scale fermentation equipment may be used. Iron limitation or low temperature incubation on a minimal media may enhance LPS antigen production by the vaccine strain.

c) Validation as a vaccine

i) **Purity**

The vaccine strain must be checked as follows:

- Staining of a smear of bacterial suspension on a glass slide using Gram stain.
- Homogeneity of culture on nonselective media.
- Metabolic requirements as indicated by biochemical tests.
- Detection of markers, and phage type.
- Agglutination with specific antiserum.

ii) **Safety**

The LD$_{50}$ (50% lethal dose) or ID$_{50}$ (50% infectious dose) may be determined in mice. Ten times the field dose of live vaccine or twice the dose for killed vaccines must be given to the target species at the recommended age and by the recommended route. The animals are observed for absence of adverse reactions. Stability and nonreversion to virulence after serial passages in susceptible species should be shown for live vaccines. It is also necessary to consider repeat vaccination. Live vaccine should be shown not to persist for long in vaccinated animals or be transmitted to milk or eggs that may be consumed, and the method of application should not present a hazard to operators.

iii) **Efficacy**

Laboratory experiments and field trials should be used to show that the vaccine is effective. The laboratory experiments consist of vaccination–challenge tests in the target species at the recommended dose and age. The efficacy data can also be used as the basis for a batch potency test.
Field trials are more difficult to undertake with respect to testing efficacy because of difficulties with standardising the challenge and providing appropriate controls.

iv) Environmental aspects

Live vaccine strains should be tested for their ability to persist in the environment and infect non-target species such as rodents and wild birds that are likely to be exposed. Prolonged survival of some live vaccines in faeces and litter may present an unacceptable environmental hazard when the material is removed from the animal houses.

2. Method of manufacture

Vaccine must be made in suitable clean rooms to which only approved personnel have access. Care must be taken to avoid cross-contamination between areas where live organisms are processed and other areas. Contamination from operators and/or the environment must be avoided and vaccine preparation should take place in a separate area from diagnostic culture work. Operators must not work with vaccine whilst ill and must not be subject to immunosuppressive conditions or medications. Personnel must be provided with protective clothing in production areas and in animal rooms.

Seed-lot cultures are prepared from the primary seed-lot, and the number of passages is dependent on the validation of the process. The vaccine may be prepared by inoculation of a suitable medium, such as nutrient broth, with a fresh culture and incubation on a shaker at 37°C for 24 hours, with or without aeration. The organisms are harvested by sedimentation or centrifugation. Alternatively, the organisms may be grown on and harvested from a solid medium, such as nutrient agar. In the case of live vaccines, the suspension is diluted in PBS, pH 7.0, and may be freeze-dried.

The time of inactivation of dead vaccines should be at least 33% more than that taken to reduce the viable number to an undetectable level. The inactivation process must be applied to the whole volume of the vaccine cell harvest.

Preservatives, excipient for lyophilisation, stabiliser for multidose containers or other substances added to or combined with a vaccinal preparation must have no deleterious effect on the immunising potency of the product.

3. In-process control

The following points require attention:

• Visual control of the suspension, homogeneity by Gram stain, culture on nonselective medium.
• Slide agglutination with specific antisera.
• Titration of bacteria by turbidimetry and/or plate count.
• Test of effective inactivation (dead vaccine) by plating on nonselective medium or use of a medium that gives optimum chance of recovery e.g. production medium with neutralisation of the inactivating compound.
• Titration of viable bacteria (living vaccine) before and after lyophilisation.

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

A laboratory test in mice that has previously shown a correlation with safety in the target species may be used to determine the LD₅₀ and/or ID₅₀. Each batch should be tested in the target species at the recommended age and route, using at least twice the field dose for killed vaccines and ten times the dose for live vaccines.

c) Potency

Potency is tested using vaccination–challenge assay in mice and/or other species, including (if practicable) the target species and immunological response in target species.
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d) Duration of immunity

The duration of immunity is likely to vary considerably between products, vaccination regimes and individual vaccinated animals. Immunity to Salmonella is normally serogroup specific. Consultation among colleagues suggests that killed vaccines will provide protection for 6 months, while live vaccines elicit stronger immunity, which may persist for 1 year or more. It should be remembered, however, that a strong challenge such as that associated with continuously occupied farms or infected rodents may overwhelm vaccinal immunity.

e) Stability

Information is lacking on the stability of killed vaccines. Stability is affected by storage conditions and by the presence of contaminating microorganisms growing in the product. The stability is assessed by potency tests repeated at appropriate time intervals. The stability of live vaccines can be assessed by performing counts of the number of viable organisms repeated at appropriate time intervals.

f) Preservatives

Chemicals with antimicrobial activity, such as thiomersal, phenol or crystal violet, are often included as preservatives in killed bacterial vaccines.

g) Precautions

Certain killed vaccines may occasionally cause abortion in pregnant animals because of their LPS content, and likewise live vaccines should be used with caution in pregnant animals. It is often necessary, however, to vaccinate pregnant animals to provide maternal immunity for their offspring. It may be useful to include endotoxin assay in the safety test programme so that the levels can be compared with those shown to be safe in the double-dose tests. Vaccines may also cause swelling at the site of injection.

5. Tests on the final product

a) Safety

Killed vaccines are assessed in a double-dose test, and live vaccines are assessed in a test using ten times the dose, in the target species.

b) Potency

If possible, the potency test should relate to the efficacy of the vaccine in the target species, and suitable criteria should be applied for passing batches. It may be possible to assess killed vaccines by the O-H antibody response produced, although it should be remembered that serum antibodies are only part of the host’s protective mechanism against Salmonella. Alternatively, the potency of the vaccine may be assessed by its effect on the LD_{50} in mice.

D. COMPETITIVE EXCLUSION

Susceptibility to Salmonella infection in poultry can be substantially reduced by spray or oral treatment prior to exposure (ideally in the hatchery) with an anaerobic culture of caecal microflora that inhibits colonisation by Salmonella. This treatment is widely used in some countries but only used as an aid to decontaminating persistently infected farms in others (1, 27).

ACKNOWLEDGEMENT

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

REFERENCES


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**NB:** There are OIE Reference Laboratories for Salmonellosis (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

Mange is a form of ectoparasitic dermatitis characterised by encrustation, alopecia, and pruritus of the skin, initiated by and maintained by a number of mite species. Mites and ticks are a subclass of the Arachnida divided into two superorders, the Parasitiformes (Mesostigmata) and the Acariformes. The Acariformes contains three orders: the Astigmata (Acaridida, not possessing stigmata), the Prostigmata (Actinedida, possessing stigmata behind the gnathosoma) and the Oribatida (Cryptostigmata). All the major mange mite species are contained within the orders Astigmata and Prostigmata. The diagnosis of mange in domestic animals is based on clinical manifestations and the demonstration of mites or their developmental stages in skin scrapings.

Identification of the agent: Mange can be the result of infestation by the astigmatid mites, e.g. Chorioptes, Knemidokoptes, Notoedres, Otodectes, Psoroptes, or Sarcoptes or the prostigmatid mites, e.g. Cheyletiella, Demodex or Psorobia. All mange mites are classified by structure. Specialised illustrated keys should be consulted in order to identify the causative organism.

Requirements for vaccines and diagnostic biologicals: There are no commercial vaccines currently available.

A. INTRODUCTION

Mange is a form of ectoparasitic dermatitis characterised by encrustation, alopecia, and pruritus of the skin, initiated by and maintained by a number of mite species. Mites and ticks make up the Acari, a subclass of the Arachnida. Within this subclass mites are further divided into two superorders: the Parasitiformes and the Acariformes. The Acariformes contains three orders: the Astigmata (acaridida, not possessing stigmata), the Prostigmata (actinedida, possessing stigmata behind the gnathosoma) and the Oribatida (cryptostigmata) (3). All the major mange mite species are contained within the orders Astigmata and Prostigmata. The Astigmata is a well defined group of slow-moving, weakly sclerotised mites, including the families of medical or veterinary importance – Sarcoptidae and Psoroptidae. The Prostigmata is the most heterogeneous of acarine orders with adults ranging in size from 100 µm to 16 mm (21). Included in the Prostigmata are the Trombiculidae (harvest mites), parasitic as larvae but free-living predators in the nymphal and adult stages, and the true mange mite families Psorergatidae (Psorobia [Psorergates] sp.), Demodicidae (Demodex sp.) and Cheyletiellidae (Cheyletiella sp.) (3).

B. DIAGNOSTIC TECHNIQUES

The diagnosis of mange in domestic animals is based on clinical manifestations and the demonstration of mites or their developmental stages in skin scrapings (19). Specialised illustrated keys should be consulted in order to identify the causative organism.

1. Identification of the agent

Mange can be the result of infestation by the astigmatid mites, e.g. Chorioptes, Knemidokoptes, Notoedres, Otodectes, Psoroptes and Sarcoptes or the prostigmatid mites, e.g. Cheyletiella, Demodex and Psorobia.

A) The Astigmata

The Astigmata are small thin-skinned mites, generally lacking obvious shields. The bases of the coxae are sunken into the body and referred to as epimeres when outside the body and apodemes when inside the body.
The empodium is claw-like and the pedicel is stalked or sessile. True claws are absent. Fertilised eggs are extruded through an antero-ventral slit, the oviporus or genital opening. Most species of medical or veterinary importance are in the division Psoroptidia, comprising the Psoroptoidea (Sarcoptidae and Psoroptidae), the Analgoidea (Knemidokoptidae, Cytoditidae and Laminosoptidae) and the Pyroglyphoidea (Pyroglyphidae). The division Acaridae contains the stored product mites, which can cause transitory mange and can be an important source of allergens.

i) Sarcoptidae

Sarcoptid (sarcoptiform) mites are obligate parasites, burrowing into the skin of mammals. They are globose mites with the ventral surface flattened and the cuticle finely striated. The chelicerae are adapted for cutting and paring.

a) Sarcoptes scabiei

The mite (Sarcoptes scabiei) is the cause of scabies, sarcoptic mange, in humans and in a wide range of domestic and wild mammals (both carnivorous and herbivorous) throughout the world, generally affecting the sparsely haired parts of the body. The number of species within the genus is still open to debate. Studies of populations of Sarcoptes mites from a wide range of hosts have suggested that there is only one type species (Sarcoptes scabiei) with a number of variants infesting a wide range of mammalian hosts (14). Recent investigations based on molecular analysis of the ITS-2 of the rRNA gene suggest that the genus Sarcoptes is monospecific (37). Humans are easily infested, particularly in crowded or poor living conditions (35).

Sarcoptes can be identified on the basis of size, shape and morphology. The body outline of adult mites is circular and approximately 250 µm in length, but as ovaries develop in the female, she can increase in size to 300 to 500 µm in length. The cuticle (integument) is striated, bearing a central patch of raised tooth and peg-like structures on the dorsum that decreases in density posterolaterally. Legs are weakly developed and in both sexes the pretarsi of legs I and II bear empodial claws but the ambulacral suckers are on long unjointed pedicels (stalks). The epimeres (apodemes) of the first pair of legs are fused in a ‘Y’ shape. Legs III and IV in the female (identified by the transverse egg laying slit [oviporus] in the middle of the ventral surface) are short and end in long setae and lack a stalked pedicel. They are located on the ventral surface and are not visible in dorsal view. Males are smaller and distinguished by the presence of a stalked pedicel on legs IV, between which there is an obvious sclerotised genital apparatus. Nymphs are similar to the female, but smaller and without an oviporus. Larvae resemble nymphs, but have only three pairs of legs. A similar mite, Trixacarus caviae, occurs on guinea-pigs, but is smaller and the anus is dorsal. Secondary immune reactions may produce a rash at sites away from the infested area.

b) Notoedres spp.

Notoedres also burrows into the host epidermis and is similar to Sarcoptes, but can be distinguished by the absence of dorsal spines and by the dorsal anus. Over 20 species of Notoedres have been described of which the cat mange mite, Notoedres cati, is of veterinary interest, producing a highly contagious and intensely pruritic mange on cats (and sometimes dogs and rabbits). Infestations usually start on the head of the cat, from where it may spread. Notoedres muris occurs on rats and N. musculi on house mice. Notoedres cati is similar to S. scabiei having unjointed stalked pedicels with terminal suckers on legs I and II in all stages, and on leg IV in the male. Notoedres cati is considerably smaller, the female being 225 µm and the male 150 µm. The anus is located on the dorsal surface and there are no projecting spines, but mid-dorsally the striae are broken into a scale-like pattern and stout setae replace the conical peg-like setae of S. scabiei.

ii) Analgoidea

The Analgoidea include 12 species of the mange mites Knemidokoptes (Knemidokoptidae), the air sac mite of poultry (Cytodites nudus: Cytoditidae) and the Laminosoptidae, represented by Laminosoptes cysticola, infesting the subcutaneous cellular tissue of turkeys and chickens.

a) Knemidokoptes sp.

Three species of Knemidokoptes are of veterinary importance: K. mutans infesting the epidermis of the legs of poultry (causing ‘scaley leg’), K. galliniae (Neoknemidokoptes galliniae, Mesoknemidokoptes laevis galliniae), the ‘depluming mite’ infesting the skin of poultry near the base of the feathers, and K. pilae infesting the ceres of cage and aviary birds (causing ‘scaly face’). Female Knemidokoptes are 400 µm long, and dorsal scales, if present, are faint and irregular. Knemidokoptes galliniae (Mesoknemidokoptes) do not have any scales (3). Two sclerotised, almost parallel, longitudinal bands are present on the anterior mid-dorsal surface, connected posteriorly by a less well developed transverse band. In the male, the epimeres (apodemes) of the first pair of legs are joined in a ‘Y’ shape, and in the female, the epimeres (apodemes) are joined in a ‘U’ shape. Stalked pulvilli are present on all legs in the male and larvae, but are absent in the
nymphal stages and females. The anus is situated terminally. The female is viviparous and there are one larval and two nymphal stages.

iii) Psoroptidae

Members of the family Psoroptidae are oval, nonburrowing mites, parasitic on mammalian skin. The cuticle (integument) is striated, legs III and IV are usually visible from above, the epimeres (apodemes) of leg I are not fused, and there are no vertical setae on the propodosoma. The male has prominent adanal suckers that engage with the copulatory tubercles of the female tritonymph (‘pubescent female’). The inverted U-shaped oviporus is prominent on the venter of the ovigerous female, posterior to leg II. Three genera, Psoroptes, Chorioptes and Otodectes are of veterinary importance.

a) Psoroptes spp.

Psoroptes spp. have strongly developed legs bearing, in all stages, funnel-shaped suckers on long three-segmented pedicels. The ovigerous mite is pearly white and 750 µm in length. The male has a pair of opisthosomal lobes and a pair of copulatory suckers. Psoroptes mites cause a debilitating dermatitis involving wool/hair loss and a pruritic scab formation. Five species of Psoroptes are recognised (33): P. ovis, a body mite causing mange in sheep, cattle and horses, P. equi, a body mite of equids, P. natalensis, a body mite of cattle and horses, P. cuniculi, the ear mite of rabbits, goats, horses and sheep and P. cervinus, an ear mite of bighorn sheep, elk and wapiti. A sixth, unvalidated, species is P. auchiniae, an ear mite of New World camelids. P. natalensis and P. equi can be distinguished morphologically by the length of the fourth outer opisthosomal seta (L_{4OOS}) of the male (33). In P. natalensis the seta is also spatulate, as is the shorter L_{4OOS} of male P. cervinus (22). It is difficult to separate P. ovis and P. cuniculi on morphological grounds. Like the genus Sarcoptes, the number of species in the genus Psoroptes is open to debate (25); P. ovis and P. cuniculi may be variants of the same species (4).

b) Chorioptes

Legs of Chorioptes mites have broad bowl-shaped ambulacra borne on very short unsegmented pedicels, except on legs III of the female, which terminate in two long setae. Ovigerous C. bovis are smaller than Psoroptes, being 400 µm in length. The male C. bovis has pedicels on all four pairs of legs, a pair of squarish to rectangular opisthosomal lobes and a pair of copulatory suckers anteriorly. The opisthosomal lobes bear two spatulate (paddle-like) hairs, together with three normal setae of varying length. The epimeres extending from the bases of legs I and II are more triangular in outline to C. bovis and the pair of spatulate opisthosomal setae are much longer. In goats, horses and camels C. texanus generally inhabits the lower parts of the leg, as does C. bovis on sheep, but in the latter case, the scrotum may also be infested and has been associated with infertility in rams. In cattle chorioptic mange commonly occurs on the base of the tail, the perineum and the back of the udder. The mite feeds at the external surface of the skin causing inflammation, exudation and scab formation. Heavy infestations can cause loss of condition, which can lead to emaciation and damage to hides (35).

c) Otodectes

Male Otodectes are similar to Chorioptes, but the opisthosomal lobes are much less distinct, copulatory suckers are present and the hairs are less spatulate. The epimeres extending from the bases of legs I and II are joined. The male has broad bowl-shaped ambulacra borne on very short unsegmented pedicels on all the legs, but only legs I and II of the female have pedicels and ambulacra. Legs III of the female terminate in two long setae and legs IV are reduced (3). Otodectes cynotis occurs in the ears, causing parasitic otitis (and occasionally elsewhere on the body) of domestic dogs, cats, foxes and ferrets together with a number of wild carnivores (34). Otodectes cynotis feeds on the external surface of the skin causing inflammation and exudation. Excessive exudation may lead to wet eczema on adjacent skin surfaces. The tympanic membrane may be perforated and otitis media and nervous signs (e.g. convulsions) can develop.

iv) Free-living astigmatid mites

Free-living astigmatid (‘forage’) mites can occur in large numbers in stored food, spilled grain, poultry feed, etc. Their ingestion can lead to gastrointestinal disturbance. Mites may also be the cause of incidental mange of mammals through contact with antigenic material present in infested feed or the environment. Workers handling infested stored food can develop reactions to the mites and suffer from pruritus, dermatitis, rhinitis or asthma (35). The cause of the reaction has not been established but is assumed to be allergic or possibly do to bites. The most obvious characteristic of the more common forage mites (Acaridae) is the possession of many ‘hairs’, which are much longer than the parasitic forms, and may be simple, branched or spatulate. In some genera (e.g. Glycyphagus) the posterior hairs often become entangled in debris. Acarus siro, probably the commonest of these mites, has shorter hairs.
B) The Prostigmata

Prostigmata mites are weakly sclerotised and, where there is a respiratory system, the stigmata open on the gnathosoma or anterior part of the propodosoma. The palpi are usually free and highly developed, either as pincer-like claspers or sensory organs. The chelicerae are usually modified for piercing (3, 35). Included in the Prostigmata are the Trombiculidae (harvest or chigger mites), parasitic as larvae, but free-living predators in the nymphal and adult stages, and the true mange mite families Psorergatidae ([Psorobia [Psoreregates] sp.], Demodicidae ([Demodex sp.] and Cheyletiella ([Cheyletiella sp.]) (3).

i) Psorobia (Psoreregates) spp.

Two species of Psorobia have been isolated from domestic animals and one species from laboratory animals: the benign ‘parasite’ P. bos from cattle, the more important P. ovis occurring in Merino sheep and Psorergates simplex (Psoreregates muricola) from laboratory animals (3). Larval Psorobia hatch with reduced legs that become progressively larger at each of three nymphal molts, and by the adult stage the legs are well developed and the mites are motile. Adults of both sexes are very small (200 µm) and can be recognised by the radially arranged legs around a more or less circular body. Each leg has an inward curving spine on each femur. Most mites are found under the host stratum corneum in the superficial layers of the skin of the sides, flanks and thighs, feeding on the exuding fluid. The infested area is dry and scruffy, wool fibres break easily with the remaining wool coming together as ragged tufts. Irritation causes the sheep to rub and kick the affected area and chew its fleece, resulting in ‘fleece derangement’ and downgrading of the wool clip.

ii) Cheyletiella spp.

Cheyletiella ([fur mites]) are large mites (385 µm) with large curved palpal claws and an obvious ‘M’ shaped respiratory peritreme or stomata at the bases of the chelicerae. Cheyletiella is an obligate parasite, inhabiting the keratin layer of the epidermis. The mites develop rapidly, giving rise to the term ‘walking dandruff’. Three species are of veterinary importance, causing mild, non-suppurative dermatitis in mammals and transitory dermatitis in humans: C. parasitivorax occurring on cats, the scapular region of rabbits and occasionally humans, C. yasguri causing a highly infectious infestation of puppies (adult dogs as carriers), and C. blakei causing a mild dermatitis in cats. Cheyletiella parasitivorax has an ovate (spade-shaped) sensory organ or seta (solenidion) on the genu of the first leg, whereas that of C. yasguri is apically cleaved (heart-shaped) (3).

iii) Demodex spp.

Demodex are easily recognised by their annulate, vermiform (‘worm-like’) shape, but may be overlooked on account of their small size 100–400 µm. Demodex inhabit the hair follicles and the sebaceous and meibomian glands of the skin of a number of wild and domesticated mammals, including humans. Male Demodex live at or near the skin surface and females in the follicles. Different species occur on different hosts, and more than one species may occur on the same host, e.g. D. folliculorum and D. brevis on humans (12). Demodectic mange is of great importance in dogs (especially short-haired breeds), goats and pigs, and of lesser importance in cattle, horses, sheep and cats. There is usually no irritation or pathological conditions, but in some individuals the number and spread of mites on the hosts increases to form clinical demodectic mange. On cattle this occurs as flat nodules in the skin with a massive enlargement of the sebaceous glands that contain vast numbers of Demodex mites. In dogs infestations may become generalised as a squamous form, with associated hair loss and thickening of the skin. Pustular demodectic mange is a more severe form in dogs and is complicated by secondary bacterial infection. Diagnosis of demodectic mange depends on the recovery of mites from deep skin scrapings. Mites can easily be identified as Demodex, but specialised keys are required for specific identification (23).

2. Diagnosis of scabies or mange

A firm diagnosis of scabies or mange must be based on the recovery and identification of the mite from the affected hosts. Visual examination of a suspect mange lesion may reveal the larger mites (e.g. Psoroptes), but in most cases it is necessary to take skin scrapings from the edge of visible lesions. Specialised illustrated keys should be consulted in order to identify the causative organism (3).

An indication of the presence of Psoroptes mites is the reaction of the host to scratching or rubbing of the affected skin by the operator, where it responds by a nibbling mouth reflex and/or scratching itself. Forced exercise followed by close penning will increase body heat and the associated percutaneous absorption of mite antigens/irritants inducing the presentation of clinical signs. Skin scrapings are taken from the affected area. In diagnosing sarcoptic scabies it must be borne in mind that the distribution of rash on the body bears no relation to the distribution of mites. Wool or hair should be clipped (and stored for the differential diagnosis of other ectoparasites or mycoses). The area selected for scraping should be the moist part or the edge of the lesion. If sarcoptic mange is suspected the scraping should be taken from the hairless area or where pruritus or pimplies...
are seen. In psoroptic mange of sheep (sheep scab) the area selected is the edge of the progressing lesion, but scrapings can be taken from any part of the lesion for *Psoroptes* infestations of rabbits or cattle. In general for mites living on the skin surface (i.e. *Psoroptes* or *Chorioptes*) scrapings should be taken with the scalpel blade held at an acute angle, shaving rather than scraping off the outer epidermis. *Demodex*, *Psorobia* or *Sarcoptes* are found burrowing into the skin and the scalpel blade should be held at right angles and the skin scraped until it oozes blood. A drop of glycerine or liquid paraffin put on the skin or scalpel blade before the skin is scraped will aid in the collection of mites (29). In pustular demodicet mange, mites are usually abundant and can be demonstrated on examination of the cheesy contents of an expressed or incised pustule. In the case of squamous lesions, a deep scraping may be necessary. In cases of ear mange (canker) in cats, dogs or rabbits the scabby material within the external ear may be detached with blunt-ended forceps. A vacuum cleaner technique for collecting mange mites has been found to be useful and more sensitive than skin scraping (21). These authors found *Cheyletiella*, *Sarcoptes*, *Psoroptes*, *Otodectes*, *Demodex* and forage mites from infested dogs, sheep or swine, using 10% KOH treatment of the vacuum collected material before microscopic examination. Occasionally a diagnosis of mange can be made when mites are found in faecal samples, from pruritic dogs for example.

In suspect cases of otoacariasis (*Otodectes* or *Psoroptes*) in the ears of cats, dogs, rabbits, sheep and goats, live mites can be observed through auroscopic examination of the external ear canal (EAC), but this technique may be difficult if applied to larger animals or large numbers of animals. In this situation it may be necessary to swap the external ear canal. Suitable sized swabs are inserted into the EAC until resistance is met, then gently twisted and removed. Care must be taken that the swabs enter the EAC and not the blind pouches of the tragus. Care should also be taken with young animals.

Skin scrapings or ear swabs should be immediately transferred into small tubes that can be securely stoppered or lidded plastic bottles. Submission in sealed envelopes is not recommended as they may dry out or be lost. Live *Chorioptes*, *Otodectes*, *Psoroptes* and *Sarcoptes* can easily be seen during direct examination of the original skin scraping or ear swab under a dissecting microscope (×40) with overhead lighting. Mites will be active after incubation of the sample at 37°C for 30 minutes and can easily be transferred to microscope slides using a mounted needle.

Mites can be mounted directly in either Berlese’s fluid (gum chloral), Vitzhumis fluid, Hoyer’s medium or Heinze’s modified PVA medium (13). Put a drop of medium on the centre of the slide and transfer the specimen into it by means of a fine brush or mounted needle, and remove air bubbles with a needle. Put the cover-slip on its edge to the side of the medium and let it down slowly using a needle and then press slightly with the needle. If there is too much medium the pressure will force some of it out. If there is too little medium, add small drops, one at a time, letting it flow under the cover-slip. After mounting, allow the slides to dry at room temperature for 5–7 days before ringing with nail-polish or another nonsoluble sealant. The drying time can be decreased by placing the slides in an oven (40–45°C) for at least 2 days.

To locate dead mites and live mites of smaller mite species (e.g. *Demodex*) the scraping will have to be processed in hot potassium hydroxide. Large amounts of material (up to 5.0 g) can be placed in a glass boiling tube and covered with 10% potassium hydroxide. The tube is then placed in a beaker of water, which is gradually warmed over a Bunsen or spirit lamp flame. Care must be taken that the slide does not get too hot, as escaping body gases in the mite may cause the mites to ‘jump’. If smoke appears on the surface of the fluid the process is too hot. These methods are necessary to clear out the opaque body contents in order to prepare the mites for microscopic examination. It is advisable to carry out both these methods in a cabinet with an extractor fan due to the caustic fumes released. Staining using lignin pink or chlorazol black can help highlight structures or identify mites embedded in host tissue (3).

Potential zoonotic problems may occur when handling infested animals or diagnostic material containing *Cheyletiella*, *Sarcoptes*, *Notoedres* or *Trixacarus*. Faeces or cuticular debris from any mite species may be a hazard to those prone to house dust mite (*Dermatophagoides pteronyssinus*) allergy.

### 3. Serological tests

An enzyme linked immunosorbent assay (ELISA) for the serological diagnosis of sarcoptic mange in dogs and pigs is used in Sweden (1, 2, 8, 9), and is currently used in the porcine scabies eradication campaigns in Sweden (18) and Switzerland (20). An ELISA kit for the serodiagnosis of sarcoptic mange in pigs and dogs (*Sarcoptes-ELISA 2001® PIG; DOG*) is now commercially available. ELISA techniques have been developed to monitor...
Psoroptes infestations of sheep, cattle and nondomesticated animals (10, 11, 16, 17, 22, 24, 36), but none is currently commercially available.

However, ELISAs are in routine use by researchers for detection of specific antibodies to S. scabiei (1, 2).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no commercial vaccines currently available to protect animals against mange mites. Mange vaccines would a) reduce the need for chemical acaricides, b) reduce environmental pollution, c) have minimal meat/milk/wool residues and d) reduce the risk of resistance. The potential for the immunological control of Psoroptes ovis has been investigated for sheep (5–7, 26, 30, 31) and cattle (27). All these studies demonstrated that soluble fractions of P. ovis, under experimental conditions, significantly reduce the pathology of sheep scab and inhibited P. ovis population increase.

REFERENCES


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CHAPTER 2.10.5.
BORDER DISEASE

SUMMARY

Border disease (BD) is a viral disease of sheep first reported in 1959 from the border region of England and Wales. Distribution of the virus is world-wide. Prevalence rates vary in sheep from 5% to 50% between countries and from region to region within countries. Clinical signs include barren ewes, abortions, stillbirths and the birth of small weak lambs. Affected lambs can show tremor, abnormal body conformation and hairy fleeces (so-called 'hairy-shaker' or 'fuzzy' lambs) and the disease has been referred to as 'hairy shaker disease'. Vertical transmission plays an important role in the epidemiology of the disease. Infection of fetuses can result in the birth of persistently infected (PI) lambs. These PI lambs are viraemic, antibody negative and constantly excrete virus. The virus spreads from sheep to sheep, with PI animals being the most potent source of infection. Sheep may also be infected following close contact with cattle excreting the closely related bovine viral diarrhoea virus (BVDV).

It is important to identify the viraemic PI animals so that they will not be used for breeding or trading purposes. Serological testing is insufficient. It is generally considered that serologically positive, nonviraemic sheep are 'safe', as latent infections are not known to occur in recovered animals.

Identification of the agent: BD virus (BDV) is a Pestivirus in the family Flaviviridae and is closely related to classical swine fever virus and BVDV. Nearly all isolates of BDV are noncytopathogenic in cell culture. There are no defined serotypes but virus isolates exhibit considerable antigenic diversity, and three distinct antigenic groups have been identified.

Apparently healthy PI sheep resulting from congenital infection can be identified by isolation and immunostaining of noncytopathogenic virus from blood or sera in laboratory cell cultures. Rapid direct methods to identify PI sheep include detection of viral antigen or viral RNA in leukocytes and immunohistochemical demonstration of viral antigen in skin biopsies. The demonstration of virus is less reliable in lambs younger than 2 months that have received colostral antibody. Acute infection is usually subclinical and viraemia is transient and difficult to detect. From dead animals, the isolation of virus from tissues of aborted or stillborn lambs is difficult, but tissues from PI sheep contain high levels of virus, which can be easily detected by isolation and direct methods.

Serological tests: Acute infection with BDV is best confirmed by demonstrating seroconversion using paired or sequential samples from several animals in the group. The enzyme-linked immunosorbent assay and virus neutralisation test are the most commonly used antibody detection methods.

Requirements for vaccines and diagnostic biologicals: There is no standard vaccine for BDV, but a commercial killed whole-virus vaccine has been produced. Ideally, such a vaccine should be suitable for administration to females before breeding for prevention of transplacental infection. The use of BVDV vaccines has been advocated, but the antigenic diversity of BD viruses must be considered.

BD viruses have contaminated several modified live veterinary vaccines produced in sheep cells or containing sheep serum. This potential hazard should be recognised by manufacturers of biological products.
**A. INTRODUCTION**

Border disease virus (BDV) is a *Pestivirus* of the family *Flaviviridae* and is closely related to classical swine fever virus (CSFV) and bovine viral diarrhoea virus (BVDV) (21). Although six species of *Pestivirus* have been proposed, there are four recognised species, namely – CSFV, BVDV types 1 and 2 and BDV (1). While CSF viruses are predominantly restricted to pigs, examples of the other three species have all been recovered from sheep, with the majority of isolates being BD viruses (27). Nearly all virus isolates of BDV are noncytopathogenic, although occasional cytopathic viruses have been isolated (23). BDV spreads naturally among sheep by the oronasal route and by vertical transmission. It is principally a cause of congenital disease in sheep and goats, but can also cause acute and persistent infections. Sheep can become infected from cattle (6). Pigs may also be infected and antibodies to BDV in pigs may interfere with tests for the diagnosis of CSF (15). This chapter describes BDV infection in sheep.

a) **Acute infections**

Healthy newborn and adult sheep exposed to BDV experience only mild or inapparent disease. Slight fever and a mild leukopenia are associated with a short-lived viraemia detectable between days 4 and 11 post-infection, after which virus neutralising antibody appears in the serum (19).

Acute infections are best diagnosed serologically using paired sera from a representative number of sheep. Occasional BDV isolates have been shown to produce high fever, profound and prolonged leukopenia, anorexia, conjunctivitis, nasal discharge, dyspnoea and diarrhoea, and 50% mortality in young lambs. One such isolate was recovered from a severe epidemic of BD among dairy sheep in 1984 (7). A second such isolate was a BDV contaminant of a live CSFV vaccine (29).

b) **Fetal infection**

The main clinical signs of BD are seen following the infection of pregnant ewes. While the initial maternal infection is subclinical or mild, the consequences for the fetus are serious. Fetal death may occur at any stage of pregnancy, but is more common in fetuses infected early in gestation. Small dead fetuses may be resorbed or their abortion may pass unnoticed as the ewes continue to feed well and show no sign of discomfort. As lambing time approaches, the abortion of larger fetuses, stillbirths and the premature births of small, weak lambs will be seen. Confirmation that an abortion or stillbirth is due to BDV is often difficult to establish, but virus may be isolated from fetal tissues in some cases. In aborted fetuses, it is also possible to detect virus by immunohistochemistry of brain, thyroid and other tissues (20). Samples of fetal fluids or serum should be tested for BDV antibody.

During lambing, an excessive number of barren ewes will become apparent, but it is the diseased live lambs that present the main clinical features characteristic of BD. The clinical signs exhibited by BD lambs are very variable and depend on the breed of sheep, the virulence of the virus and the time at which infection was introduced into the flock. Affected lambs are usually small and weak, many being unable to stand. Nervous signs and fleece changes are often apparent. The nervous signs of BD are its most characteristic feature. The tremor can vary from violent rhythmic contractions of the muscles of the hindlegs and back, to barely detectable fine trembling of the head, ears, and tail. Fleece abnormalities are most obvious in smooth-coated breeds, which develop hairy fleeces, especially on the neck and back. Abnormal brown or black pigmentation of the fleece may also be seen in BD-affected lambs. Blood samples to be tested for the presence of BDV and/or antibody should be collected into anticoagulant from suspect lambs before they have received colostrum. Once lambs have ingested colostrum, it is difficult to detect virus until they are 2 months old and maternal antibody levels have waned. However, during this period, it may be possible to detect viral antigen in skin biopsies, by immunohistochemistry, and in leukocytes.

With careful nursing, a proportion of BD lambs can be reared, although deaths may occur at any age. The nervous signs gradually decline and can have disappeared by 3–6 months of age. Weakness, and swaying of the hind-quarters, together with fine trembling of the head, may reappear at times of stress. Affected lambs often grow slowly and under normal field conditions many will die before or around weaning time. In cases where losses at lambing time have been low and no lambs with obvious signs of BD have been born, this can be the first presenting sign of disease.

Some fetal infections occurring around mid-gestation can result in lambs with severe nervous signs, locomotor disturbances and abnormal skeletons. Such lambs have lesions of cerebellar hypoplasia and dysplasia, hydranencephaly and porencephaly resulting from necrotising inflammation. The severe destructive lesions appear to be immune mediated, and lambs with such lesions frequently have high concentrations of serum antibody to BDV. Most lambs infected in late gestation are normal and healthy and are born free from virus but with BDV antibody. Some such lambs can be weak and may die in early life (2).
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c) Persistent viraemia

When fetuses survive an infection that occurs before the onset of immune competence, they are born with a persistent viraemia. The ovine fetus can first respond to an antigenic stimulus between approximately 60 and 85 days of its 150-day gestation period. In fetuses infected before the onset of immune competence, viral replication is uncontrolled and 50% fetal death is common. In lambs surviving infection in early gestation, virus is widespread in all organs. Such lambs appear to be tolerant of the virus and have a persistent infection, usually for life. A precolostral blood sample will be virus positive and antibody negative. Typically, there is no inflammatory reaction and the most characteristic pathological changes are in the central nervous system (CNS) and skin. Throughout the CNS, there is a deficiency of myelin, and this causes the nervous signs. In the skin, primary wool follicles increase in size and the number of secondary wool follicles decreases, causing the hairy or coarse fleece.

Persistently viraemic sheep can be diagnosed by virus isolation/detection in a blood sample. Viraemia is readily detectable at any time except within the first 2 months of life, when virus is masked by colostral antibody; however, the virus may be identified in washed leukocytes during this period, and in animals older than 4 years, some of which develop low levels of anti-BDV antibody (13). Although virus detection in blood during an acute infection is difficult, persistent viraemia should be confirmed by retesting animals after an interval of at least 3 weeks.

Some viraemic sheep survive to sexual maturity and are used for breeding. Lambs born to these infected dams are always persistently viraemic. Persistently viraemic sheep are a continual source of infectious virus to other animals and their identification is a major factor in any control programme. Sheep being traded should be screened for the absence of BDV viraemia.

Usually persistently infected (PI) rams have poor quality, highly infective semen and reduced fertility. All rams used for breeding should be screened for persistent BDV infection on a blood sample. Semen samples can also be screened for virus, but virus isolation is much less satisfactory than from blood due to the toxicity of semen for cell cultures. Reverse-transcription polymerase chain reaction (RT-PCR) for detecting pestivirus nucleic acid may be justifiable on semen from some rams.

d) Late-onset disease in persistently viraemic sheep

Some PI sheep housed apart from other animals spontaneously develop intractable scour, wasting, excessive ocular and nasal discharges, sometimes with respiratory distress. At necropsy such sheep have gross thickening of the distal ileum, caecum and colon resulting from focal hyperplastic enteropathy. Cytopathic BDV can be recovered from the gut of these lambs. With no obvious outside source of cytopathic virus it is most likely that such virus originates from the lamb's own virus pool. Other PI sheep in the group do not develop the disease. This syndrome, which has also been recognised in occasional field outbreaks of BD, has several similarities with bovine mucosal disease (13).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent (the prescribed test for international trade)

There is no designated OIE reference laboratory for BDV, but the reference laboratories for BVDV or CSFV will be able to provide advice (see Table given in Part 3 of this Terrestrial Manual). One of the most sensitive proven methods for identifying BDV remains virus isolation. Direct immunofluorescence or other immunohistochemical techniques on frozen tissue sections as well as antigen detecting enzyme-linked immunosorbent assay (ELISA) and RT-PCR are also valuable methods for identifying BDV-infected animals.

a) Virus isolation

It is essential that laboratories undertaking virus isolation have a guaranteed supply of pestivirus-free susceptible cells and fetal bovine serum (FBS), or equivalent, that contain no anti-pestivirus activity and no contaminating virus. It is important that a laboratory quality assurance programme be in place.

The virus can be isolated in a number of primary or secondary ovine cell cultures (e.g. kidney, testes, lung). Ovine cell lines for BDV growth are rare. Semicontinuous cell lines derived from fetal lamb muscle (FLM), whole embryo (19) or sheep choroid plexus can be useful, but different lines vary considerably in their susceptibility to the virus. Ovine cells have been used successfully for the isolation and growth of BD viruses and BVDV types 1 and 2 from sheep. In regions where sheep may become infected with BVD viruses from cattle, a virus isolation system using both ovine and bovine cells could be optimal. Several bovine cell cultures may be suggested, including testicular, embryonic tracheal or turbinate cells, or a susceptible continuous kidney cell line. However, bovine cells are insensitive for the primary isolation and growth of some BD viruses, so reliance on bovine cells alone is inadvisable.
From live animals, serum can be tested for the presence of infectious virus, but the most sensitive way to confirm pestivirus viraemia is to wash leukocytes repeatedly (at least three times) in culture medium before co-cultivating them with susceptible cells for 5–7 days. Cells are frozen and thawed once and an aliquot passed on to further susceptible cells grown on cover-slips. The cells are stained, 3 days later, for the presence of pestivirus using an immunofluorescence or immunoperoxidase test. Tissues should be collected from dead animals in virus transport medium (10% [w/v]). In the laboratory, the tissues are ground, centrifuged to remove debris, and the supernatant passed through 0.45 µm filters. Spleen, thyroid, thymus, kidney, brain, lymph nodes and gut lesions are the best organs for virus isolation.

Semen can be examined for the presence of BDV, but raw semen is strongly cytotoxic and must be diluted, usually at least 1/10 in culture medium. As the major threat of BDV-infected semen is from PI rams, blood is a more reliable clinical sample than semen for identifying such animals. There are many variations in virus isolation procedures. All should be optimised for maximum sensitivity using a standard reference virus preparation and, whenever possible, recent BDV field isolates. A practical tube isolation procedure is outlined below:

i) Test tube cultures with subconfluent or newly confluent monolayers of susceptible ovine cells are washed at least twice with Hanks balanced salt solution to remove growth medium before being inoculated with 0.2 ml of sample and adsorbed for 2 hours at 37°C.

ii) Cultures are washed with 2 ml medium. This is discarded and 1 ml of culture maintenance medium is added.

iii) Cultures are incubated for 5–7 days at 37°C. They are examined microscopically on a daily basis and evidence of cytopathic effect (CPE) is recorded.

iv) The tubes are frozen at –70°C, and then thawed, as before, for passage to fresh tube cultures containing cells growing on cover-slips.

v) Cover-slips are removed 3–4 days later, fixed in cold acetone for 15 minutes and stained using an indirect or direct immunofluorescence method. Essential controls must include known negative cells and cells growing standard cytopathic and noncytopathic BDV strains.

vi) The cover-slips are examined under a UV microscope for the diffuse cytoplasmic fluorescence that is characteristic of pestiviruses.

Alternatively, frozen and thawed cultures can be added to cells growing on chamber slides and stained by direct immunofluorescence test as above. Immunoperoxidase staining can also be used on cover-slips or chamber slides as well as microtitre plates (see method under virus neutralisation [VN] test below). Frozen and thawed cultures can also be tested in an antigen detection ELISA system.

b) Immunohistochemistry

Viral antigen demonstration is possible in most of the tissues of PI animals (4, 20). This should be done on acetone-fixed frozen tissue sections (cryostat sections) using appropriate antibodies. Tissues with a high amount of viral antigen are brain, thyroid gland and oral mucosa. Skin biopsies have been shown to be useful for in-vivo diagnosis of persistent BDV infection.

c) Enzyme-linked immunosorbent assay for antigen detection

The first ELISA for pestivirus antigen detection was described for detecting viraemic sheep. This has now been modified into a double monoclonal antibody (MAb) capture ELISA for use in sheep and cattle. Two capture MAbs are bound to wells in microtitre plates, and two other MAbs, conjugated to peroxidase, serve as detector MAbs (9). The test is most commonly employed to identify PI viraemic sheep using washed, detergent-lysed blood leukocytes. The sensitivity is close to that of virus isolation and it is a practical method for screening high numbers of blood samples. As with virus isolation, high levels of colostral antibody can mask persistent viraemia. The ELISA is more effective than virus isolation in the presence of antibody, but may give false-negative results in viraemic lambs younger than 2 months old. The ELISA is usually not sensitive enough to detect acute BDV infections on blood samples. As well as for testing leukocytes, the antigen ELISA can also be used on tissue suspensions, especially spleen, from suspected PI sheep and, as an alternative to immunofluorescence and immunoperoxidase methods, on cell cultures. Several pestivirus ELISA methods have been published and commercial kits are now available for detecting BDV. Validation of these kits is in process.

d) Nucleic acid recognition methods

The complete genomic sequences of two BD viruses have been determined and compared with those of other pestiviruses (3, 17). Phylogenetic analysis shows BD viruses to be more closely related to CSFV than to BVDV (1, 22, 27). RT-PCR for diagnosing pestivirus infection is now used widely. A basic nested RT-PCR protocol involves the following stages:
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i) Total RNA is isolated by phenol-chloroform, TRIZOL, guanidine isothiocyanate (GITC), or a spin column method.

ii) cDNA is synthesised using random hexamers or antisense primers.

iii) Using panpestivirus primers from the 5'-non-coding region, the nested PCR uses approximately 25 cycles in the first amplification and 30–35 cycles in the second amplification.

iv) TaqMan or another probe system is used to identify the pestivirus product.

Primer choice is critical. Panpestivirus primers are valuable for detecting and typing all species of Pestivirus (18, 26). Specific primers for the rapid recognition of BD viruses have also been described (11, 28). Using a closed one tube RT-PCR with fluorescent probes reduces the potential for cross-contamination of diagnostic samples (12). Important applications include the detection of viral RNA in fetal tissues and in cell culture constituents or vaccines (25); validation of the RT-PCR is in process. It may also prove valuable for detecting virus when BDV-specific antibodies are present. The precautions to be taken with RT-PCR have been covered in Chapter I.1.4, Validation and Quality Control of Polymerase Chain Reaction Methods used for the Diagnosis of Infectious Diseases.

2. Serological tests

Antibody to BDV is usually detected in sheep sera using VN or an ELISA. The less sensitive agar gel immunodiffusion (AGID) test may also be used. Control positive and negative reference sera must be included in every test. These should give results within predetermined limits for the test to be considered valid. Single sera can be tested to determine the prevalence of BDV in a flock, region or country. For diagnosis, however, acute and convalescent sera are the best samples for confirming acute BDV infection. Repeat sera from one animal should always be tested alongside each other on the same plate.

a) Virus neutralisation test

A standard cytopathic strain of BDV (e.g. Moredun strain) can be used for the VN test with semicontinuous cells such as FLM. An outline protocol is given below.

i) Test and control sera are heat-inactivated for 30 minutes at 56°C.

ii) From a starting serum dilution of 1/4, serial twofold dilutions of the test sera are made in cell culture growth media in a cell culture-grade flat-bottomed 96-well microtitre plate. For each sample two or four wells are used at each dilution depending on the accuracy required. The range of dilution can also vary. It is common to screen sera initially at a dilution of 1/4 and titrate out positive sera. To screen sera a minimum of four wells is required. The standard working volume is 25 µl: 25 µl of the diluted serum is added to each well; 25 µl of media is added to each of the lower two control wells and 25 µl of media containing 100 TCID50 (50% tissue culture infective dose) of virus is added to each of the two upper test wells. Control positive and negative sera and a virus titration are included in every test.

iii) Plates are sealed with nontoxic plate sealers or lids, and incubated at 37°C for 1 hour.

iv) 100 µl of a cell suspension with a count of 2 × 10^5 cells/ml is added to every well. The FBS or equivalent serum used for cell growth must be free from antibody to BDV.

v) The plate is sealed or incubated in a humid chamber in 5% CO2 for 4 days at 37°C.

vi) The wells are examined microscopically for CPE. In the control wells of the test sera, CPE will be due to toxicity. Further dilution of toxic sera can be attempted, but it may not be possible to obtain reliable results with occasional sera. The VN titre for each serum is the dilution at which the virus is neutralised in 50% of the wells. This can be calculated by the Spearman–Kärber method. A seronegative animal will show no neutralisation at the lowest dilution (i.e. 1/4).

The choice of test virus is difficult due to antigenic diversity among pestiviruses (8, 14). Standard strains of cytopathic BVD viruses and bovine cells can be used. Oregon C24V results correlate better with Moredun BDV than results with the NADL strain. No single strain is ideal. A local strain that gives the highest antibody titre with a range of positive sheep sera should be used. The VN test can also be used with noncytopathic viruses when the following immunoperoxidase staining system is used after step v above:

i) The culture medium is removed and the cells are washed gently with warm phosphate buffered saline (PBS), air-dried and cooled to 4°C.

ii) The cells are fixed by quickly adding to all wells 95% acetone (in water) previously cooled to −20°C. The plates are held at −20°C for 30 minutes and should not be stacked or allowed to warm as etching of the plastic may occur.

iii) The acetone is removed and the plates are dried quickly in a cool environment.
iv) 50 µl of BDV antiserum is added to all wells at a predetermined dilution in PBS with 1% Tween 80 (PBST). The plates are incubated at 37°C for 30 minutes in a humid atmosphere.

v) The plates are emptied and washed three times with PBST.

vi) The wells are drained and an appropriate anti-species serum conjugated to peroxidase at a predetermined dilution is added, and the plates are left for 30 minutes at 37°C in a humid atmosphere.

vii) The plates are emptied and washed three times with PBST.

viii) The plates are drained well and 50 µl of activated substrate, e.g. 3-amino-9-ethyl carbazole (AEC) is added. AEC stock solution is: AEC (0.1 g) dissolved in dimethyl formamide (15 ml). For use add stock (0.3 ml) to membrane-filtered 0.05 M acetate buffer, pH 5.0, (4.7 ml), and then add 30% H₂O₂ (5 µl). NB: This solution is toxic and should be handled with adequate precautions.

ix) The plates are incubated at room temperature and known virus-positive control wells are monitored for development of specific red-brown cytoplasmic staining. When staining is complete the substrate is removed carefully and the wells are washed thoroughly with tap water. Leaving the tap water in the wells, the plates are examined microscopically for virus-containing wells.

x) The VN titre is calculated as above using the Spearman–Kärber method.

xi) Alternatively, the test can be performed using direct fluorescein-isothiocyanate conjugate staining.

Occasionally there may be a need to determine whether antibody in a flock is against a virus belonging to a particular Pestivirus serogroup. A differential VN test can be used in which sera are titrated out against representative viruses from each of the four Pestivirus groups, i.e. BDV, BVDV types 1 and 2, and CSFV. Maximum titre will identify the infecting serotype and the spectrum of cross-reactivity with the other serotypes will also be revealed.

b) Enzyme-linked immunosorbent assay

An MAb-capture ELISA for measuring BDV antibodies has been described. Two panpestivirus MAbs that detect different epitopes on the immunodominant nonstructural protein NS 2/3 are used to capture detergent-lysed cell-culture grown antigen. The results correlate qualitatively with the VN test (10).

Antigen is prepared as follows: Use eight 225 cm² flasks of newly confluent FLM cells; four flasks will be controls and four will be infected. Wash the flasks and infect four with a 0.01–0.1 m.o.i. (multiplicity of infection) of Moredun cytopathic BDV. Adsorb the virus for 2 hours at 37°C. Add maintenance media containing 2% FBS (free from BDV antibody), and incubate cultures for 4–5 days until CPE is obvious. Pool four control flask supernatants and separately pool four infected flask supernatants. Centrifuge at 3000 g for 15 minutes to pellet cells. Discard the supernatants. Retain the cell pellets. Wash the flasks with 50 ml of PBS and repeat the centrifugation step as above. Pool all the control cell pellets in 8 ml PBS containing 1% nonidet P40 and return 2 ml to each control flask to lyse the remaining attached cells. Repeat for infected cells. Keep the flasks at 4°C for at least 2 hours agitating the small volume of fluid on the cells vigorously every 30 minutes to ensure total cell detachment. Centrifuge the control and infected antigen at 12,000 g for 5 minutes to remove the cell debris. Supernatant antigens are stored at –70°C in small aliquots.

**Test procedure**

i) The two MAbs are diluted to a predetermined dilution (commonly 1/4000) in 0.05 M bicarbonate buffer, pH 9.6. All wells of a suitable ELISA-grade microtitre plate (e.g. Nunc maxisorb, Greiner 129b) are coated overnight at 4°C.

ii) After washing three times in PBST, a blocking solution of PBST containing 10% horse serum (PBSTH) is added to all wells, which are incubated at 37°C for 1 hour.

iii) The antigen is diluted to a predetermined dilution in PBSTH and alternate rows of wells are coated with virus and control antigens for 1 hour at 37°C. The plates are then washed three times in PBST before addition of test sera.

iv) Test sera are diluted 1/50 in PBSTH and added to duplicate virus and duplicate control wells for 1 hour at 37°C. The plates are then washed three times in PBST.

v) Anti-ovine IgG peroxidase conjugate is diluted to a predetermined dilution in PBSTH and added to all wells for 1 hour at 37°C. The plates are washed three times in PBST.

vi) A suitable activated enzyme substrate, such as ortho-phenylene diamine (OPD) or tetramethyl blue (TMB) is added noting the manufacturer’s toxicity warning. After colour development, the reaction is stopped with sulphuric acid and the absorbance read on an ELISA plate reader. The mean value of the two control wells is subtracted from the mean value of the two virus wells to give the corrected absorbance for each serum. Results are expressed as corrected absorbance with reference to the
corrected absorbance of known positive and negative sera. Alternatively, ELISA titres can be extrapolated from a standard curve of a dilution series of a known positive reference serum.

If antigens of sufficient potency can be produced the MAb capture stage can be omitted. In this case alternate rows of wells are coated with virus and control antigen diluted to a predetermined dilution (commonly 1/100) in 0.05 M bicarbonate buffer, pH 9.6, overnight at +4°C. The plates are washed and blocked as in step ii above. After washing, diluted test sera are added and the test proceeds from step iv as above.

c) Agar gel immunodiffusion test

The AGID test was first used to demonstrate an immunological relationship between BD, BVD and CSF viruses.

The Oregon C24V strain of BVDV grown on calf testis cells has been used to detect antibody in sheep. Suitable antigen can be prepared using medium harvested from cells showing early CPE. Concentration of the medium approximately 100-fold by dialysis against polyethylene glycol (PEG) is required. Alternatively, PEG 6000 can be added to sonicated virus/cell suspensions at the rate of 8% (w/v). After constant stirring overnight at 4°C, the precipitate is removed by centrifugation at 1800 g for 1 hour. The supernatant is decanted thoroughly and the precipitate resuspended to 1% of the original virus/cell culture volume in distilled water. The resuspended precipitate is centrifuged at 286,000 g for 2 hours and the supernatant withdrawn for use as antigen. The precipitate is discarded.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

To be useful, a BDV vaccine should be effective when administered to female sheep before breeding to prevent transplacental infection. Experimental and commercial inactivated whole virus BDV vaccines have been produced in Europe (5, 24).

Pestivirus contaminants of modified live virus vaccines have been found to be a cause of serious disease following their use in pigs, cattle, sheep and goats. Contaminated vaccines have included those used for the control of Aujesky’s disease, CSF, rotavirus, coronavirus, rinderpest, sheep pox and contagious pustular dermatitis. The insidious ability of pestiviruses to cross the placenta, and thus establish PI animals, gives them the potential to contaminate vaccines through cells, serum used as medium supplement or seed stock virus. As nearly all isolates of pestiviruses are noncytopathic, they will remain undetected unless specific tests are carried out.

1. Seed management

a) Characterisation of the seed

An ideal vaccine should contain a strain or strains of virus that give protection against all sheep pestiviruses. Recent evidence is that three antigenically distinguishable groups of pestiviruses infect sheep. One group is represented by the Moredun reference strain of BDV; the second group contains viruses similar to the majority of cattle BVDV strains (BVDV type 1); and the third group contains the less common BVDV (type 2) strains (30). Further cross-protection studies are required to determine the significance of these findings. Nevertheless it would appear that any BDV vaccine should contain at least a representative of the BDV and BVDV (type 1) groups. Characterisation of the biologically cloned vaccine viruses should include typing with MAbs and genotyping (16).

b) Culture

A variety of ruminant cell cultures can be used. Optimal yields depend on the cell type and isolate used. A commercial BDV vaccine containing two strains of virus is prepared on ovine cell lines (5). Cells must be produced according to a seed-lot system from a master cell seed (MCS) that has been shown to be free from all contaminating microorganisms. Vaccine should only be produced in cells fewer than 20 passages from the MCS. Control cells from every passage should be checked for pestivirus contamination.

c) Validation as a vaccine

All vaccines should pass standard tests for safety and efficacy. Safety testing of inactivated BDV vaccines should include monitoring of all vaccine components for contaminating pestiviruses.

Efficacy tests of BDV vaccines should demonstrate their ability to prevent transplacental spread of virus. Effective challenge of vaccinated pregnant ewes at 50–60 days gestation has been achieved by intranasal installation of virus or by mixing with PI sheep (5).
2. **Method of manufacture**

Inactivated vaccines have been prepared using conventional laboratory techniques with stationary or rolled cell cultures. Inactivants have included formalin and beta-propiolactone. Adjuvants have included aluminium hydroxide and oil (5, 24).

3. **In-process control**

Cultures should be inspected daily to ensure they are free from gross bacterial contamination and that any CPE observed is appropriate to the cytopathic virus being grown. No CPE should be observed in cultures being used to grow noncytopathic strains of virus.

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**

Samples from inactivated vaccines should be tested rigorously for viable virus. Samples of the product should be passaged several times in sensitive cell cultures to ensure absence of live BDV. This *in-vitro* monitoring can be augmented by injecting two BDV-seronegative sheep with 20 doses of unformulated antigen as part of a standard safety test. Presence of live virus will result in the development of a more convincing serological response than will occur with inactivated virus alone. The sheep sera can also be examined for antibody to other prescribed agents.

c) **Potency**

Vaccine potency is also best tested in seronegative sheep in which the development and level of antibody is measured. An indirect measure of potency is given by the level of virus infectivity prior to inactivation. The antigen content following inactivation can be assayed by MAb-capture ELISA and related to the results of established *in-vivo* potency results. As recommended for potency testing of BVDV vaccine in cattle it should be demonstrated that the vaccine can prevent transplacental transmission of BDV in pregnant sheep.

d) **Duration of immunity**

No information is available on duration of immunity following vaccination. Inactivated vaccines are unlikely to provide sustained levels of immunity and it is likely that after an initial course of two or three injections annual booster doses may be required. Insufficient information is available to determine any correlation between vaccinal antibody titres in the dam and fetal protection.

e) **Stability**

There is little information on the stability of BDV vaccines. Inactivated vaccines could be expected to have at least a 1 year shelf life when protected from light and stored at 4°C.

f) **Preservatives**

Preservatives may be added to multidose vaccine containers subject to the approval of the Control Authority.

g) **Precautions (hazards)**

BDV is not considered to be a hazard to human health. Standard good microbiological practice should be used when handling the virus.

5. **Tests on the final product**

a) **Safety**

*In-vitro* test only.

b) **Potency**

*In-vitro* antigen content test.
REFERENCES


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CHAPTER 2.10.6

BOVINE VIRAL DIARRHOEA

SUMMARY

Cattle of all ages are susceptible to infection with bovine viral diarrhoea virus (BVDV) (see also Appendix 3.2.1. in the Terrestrial Animal Health Code). Distribution of the virus is world-wide. The clinical signs range from subclinical to the fulminating fatal condition called mucosal disease. Acute infections may result in transient diarrhoea or pneumonia, usually in the form of group outbreaks. Acute forms of the disease associated with high mortality have also been described, often, but not always, associated with a haemorrhagic syndrome. However, most infections in the young calf are mild and go unrecognised clinically. The virus spreads mainly by contact between cattle. Vertical transmission plays an important role in its epidemiology and pathogenesis.

Infections of the bovine fetus may result in abortions, stillbirths, teratogenic effects or persistent infection in the neonatal calf. Persistently viraemic animals may be born as weak, unthrifty calves or may appear as normal healthy calves and be unrecognised clinically. Some of these animals may later develop mucosal disease with anorexia, gastrointestinal erosions, and profuse diarrhoea, leading invariably to death. Mucosal disease can arise only in persistently infected animals.

It is important to avoid the trade of viraemic animals. It is generally considered that serologically positive, nonviraemic cattle are ‘safe’, providing that they are not pregnant. Antibody-positive pregnant cattle carrying persistently infected fetuses are important transmitters of the virus between herds. About 15% of persistently viraemic animals have antibody to the NS/2 protein and a lower percentage to the E2 glycoprotein. Therefore, seropositivity cannot be equated with ‘safety’. Latent infections are not generally thought to occur following recovery from acute infection, though semen from acutely infected animals may be suspect.

Identification of the agent: BVDV is a pestivirus in the Flaviviridae and is closely related to classical swine fever and ovine Border disease viruses. BVDV occurs in two forms: noncytopathogenic and cytopathogenic. There are two antigenically distinct genotypes (types 1 and 2), and virus isolates within these groups exhibit considerable biological and antigenic diversity.

Persistently viraemic healthy animals resulting from congenital infection can be readily identified by isolation of noncytopathogenic virus in cell cultures from blood or serum. It is necessary to use an immune-labelling method to detect the growth of virus in the cultures. Alternative methods based on direct detection of viral antigen or viral RNA in leukocytes are also available. Persistence of virus should be confirmed by resampling after an interval of at least 3 weeks. These animals will usually have no or low levels of antibodies to BVDV.

Viraemia in acute cases is transient and can be difficult to detect. In fatal cases of haemorrhagic disease, virus can be isolated from tissues post-mortem. Confirmation of mucosal disease can be made by isolation of the cytopathogenic biotype of BVDV, particularly from intestinal tissues. Noncytopathogenic virus may also be detected, especially in blood.

Serological tests: Acute infection with BVDV is best confirmed by demonstrating seroconversion using sequential paired samples from several animals in the group. The testing of paired (acute and convalescent samples) should be done a minimum of 14 days apart and samples should be tested side by side. The enzyme-linked immunosorbent assay for antibody and the virus neutralisation test are the most widely used.

Requirements for vaccines and diagnostic biologicals: There is no standard vaccine for BVD, but a number of commercial preparations are available. Modified live virus vaccine should not be
administered to pregnant cattle (or to their sucking calves) due to the risk of transplacental infection. There is also a risk of inducing mucosal disease in persistently infected animals. Killed virus vaccines generally require booster vaccinations. An ideal vaccine should be able to prevent transplacental infection in pregnant cows.

BVDV is a particularly important hazard to the manufacture of biological products for other diseases due to the high frequency of contamination of batches of fetal calf serum used as a culture medium supplement.

A. INTRODUCTION

Bovine viral diarrhoea virus (BVDV) is a pestivirus in the Flaviviridae and is closely related to classical swine fever and ovine border disease viruses (20). Two antigenically distinct genotypes of BVDV exist, types 1 and 2, with further subdivisions discernable by genetic analysis (66). The two genotypes may be differentiated from each other, and from other pestiviruses, by monoclonal antibodies (MAbs) directed against the E2 and E RNS major glycoproteins, or by genetic analysis (57, 60). Multiplex polymerase chain reaction (PCR) enables virus typing direct from blood samples (30). Type 1 virus is generally more common although the prevalence of type 2 is reported to be almost as high as type 1 in North America. BVDV of both genotypes may occur in noncytopathogenic and cytopathogenic forms (biotypes), classified according to whether or not it produces visible change in cell cultures. Usually, it is the noncytopathogenic biotype that circulates in cattle populations. Each biotype has a specific role in a variety of clinical syndromes – acute, congenital and chronic infections (4, 10). Type 2 viruses are usually noncytopathogenic and have been associated with outbreaks of severe acute infection (15). Clinically inapparent infections are common with both genotypes.

B. DIAGNOSTIC TECHNIQUES

a) Acute infections

Acute infections of cattle occur particularly in young animals, and may be clinically inapparent or associated with diarrhea (1). Affected animals may be predisposed to concurrent infections, for example respiratory disease, perhaps due to an immunosuppressive effect of the virus. Bulls may suffer a temporary depression of fertility and can show transient shedding of virus in the semen (54). Cows may also suffer from infertility, likely associated with changes in ovarian function (31) and secretions in gonadotrophin and progesterone (27). During acute infections, a brief viraemia may be detectable and nasal shedding of virus may occur. There may also be a transient leukopenia, thrombocytopenia or temperature response, but these can vary greatly among animals. A serological response is the most certain means of diagnosing a previous infection. The clinical picture is generally one of high morbidity and low mortality, though more severe disease is sometimes seen (11). In particular, outbreaks of a severe form of acute disease with haemorrhagic lesions, thrombocytopenia and high mortality have been reported sporadically from some countries (1, 5) and infection with Type 2 viruses in particular has been demonstrated to cause altered platelet function (68). Other acute outbreaks may show fever, pneumonia, diarrhoea and sudden death in any age group, with haemorrhagic signs (15).

b) Congenital infection

If noncytopathogenic virus infects the bovine fetus, this may result in abortion, stillbirth, teratogenic effects or a congenital infection that persists in the neonatal calf (1, 10, 23, 50). Confirmation that an abortion is caused by BVDV is often difficult to establish (61), but virus may be isolated from fetal tissue in some cases, or viral antigen or genome may be demonstrated. An attempt should also be made to detect specific antibody in samples of fetal fluids or serum, or in the supernatant fluid from a tissue suspension. Stillbirths or teratogenic effects may be associated with an active fetal immune response to the virus during mid-to-late gestation. The dam will often have high antibody titres (>1/2000) to BVDV, which is suggestive of fetal infection and is probably due to the fetus providing the dam an extended challenge of virus.

Although congenital infection with BVDV often leads to abortion, it is not always recognised in the field. Infection during the first third of the gestation period can result in the abortion of a conceptus that is small and goes unnoticed by the farmer. The cow would return to service and the failure to maintain pregnancy would be classified as an example of early embryonic death. Another possible outcome of infection is the death and subsequent resorption of fluids from the fetus that results in mummification. It is frequently observed that aborted fetuses have subcutaneous oedema and copious pleural and peritoneal effusions. There may also be congenital abnormalities that result in growth retardation and in selective central nervous system (CNS) defects, such as cerebellar hypoplasia and dysmyelination (62), and eye defects, such as cataracts. Sometimes there are skeletal defects, the most advanced of which is arthrogryposis.
Stillborn calves are a common sequel to congenital infection. The calves usually appear to be fully developed at parturition, but fail to survive. After day 150 of pregnancy, the immune system of the fetus will be developed and infection of the fetus will usually result in an antibody response and the birth of a normal calf.

c) Persistent viraemia

When infections of the fetus occur before approximately 110 days of gestation and before immunocompetence, the calf may be born with a persistent viraemia. Identification of these animals is readily made by detection of noncytopathogenic BVDV in blood. The virus can also be identified in the skin by immunohistochemistry. Furthermore, animals with a persistent viraemia will also lack specific antibody, but diagnosis in the young calf, up to approximately 3 months of age, may be confused by the presence of maternal antibody to BVDV. Maternal antibody may also interfere with virus isolation. In older animals with persistent viraemia, low levels of antibody may be present due to their ability to seroconvert to strains of BVDV (including vaccines) ‘heterologous’ (antigenically different) from the persisting virus (11). To confirm a diagnosis of persistent viraemia, animals should be retested after an interval of at least 3 weeks.

There are no pathognomonic lesions in the viraemic calf. The clinical signs vary from the apparently normal healthy animal to the weak, unthrifty calf that has difficulty in standing and suckling. These latter calves can show CNS defects, such as muscular tremors, incoordination and blindness. They often die within days of birth, thus contributing to the ‘weak calf syndrome’.

Some viraemic animals survive to sexual maturity and are retained for breeding. Calves born to these infected dams are always persistently viraemic, and are often weak at birth and fail to thrive. Persistently viraemic animals are a continual source of infective virus to other cattle, and thus their rapid identification and removal from the herd are required. Animals being traded should first be screened for the absence of persistent BVD viraemia.

Bulls that are persistently infected usually have poor quality, highly infective semen and, as a result, reduced fertility (41, 59). All bulls used for natural or artificial insemination should be screened for persistent BVD infection.

Female cattle used as embryo recipients should always test negative for BVD viraemia before first use. Donor cows that are persistently infected with BVDV also represent a potential source of infection, as oocysts without an intact zona pellucida are shown to be susceptible to infection in vitro (65). However, a limited study of two persistently infected animals revealed that the majority of oocysts were BVDV-negative (63). Embryos may also become contaminated following acute infection of the donor (2). Biological materials used for in-vitro fertilisation techniques (bovine serum, bovine cell cultures) have a high risk of contamination and should be screened for BVDV (8). Recent incidents of apparent introduction of virus via such techniques (21, 43) have highlighted this risk. It is considered essential that serum supplements used in media should be sterilised as detailed in Article 3.3.1.5 of the OIE Terrestrial Animal Health Code (Terrestrial Code) and outlined in Section B.1.a. of this chapter. Importing countries may consider requesting additional tests to confirm sterilisation, detailed in Article 3.3.1.6 of the Terrestrial Code.

d) Mucosal disease

It is well established that persistently viraemic animals may later succumb to mucosal disease (10); however, cases are very rare. This syndrome has been shown to be associated with the presence of the cytopathogenic biotype, which can arise either through superinfection (4, 13), recombination between noncytopathogenic biotypes, or mutation of the persistent biotype (45). Consequently, confirmatory diagnosis of mucosal disease should include the isolation of cytopathogenic virus from affected cattle. This biotype may sometimes be isolated from blood, but it can be recovered more consistently from a variety of other tissues, in particular intestinal and Peyer’s patch tissue (16). Virus isolation is also readily accomplished from spleen. This is easy to collect and is seldom toxic for cell culture after preparation for viral isolation. Isolation from gut samples may be difficult if autolysis has occurred; in this case suspensions from lymph nodes or tonsil should then be tested. Noncytopathogenic virus can also be detected, particularly from blood or blood-associated organs. Cryostat tissue sections from mucosal disease cases can be stained for viral antigen by immunofluorescence or immunoperoxidase labelling.

Mucosal disease is invariably fatal. Its onset may be so rapid that the first signs seen are dead or moribund animals. However, it is more common for animals to become anorexic over a period of several days, to be disinclined to move and to show signs of abdominal pain. They can develop a profuse diarrhoea and rapidly lose bodily condition. Erosions can often be seen in the mouth, particularly along the gingival margin. Lacrimation and excessive salivation occur. Generally, cases of mucosal disease are sporadic and rare.

Post-mortem examination reveals erosions in the mucosa at various sites along the gastrointestinal tract. The most noticeable are those overlying the lymphoid Peyer’s patches in the small intestine and in the
colonic tonsils. On histological examination, there is a clear demonstration of destruction of the lymphoid tissue within the gut-associated lymphoid tissue. Most of the Peyer’s patch lymphoid cells have been lysed and replaced by inflammatory cells, debris and cells from the overlying collapsed epithelium.

Severe acute BVD infection can be clinically similar to mucosal disease and confusion can arise, particularly when a number of animals are so affected. Mucosal disease can occur among cohorts of persistently infected animals when oestrus synchronisation has been carried out. Differentiation requires a careful examination of case histories and testing for antibody as well as antigen or virus among infected and any recovered animals. Seroconversion among recovered animals is indicative of acute infection, whereas two antigen or virus positive results on samples from an affected animal, taken 3 weeks apart, is diagnostic of mucosal disease. Generally, animals with mucosal disease are antibody negative, though low levels of antibody can sometimes be detected.

1. Identification of the agent (the prescribed test for international trade)

All test methods must be validated by testing on known noninfected and infected populations of cattle, including animals with low- and high-titre viraemias. Methods based on MAb-binding assays or on nucleic acid recognition must be shown to detect the full range of antigenic and genetic diversity found among BVD viruses. There are two designated OIE Reference Laboratories for BVD (see Table given in Part 3 of this Terrestrial Manual); the reference laboratories for classical swine fever could also be approached to offer advice.

a) Virus isolation

The virus may be isolated in a number of bovine monolayer cell cultures (e.g. kidney, lung, testis or turbinate). Growth of both biotypes is usually satisfactory. Noncytopathogenic BVDV is a common contaminant of fresh bovine tissue, and cell cultures must be checked for freedom from adventitious virus by regular testing (7, 25). Primary or secondary cultures can be frozen as cell suspensions in liquid nitrogen. These can then be tested over a series of passages, or seeded to other susceptible cells and checked before routine use. Such problems may be overcome by the use of continuous cell lines, which can be obtained BVD-free (7).

The fetal bovine serum that is selected for use in cell culture must also be free not only from virus, but also and of equal importance, from BVDV neutralising antibody (25). Heat treatment (56°C for 30–45 minutes) is inadequate for the destruction of BVDV in contaminated serum; irradiation at 25 kiloGrays (2.5 Mrad) is more certain. Commercial batches of fetal bovine serum mostly test positive by PCR even after the virus has been inactivated by irradiation. Where appropriate, horse serum can be substituted for bovine fetal serum, although it is often found to have poorer cell-growth-promoting characteristics.

Buffy coat cells, whole blood, washed leukocytes or serum are suitable for isolation of the virus from live animals. Maternal antibody may interfere with isolation from serum in young calves. Tissue suspensions from post-mortem cases should be prepared by standard methods. Semen can also be examined, but a blood sample from the donor bull is preferable if it can be obtained. There is a report of an atypical persistent shedding of BVDV in semen from a bull that was not viraemic (67). Raw semen is cytotoxic and must be diluted in culture medium. Extended semen can usually be inoculated directly on to cell monolayers, but may occasionally cause cytotoxicity. For these reasons, it is important to monitor the health of the cells by microscopic examination at intervals during the incubation.

There are many variations of procedure in use for virus isolation. All should be optimised to give maximum sensitivity of detection of a standard virus preparation. This may include one or more in-vitro passage(s). Conventional methods for virus isolation are used, with the addition of a final immune-labelling step (fluorescence or enzymatic) to detect growth of noncytopathogenic virus. Thus tube cultures should include flying cover-slips, while plate cultures can be fixed and labelled directly in the plate. Examples are given below.

- Microplate immunoperoxidase method for mass screening for virus detection in serum samples (49)
  i) 10 µl of the serum sample is placed into each of four wells of a 96-well tissue-culture grade microplate. This is repeated for each sample. Known positive and negative controls are included.
  ii) 100 µl of a cell suspension of 150,000 cells/ml in medium without fetal calf serum (FCS) is added to all wells. NB: the sample itself acts as the cell-growth supplement. If testing samples other than serum, use medium with 10% FCS.
  iii) The plate is incubated at 37°C for 4 days, either in a 5% CO₂ atmosphere or with the plate sealed.
  iv) Each well is examined microscopically for evidence of cytopathic effect (CPE), or signs of cytotoxicity.
  v) The plate is emptied by gentle inversion and rinsed in phosphate buffered saline (PBS).
vi) The plate is fixed as follows: the plate is dipped into a bath of 20% acetone in PBS, emptied immediately, and then transferred to a fresh bath of 20% acetone in PBS for 10 minutes. The plate is drained thoroughly and as much fluid as possible is removed by tapping and blotting. The plate is dried thoroughly for at least 3 hours at a temperature of 25–30°C (e.g. using radiant heat from a bench lamp). NB: the drying is part of the fixation process.

Alternative fixation methods include paraformaldehyde or heat (see Chapter 2.1.13. Classical swine fever, Section B.2.b.viii).

vii) The fixed cells are rinsed by adding PBS to all wells.

viii) The wells are drained and the BVD antibody (50 µl) is added to all wells at a predetermined dilution in PBS containing 1% Tween 80 (PBST) and 5% horse serum. (Horse serum may be added to reduce nonspecific staining.) The plate is incubated at 37°C for 15 minutes.

ix) The plate is emptied and washed three times in PBST.

x) The plate is then drained and appropriate antispecies serum conjugated to peroxidase is added at a predetermined dilution in PBST (50 µl per well) for 15 minutes at 37°C.

xi) The plate is emptied and washed three times in PBST.

xii) The plate is rinsed in distilled water. All fluid is tapped out from the plate.

xiii) Freshly prepared hydrogen peroxide substrate with a suitable chromogen, e.g. 3-amino-9-ethyl carbazole (AEC) is added. The stock solution is: AEC (0.1 g) dissolved in dimethyl formamide (15 ml). For use, the stock (0.3 ml) is added to 0.05 M acetate buffer (5 ml, pH 5.0), and then 30% H₂O₂ (5 µl is added). An alternative substrate can be made, consisting of 9 mg diaminobenzidine tetrahydrochloride and 6 mg sodium perborate tetrahydrate dissolved in 15 ml of PBS. Though the staining is not quite so intense, these chemicals have the advantage that they can be shipped by air.

xiv) The plate is examined microscopically. Virus-positive cells show red-brown cytoplasmic staining.

- Tube method for tissue or buffy coat suspensions, or semen samples

  NB: this method can also be conveniently adapted to 24-well plastic dishes.

i) Tissue samples are ground up and a 10% suspension in culture medium is made. This is then centrifuged to remove the debris. Raw semen is diluted 1/10 in culture medium.

ii) Test tube cultures (with cover-slips) with newly confluent or subconfluent monolayers of susceptible bovine cells are inoculated with 0.1 ml of the sample. The culture is left to adsorb for 1 hour at 37°C.

iii) The culture is washed with 1 ml of medium; this is then discarded and 1 ml of culture maintenance medium is added.

iv) The culture is incubated for 4–5 days at 37°C, and examined microscopically for evidence of CPE or signs of cytotoxicity.

v) Culture may then either be frozen and thawed for passage to fresh cultures, or the cover-slip may be removed, fixed in acetone and stained with direct immunofluorescent conjugate to BVDV. In this case, examine under a fluorescent microscope for diffuse, cytoplasmic fluorescence characteristic of pestiviruses.

Alternatively, cultures may be freeze/thaw harvested and passed on to microtitre plates for culture and staining by the immunoperoxidase method (see section on microplate immunoperoxidase method for mass screening of serum samples above) or by the immunofluorescent method described here.

b) Enzyme-linked immunosorbent assay for antigen detection

Several methods for the enzyme-linked immunosorbent assay (ELISA) for antigen detection have been published (e.g. ref. 26) and a number of commercial kits are available. Most are based on the sandwich ELISA principle, with a capture antibody bound to the solid phase, and a detector antibody conjugated to a signal system, such as peroxidase. Both monoclonal- and polyclonal-based systems are described. The test is suitable for detection of persistently infected animals, and usually measures BVD antigen in lysates of peripheral blood leukocytes; the new generation of antigen-capture ELISAs (E²RIS capture ELISAs) is able to detect BVD antigen in blood as well as in plasma or serum samples. The best of the methods gives a sensitivity similar to virus isolation, and may be preferred in those rare cases where persistent viraemia is combined with seropositivity. The ELISA can have low sensitivity in the presence of BVDV clostral antibodies. In the presence of antibodies, reverse transcription PCR (RT-PCR) should be used as it is the most sensitive detection method for this circumstance. The antigen ELISA appears to be less useful for virus detection in acute BVD infections.
c) **Immunohistochemistry**

Enzyme-labelled methods are useful to detect BVDV antigen in tissue sections (69), particularly where suitable MAbs are available. It is important that the reagents and procedures used be fully validated, and that nonspecific reactivity be eliminated. For persistently infected cattle almost any tissue can be used, but particularly good success has been found with lymph nodes, thyroid gland, skin, brain, abomasum and placenta. Skin biopsies have shown to be useful for in-vivo diagnosis of persistent BDV infection.

d) **Nucleic acid detection**

The RT-PCR method can be adapted to the detection of BVD viral RNA for diagnostic purposes (9, 32, 40, 42). This may have a special value where low-level virus contamination is suspected, for example in screening batches of FCS, or biological products such as vaccines (34). Caution is needed in the interpretation of results, as the detection of viral RNA does not imply per se that infective virus is present. A multiplex PCR can be used to amplify and type virus from cell culture, or direct from blood samples, by producing different sized PCR products (30). Newer methodologies incorporate the use of probes, which confirm the identity of the PCR product, provide automated reading and can also differentiate among pestiviruses (48). Testing for virus after inoculation of cell cultures using PCR should be avoided as it may give positive results if commercial bovine fetal serum has been used in the growth medium. Primers should be selected in conserved regions of the genome, such as the 5'-noncoding region, or the NS3 (p80 gene). Molecular tests can be prone to contamination in unskilled hands. Stringent precautions should therefore be taken to avoid DNA contamination in the test system, and rigorous controls must be mounted (see Chapter I.1.4, Validation and Quality Control of Polymerase Chain Reaction Methods used for the Diagnosis of Infectious Diseases).

The RT-PCR technique is also sensitive enough to enable the detection of persistently infected lactating cows in a herd of up to 100 animals or more, by testing the somatic cells within bulk milk (22, 58). A positive result indicates that at least one such animal is present in the milking herd. Follow-up virus isolation or antigen detection tests are required to identify the individual(s).

Viral nucleic acid in tissues can be detected by in situ hybridisation with enzyme-linked riboprobes (19). This is a sensitive technique that can be applied to formalin-fixed paraffin-embedded tissue, thereby allowing a retrospective analysis.

2. **Serological tests**

Antibody to BVDV can be detected in cattle sera by a standard virus neutralisation (VN) test or by ELISA, using one of several published methods (24, 36, 39, 55). C ontrol positive and negative standard sera must be included in every test. These should give results within predetermined limits for the test to be considered valid. ELISA for antibody in bulk milk samples can give a useful indication of the BVD status of a herd (52). A high ELISA value of 1.0 or more absorbance units indicates a high probability of the herd having been exposed to BVDV in the recent past, most likely through one or more persistently viraemic animals being present. In contrast, a very low or negative value ( 0.2) indicates that it is unlikely that persistently viraemic animals are present. Further categorisation has been suggested for intermediate values, but this is dependent on the husbandry system in use. In at least one study, ELISA values have been proven to be an unreliable indicator of the presence of persistently infected animals on farms (70). Determination of the antibody status of a small number of young stock (9–18 months) has also been suggested as an indicator of recent exposure to BVDV (35), but these are likewise dependent on the degree of contact between different groups of animals in the herd. Rapid 'spot tests' can be used for initial screening as part of BVD control and eradication schemes (44).

a) **Virus neutralisation test**

Because it makes the test easier to read, most laboratories use highly cytopathogenic laboratory-adapted strains of BVDV for VN tests, although immune-labelling techniques are now available that allow simple detection of the growth or neutralisation of noncytopathogenic strains where this is considered desirable. No single strain is likely to be ideal for all circumstances, but in practice one should be selected that detects the highest proportion of serological reactions in the local cattle population. Two widely used cytopathogenic strains are ‘Oregon C24V’ and ‘NADL’. Low levels of antibody to BVD type 2 virus may not be detectable by a neutralisation test that uses type 1 strain of the virus, and vice versa (29). It is important that BVD type 1 and BVD type 2 be used in the test and not just the one that the diagnostician thinks is present as this can lead to under reporting.

An outline protocol for a microtitre VN test is given below (24):

i) The test sera are heat-inactivated for 30 minutes at 56°C.

ii) From a starting dilution of 1/5, serial twofold dilutions of the test sera are made in a cell-culture grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For each sample, two or
four wells are used at each dilution depending on the degree of precision required. Control positive and negative sera should also be tested.

iii) An equal volume (e.g. 50 µl) of a stock of cytopathogenic strain of BVDV containing 100 TCID$_{50}$ (50%) tissue culture infective dose is added to each well. A back titration of virus stock is also done in some spare wells to check the potency of the virus (acceptance limits 30–300 TCID$_{50}$).

iv) The plate is incubated for 1 hour at 37°C.

v) A flask of suitable cells (e.g. bovine turbinate, bovine testis) is trypsinised and the cell concentration is adjusted to $3 \times 10^5$/ml. 50 µl of the cell suspension is added to each well of the microtitre plate.

vi) The plate is incubated at 37°C for 4 days, either in a 5% CO$_2$ atmosphere or with the plate sealed.

vii) The wells are examined microscopically for CPE. The VN titre for each serum is the dilution at which the virus is neutralised in 50% of the wells. This can be calculated by the Spearman–Kärber method. A seronegative animal will show no neutralisation at the lowest dilution (1/5), equivalent to a final dilution of 1/10.

b) Enzyme-linked immunosorbent assay

Both indirect and blocking types of test can be used (36, 39, 55). A number of commercial kits are available. The chief difficulty in setting up the test lies in the preparation of a viral antigen of sufficient potency. The virus must be grown under optimal culture conditions using a highly permissive cell type. Any serum used in the medium must not inhibit growth of BVDV. The optimal time for harvest should be determined experimentally for the individual culture system. The virus can be concentrated and purified by density gradient centrifugation. Alternatively, a potent antigen can be prepared by treatment of infected cell cultures with detergents, such as Nonidet P40, Mega 10, Triton X-100 or 1-octyl-beta-D-glucopyranoside (OGP).

Some workers have used fixed, infected whole cells as antigen. In future, increasing use may be made of artificial antigens manufactured by expressing specific viral genes in bacterial or eukaryotic systems (64). Such systems should be validated by testing sera specific to a wide range of different virus strains. In the future, this technology should enable the production of serological tests complementary to subunit or marker vaccines, thus enabling differentiation between vaccinated and naturally infected cattle.

An example outline protocol for an indirect ELISA is given below (24).

i) Roller cultures of secondary calf testis cells with a high multiplicity of infection (about one), are inoculated with BVDV strain Oregon C24V, overlaid with serum-free medium and incubated for 24 hours at 37°C.

ii) The cells are scraped off and pelleted. The supernatant medium is discarded. The pellet is treated with two volumes of 2% OGP in PBS for 15 minutes at 4°C, and centrifuged to remove the cell debris. The supernatant antigen is stored in small aliquots at −70°C, or freeze-dried. Non-infected cells are processed in parallel to make a control antigen.

iii) The antigen is diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6. Alternate rows of an ELISA-grade microtitre plate are coated with virus and control antigens overnight at 4°C. The plates are then washed in PBS with 0.05% Tween 20 or Tween 80 (PBST) before use in the test.

iv) Test sera are diluted 1/50 in serum diluent (0.5 M NaCl; 0.01 M phosphate buffer; 0.05% Tween 20; 0.001 M ethylene diamine tetra-acetic acid; 1% polyvinyl pyrrolidone, pH 7.2) and added to virus- and control-coated wells for 1 hour at 37°C. The plates are then washed five times in PBST.

v) Rabbit anti-bovine IgG peroxidase conjugate is added at a predetermined dilution (in serum diluent) for 1 hour at 37°C, then the plates are again washed five times in PBST.

vi) A suitable enzyme substrate is added, such as hydrogen peroxide/tetramethyl benzidine. After colour development, the reaction is stopped with sulphuric acid and the absorbance is read on an ELISA plate reader. The value obtained with control antigen is subtracted from the test reaction to give a net absorbance value for each serum.

vii) It is recommended to convert net absorbance values to sample:positive ratio (or percentage positivity) by dividing net absorbance by the net absorbance on that test of a standard positive serum that has a net absorbance of about 1.0. This normalisation procedure leads to more consistent and reproducible results.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Infection via the oropharynx and respiratory tract is probably the most important route of transmission of BVDV on farms. Protection against spread in this way would have a beneficial effect on controlling disease due to the
Chapter 2.10.6. – Bovine viral diarrhoea

virus, particularly in the young animal. The formulation of a vaccine that will provide protection to the fetus is also required in order to prevent the wide range of syndromes that result from in utero infection (12).

A standard vaccine for protection against infection has not yet been developed, but a number of commercial preparations are available in, for example, Europe and North America. Traditionally, BVD vaccines have been based on a cytopathogenic strain of the virus and fall into two classes: modified live virus or inactivated vaccines.

Although live virus vaccines are available in some countries, they should be used under careful veterinary control because a cytopathogenic strain may precipitate mucosal disease by superinfection of persistently viraemic animals, while in pregnant cattle, a noncytopathogenic component of the vaccine may cross the placenta and infect the fetus as described in Section B.b. Live virus vaccine may also be immunosuppressive and precipitate other infections. On the other hand, modified live virus vaccines may only require a single dose. Properly constituted vaccines containing killed virus are safe to use but, to obtain satisfactory levels of immunity, they usually require booster vaccinations, which may be inconvenient. A combined vaccination protocol using inactivated followed by live vaccine may reduce the risk of adverse reaction to the live strain (28).

Experimental inactivated vaccines based on baculovirus-expressed BVD viral glycoprotein E2 have been described. They offer a future prospect of ‘marker vaccines’ when used in connection with a complementary serological test (14).

BVDV is particularly important as a hazard in the manufacture of biological products for other diseases because of the high frequency of contamination of batches of FCS used as a culture medium supplement (34). Particular attention should be paid to sera designed for administration to animals, or used as a growth supplement in embryo transfer or in-vitro fertilisation procedures. Serum used for such purposes should be treated so as to assure sterility. It is recommended that post-treatment tests, such as are detailed in Chapter I.1.5, be used to ensure that serum is free of BVDV.

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

An ideal vaccine should contain a strain (or strains) of virus that has been shown to give protection against the wide diversity of antigenicity that has been demonstrated by BVDV. A good appreciation of the antigenic characteristics of individual strains can be obtained by screening with panels of MAbs (56). The identity of the seed virus should be confirmed by sequencing (60).

The emergence of genotype 2 BVD has raised questions regarding the degree of protection conferred by type 1 vaccines against genotype 2. An in-vitro study of the neutralising ability of sera induced by one vaccine revealed broad reactivity with diverse strains from Europe and the USA, including type 2 strains (33). Other work has shown that vaccine derived from one genotype can afford a degree of protection from the other (17, 18, 47). However, the efficacy of vaccination of whatever genotype, particularly with a killed vaccine, in preventing transplacental transmission is less predictable, as viraemia is rarely completely prevented.

Isolates of cytopathic virus are often mixed with the noncytopathic biotype. The separation and purification of the two biotypes from an initial mixed culture depends on either three cycles of a limiting dilution technique for the noncytopathogenic virus, or three cycles of plaque selection for the cytopathogenic virus. Purity of the cytopathogenic virus should be confirmed by at least one additional passage at limiting dilution. When isolates have been cloned, their identity should be confirmed by direct or indirect staining with specific antibody linked to fluorescein or enzyme.

b) Method of culture

Both biotypes will grow in a variety of cell cultures of bovine origin. Standard procedures may be used, with the expectation for harvesting noncytopathogenic virus on days 5–7 and cytopathogenic virus on days 2–4. The details for optimal yield depend on several factors, including the cell culture and isolate used and the initial seeding rate of virus (38).

c) Validation as a vaccine

All vaccines should pass standard tests for safety and efficacy. It is crucial to ensure that the cell cultures and fetal bovine serum included in culture medium be free from adventitious BVDV and antibody (described
in Section B), and other microorganisms. Live vaccines must either be demonstrated to be safe in pregnant cattle (i.e. no transmission to the fetus), or should be licensed with a warning not to use them in pregnant animals. Live vaccines containing cytopathogenic strains should have an appropriate warning of the risk of inducing mucosal disease in persistently infected cattle.

Efficacy tests of BVD vaccines in non-pregnant cattle are limited by the difficulty of establishing a satisfactory challenge model. Tests should include as a minimum the demonstration of seroconversion following vaccination, a reduction in virus shedding after challenge in vaccinated cattle, and a diminution in measurable clinical parameters, such as rectal temperature response and leukopenia (3, 12, 38). Vaccines intended for use in adult breeding cattle should be evaluated for their efficiency in reducing transplacental transmission, ideally achieving complete prevention. In this case, a suitable challenge system can be established by intranasal inoculation of noncytopathogenic virus into pregnant cows at under 90 days gestation (12). Usually this system will reliably produce persistently viraemic offspring in non-immune cows.

2. Method of manufacture

There is no standard method for the manufacture of a BVD vaccine, but conventional laboratory techniques with stationary, rolled or suspension (micro-carriers) cell cultures may be used. Inactivated vaccines can be prepared by conventional methods, such as binary ethylenimine or beta-propiolactone inactivation (38, 53). A variety of adjuvants may be used (38, 51).

3. In-process control

Cultures should be inspected regularly to ensure that they are free from contamination, and to monitor the health of the cells and the development or absence of CPE, as appropriate.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter I.1.5.

b) Safety

It is essential that all the infectivity be removed during preparation of an inactivated vaccine, and samples should be subjected to several passages in cell culture to ensure the absence of live BVDV. It may also be necessary to ensure the absence of various proscribed agents (prior to inactivation) before use of the vaccine is permitted.

c) Potency

Ideally, the potency of the vaccine should be determined by inoculation into seronegative and virus negative calves, followed by monitoring of the antibody response; however, this is prohibitively expensive for batch control. Antigen content can be assayed by ELISA and adjusted as required to a standard level for the particular vaccine (3, 46). Standardised assay protocols applicable to all vaccines do not exist. Live vaccine batches may be assayed by infectivity titration.

d) Duration of immunity

There are few published data on the duration of antibody following vaccination with a commercial product. Protocols for their use usually recommend a primary course of two inoculations and boosters at yearly intervals. Only limited data are available on the antibody levels that correlate with protection against respiratory infections (6, 37) or in utero infection (12).

e) Stability

There are no accepted guidelines for the stability of BVD vaccines, but it can be assumed that attenuated virus vaccine (freeze-dried) should remain potent for at least 1 year if kept at 4°C. Inactivated virus vaccine could have a longer shelf life at 4°C. Lower temperatures could prolong shelf life for either type, but adjuvants in killed vaccine may preclude this.

f) Precautions

BVDV is not considered to be a human health hazard. Standard good microbiological practice should be adequate for handling the virus in the laboratory.
5. Tests on the final product

a) Safety tests
The safety of the final product formulation of both live and inactivated vaccines should be assessed in susceptible calves for any local reactions following administration, and in pregnant cattle for their effects on the unborn calf. Tests for individual batches are described in Section C.4.b.

b) Potency tests for antigenicity
BVD vaccines must be demonstrated to produce adequate immune responses, as outlined in Section C.4.c. above, when used in their final formulation according to the manufacturer’s published instructions. *In-vitro* assays (Section C.4.c.) may be used to monitor individual batches.

**REFERENCE**


* * *

NB: There is an OIE Reference Laboratory for Bovine viral diarrhoea (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 2.10.7.
WEST NILE ENCEPHALITIS

SUMMARY

West Nile virus (WNV) is a member of the genus Flavivirus in the family Flaviviridae. The arbovirus is maintained in nature by cycling through birds and mosquitoes; numerous avian and mosquito species support virus replication. For many avian species, WNV infection causes no overt signs while other birds, such as American crows (Corvus brachyrhynchos) and Blue Jays (Cyanocitta cristata), succumb to fatal systemic illness. Among mammals, clinical disease is primarily exhibited in horses and humans.

Clinical signs of WNV infection in horses arise from viral-induced encephalitis or encephalomyelitis. Infections are dependent on mosquito transmission and are seasonal in temperate climates, peaking in the early autumn in the Northern Hemisphere. Affected horses frequently demonstrate mild to severe ataxia. Signs can range from slight incoordination to recumbency. Some horses exhibit weakness, muscle fasciculation, and cranial nerve deficits. Fever is not a consistently recognised feature of the disease in horses.

Identification of the agent: Bird tissues generally contain higher concentrations of virus than equine tissues. Brain and spinal cord are the preferred tissues for virus isolation from horses. In birds, kidney, heart, brain or intestine can yield virus isolates. Cell cultures (using, for example, rabbit kidney or Vero cells) are used most commonly for virus isolation. WNV is cytopathic in susceptible culture systems. Viral nucleic acid and viral antigens can be demonstrated in tissues of infected animals by reverse-transcription polymerase chain reaction (RT-PCR) and immunohistochemistry, respectively. The most sensitive method for identifying WNV in equine tissues is a nested format of the RT-PCR procedure.

Serological tests: Antibody can be identified in equine serum by IgM capture enzyme-linked immunosorbent assay (IgM capture ELISA), haemagglutination inhibition (HI), IgG ELISA or plaque reduction neutralisation (PRN). The HI and PRN methods are most commonly used for identifying WN antibody in avian serum. In some serological assays, antibody cross-reactions with related flaviviruses, such as St Louis encephalitis virus or Japanese encephalitis virus, may be encountered.

Requirements for vaccines and diagnostic biologicals: A formalin-inactivated WNV vaccine derived from tissue culture and a WNV live canarypoxvirus vectored vaccine are available for use in horses.

A. INTRODUCTION

West Nile virus (WNV) is a zoonotic mosquito-transmitted arbovirus belonging to the genus Flavivirus in the family Flaviviridae. The genus Flavivirus also includes Japanese encephalitis virus (see Chapter 2.5.14.), St Louis encephalitis virus, and Kunjin virus, among others (5). WNV has a wide geographical range that includes portions of Europe, Asia, Africa, Australia and North America. Migratory birds are thought to be responsible for virus dispersal, including reintroduction of WNV from endemic areas into regions that experience sporadic outbreaks (5). WNV is maintained in a mosquito–bird–mosquito transmission cycle, whereas humans and horses are considered dead end hosts. Genetic analysis of WN isolates separates strains into two clades. Lineage 1 isolates are found in northern and central Africa, Israel, Europe, India, Australia (Kunjin virus) and North America. Lineage 2 strains are endemic in central and southern Africa and Madagascar, with co-circulation of both virus lineages in central Africa (2, 6). While recent human and equine outbreaks have been due to lineage 1 viruses, strains from each lineage have been implicated in human and animal disease.
Chapter 2.10.7. – West Nile encephalitis

WNV was recognised as a human pathogen in Africa during the first half of the 20th century. Although several WN fever epidemics were described, encephalitis as a consequence of human WN infection was rarely encountered prior to 1996, but since then, outbreaks of human West Nile encephalitis have been reported from Romania, Russia, Israel and North America (3, 10, 11). During the 1960s, West Nile encephalitis of horses was reported from Egypt and France (17, 19). Since 1998, outbreaks of equine WN encephalitis have been reported from Italy, France, and North America (7, 14, 15). In North America, from 1999 to 2002, the virus range dramatically expanded from a discrete region along the East Coast of New York State to include most of continental United States of America (USA), and Canada, with increasing numbers of horses and wild birds affected each year (15, 16, 22).

The incubation period for equine WN encephalitis following mosquito transmission is estimated to be 3–15 days. A fleeting viraemia of low virus titre precedes clinical onset (4, 19). WN encephalitis occurs in only a small per cent of infected horses; the majority of infected horses do not display clinical signs (15). The disease in horses is frequently characterised by mild to severe ataxia. Additionally, horses may exhibit weakness, muscle fasciculation and cranial nerve deficits (7, 15, 16, 20). Fever is an inconsistently recognised feature. Treatment is supportive and signs may resolve or progress to terminal recumbency. The mortality rate is approximately one in three clinically affected horses. Differential diagnoses include other arboviral encephalidites (e.g. eastern, western or Venezuelan equine encephalomyelitis, Japanese encephalitis), equine protozoal myelitis (Sarcocystis neurona), equine herpesvirus-1, Borna disease and rabies.

Although many species of birds, including chickens, are resistant to disease, the outcome of infection is generally fatal in susceptible birds. Birds may exhibit neurological signs prior to death (21). WN has been associated with sporadic disease in small numbers of other species, including squirrels, chipmunks, bats, dogs, cats, reindeer, sheep, alpacas, alligator and a harbour seal during intense periods of local viral activity. Most human infections occur by natural transmission from mosquitoes, but laboratory acquired infections have been reported. In clinically suspicious cases, diagnostic specimens from all animals, particularly birds, should be handled at containment level 3 following appropriate laboratory procedures (see Chapter I.1.6. Human safety in the veterinary microbiology laboratory) (18).

Due to the occurrence of inapparent WN infections, diagnostic criteria must include a combination of clinical assessment and laboratory tests.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Culture

Specimens for virus isolation include brain and spinal cord from encephalitic horses (15, 16); a variety of bird tissues including heart, brain or intestine may be used with success (21). In general, virus isolates are obtained more easily from avian specimens. Virus may be propagated in susceptible cell cultures, such as rabbit kidney (RK-13) and African green monkey kidney (Vero) cells, or embryonating chicken eggs. Intracerebral inoculations of newborn mice are less likely to yield virus isolates from mammalian tissues than cell culture methods. More than one cell culture passage may be required to observe cytopathic effect (CPE). Confirmation of WNV isolates is achieved by indirect fluorescent antibody staining of infected cultures or nucleic acid detection methods (see below).

b) Immunological methods

Immunohistochemical (IHC) staining of formalin-fixed avian tissues is a reliable method for identification of WN infection in birds. The specificity of identification (e.g. flavivirus specific or WN specific) depends on the selection of detector antibody. The brain and spinal cord tissues of horses with WN encephalitis are inconsistently positive in IHC tests; approximately 50% of equine WN encephalitis cases yield false-negative results. Failure to identify WNV antigen in equine central nervous system does not rule out infection.

c) Nucleic acid recognition methods

Nucleic acid detection by reverse-transcription polymerase chain reaction (RT-PCR) significantly enhances the identification of WN-infected tissues, particularly when a nested PCR approach is applied to fresh, unfixed, equine brain and spinal cord specimens (12). The RT-nested PCR method to detect WNV nucleic acid encoding a portion of the E gene is described below. This method was developed using a 1999 North American isolate and has been successful in detecting WNV RNA in animal tissues during recent North American outbreaks. St Louis encephalitis virus is not detected by this method. Lineage 1 West Nile viruses from China (People’s Rep. of), France, Egypt, Israel, Italy, Kenya, Mexico and Russia demonstrate a highly
conserved nucleotide sequence in the target region, regardless of species of origin. Analysis of sequence information for the Uganda 1937 Lineage 2 strain (GenBank M12294) in the region targeted by the PCR primers indicate that amplification of lineage 2 strains of WNV would not be expected. Other viruses from the Japanese encephalitis serogroup have not been examined. Non-nested methods, including real-time PCR, pose less risk of laboratory cross-contamination and may be applied successfully to avian tissue samples (13). Tissues selected for PCR are the same as those selected for virus isolation attempts.

- **Reverse-transcription nested polymerase chain reaction (RT-nPCR) procedure**

The RT-nPCR described here includes several procedures: extraction of RNA, reverse transcription to generate DNA from RNA and first stage PCR, second stage PCR using ‘nested’ primers and, finally, detection of the appropriately sized amplicon by gel electrophoresis. WNV protein gene regions of 445 bp (base pairs) and 248 bp are amplified in the first-stage and nested procedures, respectively. The kits and reagents described below are provided as examples. Equivalent products may be available from other sources. Extreme care in handling all materials and inclusion of proper controls are essential to ensure accurate results. The precautions to be taken have been covered in Chapter I.1.4, Validation and Quality Control of Polymerase Chain Reaction Methods used for the Diagnosis of Infectious Diseases. Duplicate samples of each diagnostic specimen should be processed and tested to increase confidence in test results. Use appropriate precautions when handling hazardous reagents such as ethidium bromide.

- **Extraction of viral RNA**

From 50 to 100 mg of tissue, extract total RNA using Trizol® reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer’s instructions. Also extract total RNA from WNV control stock virus containing 10–100 tissue culture infective dose (TCID<sub>50</sub>) per 100 µl volume.

- **Reverse transcription and first stage PCR**

  First stage primers:
  1401: 5'-ACC-AAC-TAC-TGT-GGA-GTC-3'
  1845: 5'-TTC-CAT-CTT-CAC-TCT-ACA-CT-3'

  i) Suspend extracted RNA samples in 12 µl RNase-free water.
  ii) Denature at 70°C for 10 minutes.
  iii) Add 2 µl of each denatured RNA sample to 48 µl of RT-PCR mixture containing a final composition of:
      10 mM Tris/HCl, pH 8.3
      50 mM KCl
      2.0 mM MgCl<sub>2</sub>
      0.8 mM deoxynucleoside triphosphate (dNTP) pool
      25 units M-MLV (Moloney murine leukaemia virus) RT
      1.25 units RNase inhibitor
      1.25 units AmpliTaq Gold™ (Applied Biosystems, Foster City, CA, USA)
      37.5 pmol of the first stage primers.

      Include ‘no RNA’ controls using 2 µl RNase-free water in place of denatured RNA.
  iv) Incubate reaction tubes at 45°C for 45 minutes.
  v) Incubate reactions tubes at 95°C for 11 minutes.
  vi) PCR amplification through 35 cycles:
      Denaturation at 95°C for 30 seconds,
      Primer annealing at 55°C for 45 seconds,
      Primer extension at 72°C for 60 seconds (for the 35th cycle, primer extension at 72°C for 5 minutes).
  vii) Hold samples at 4°C until second stage PCR.

- **Second stage (nested) PCR**

  Second stage primers:
  1485: 5’-GCC-TTC-ATA-CAC-ACT-AAA-G-3’
  5’-CCA-ATG-CTA-TCA-CAG-ACT-3’

  i) For each sample and control, add 1.5 µl of the first-stage amplification product to 48.5 µl of PCR mixture with a final composition of:
      10 mM Tris/HCl, pH 8.3
      50 mM KCl
      2.0 mM MgCl<sub>2</sub>
      0.8 mM deoxynucleoside triphosphate (dNTP) pool
      1.25 units AmpliTaq Gold™ (Applied Biosystems, Foster City, CA, USA)
      37.5 pmol each of the nested primers.
ii) Incubate reactions tubes at 95°C for 11 minutes.

iii) PCR amplification through 35 cycles:
- Denaturation at 95°C for 30 seconds,
- Primer annealing at 55°C for 45 seconds,
- Primer extension at 72°C for 60 seconds (for the 35th cycle, primer extension at 72°C for 5 minutes).

iv) Hold samples at 4°C or −20°C until electrophoresis.

• Analysis of PCR products by gel electrophoresis
i) Prepare a 2.5% NuSieve® 3/1 (FMC Bioproducts, Rockland, Maine, USA) agarose solution in 0.045 mM Tris/borate, pH 8.6, 1.5 mM EDTA (ethylene diamine tetra-acetic acid) (1 TBE buffer). Boil the agarose on a hotplate or in a microwave oven until completely dissolved. Cool the agarose to 45–55°C. Add 5.0 µl ethidium bromide solution (10 mg/ml) per 100 ml warm agarose and pour agarose gel with comb. Allow to solidify then remove comb.

ii) Add 30 µl ethidium bromide solution (10 mg/ml) per 600 ml 1 TBE tank buffer. Position gel in apparatus and fill buffer tanks.

iii) Mix 15 µl of each sample and control with 5 µl gel loading solution (e.g. Sigma product G-2526, St Louis, MO, USA) Include 100 bp DNA ladder (e.g. Life Technologies, Grand Island, NY, USA product 15268-019, range 100–1500 bp) in at least one gel well. Load samples into agar wells and electrophorese at 65–75 volts until the gel loading dye has travelled approximately two-thirds the length of the gel.

iv) Visualise and photograph gel under ultraviolet illumination.

• Interpretation of the test
For the PCR test to be valid, the positive controls must show the appropriate size band (248 bp). The ‘no RNA’ controls should have no bands. Samples are considered to be positive if there is a band of the same size as the positive control. Duplicate samples should both show the same reaction. If there is a disparity, the test should be repeated, starting with extraction from tissue. If further validation is required, the final nested PCR product can be sequenced and compared with the published sequences of WNV from GenBank.

2. Serological tests
Antibody can be identified in equine serum by IgM capture enzyme-linked immunosorbent assay (IgM capture ELISA), hemagglutination inhibition (HI), IgG ELISA or plaque reduction neutralisation (PRN) (1, 9). The IgM capture ELISA described below is particularly useful to detect antibodies resulting from recent natural exposure to WNV. Equine WN-specific IgM antibodies are usually detectable from 7–10 days post-infection to 1–2 months post-infection. Most horses with WN encephalitis test positive in the IgM capture ELISA at the time that clinical signs are first observed. WNV neutralising antibodies are detectable in equine serum by 2 weeks post-infection and can persist for more than 1 year. The HI and PRN methods are most commonly used for identifying WN antibody in avian serum. In some serological assays, antibody cross-reactions with related flaviviruses, such as St Louis encephalitis virus or Japanese encephalitis virus, may be encountered. The PRN test is the most specific among WN serological tests; when needed, serum antibody titres against related flaviviruses can be tested in parallel. Finally, WN vaccination history must be considered in interpretation of serology results, particularly in the PRN test and IgG ELISA. IgM capture ELISA may be used to test avian or other species provided that species-specific capture antibody is available (e.g. anti-chicken IgM). The PRN test is applicable to any species, including birds.

a) Equine IgM capture ELISA
WNV and normal antigens for the IgM capture ELISA may be prepared from mouse brain (see Chapter 2.5.3), tissue culture or recombinant cell lines (8). Commercial sources of WNV testing reagents are available in North America. Characterised equine control serum, although not an international standard, can be obtained from the National Veterinary Services Laboratories, Ames, Iowa, USA. Virus and control antigens should be prepared in parallel for use in the ELISA. Antigen preparations must be titrated with control sera to optimise sensitivity and specificity of the assay. Equine serum samples are tested at a dilution of 1/400 and equine cerebrospinal fluid samples are tested at a dilution of 1/2 in the assay. To ensure specificity, each serum sample is tested for reactivity with both virus antigen and control antigen.

• Test procedure
i) Coat flat-bottom 96-well ELISA plates (e.g. Immulon 2HB, Dynex Technologies, Chantilly, VA, USA) with 100 µl/well anti-equine IgM diluted in 0.5 M carbonate buffer, pH 9.6, according to the manufacturer’s suggested dilution for use as a capture antibody.
ii) Incubate plates overnight at 4°C in a humid chamber. Coated plates may be stored for several weeks.

iii) Prior to use, wash plates twice with 200–300 µl/well 0.01 M phosphate buffered saline, pH 7.2, containing 0.05% Tween 20 (PBST).

iv) Block plates by adding 300 µl/well freshly prepared 5% nonfat dry milk in PBST and incubate 60 minutes at room temperature. After incubation, remove blocking solution and wash plates three times with PBST.

v) Test and control sera are diluted 1/400 (cerebrospinal fluid is diluted 1/2) in PBST and 50 µl/well of each sample is added to duplicate sets of wells (total of four wells per sample) on the plate. Include control positive and negative sera prepared in the same manner as samples.

vi) Cover the plates and incubate 75 minutes at 37°C in a humid chamber.

vii) Remove serum and wash plates three times in PBST.

viii) Dilute virus and normal antigens in PBST and add 50 µl of virus antigen to one set of wells per test and control sera and add 50 µl normal antigen to the second set of wells per test and control sera.

ix) Cover the plates and incubate overnight at 4°C in a humid chamber.

x) Remove antigens from the wells and wash the plates three times in PBST.

xi) Dilute horseradish peroxidase conjugated anti-Flavivirus monoclonal antibody¹ in PBST according to manufacturer’s directions and add 50 µl per well.

xii) Cover the plates and incubate at 37°C for 60 minutes.

xiii) Remove conjugate and wash plates six times in PBST.

xiv) Add 50 µl/well freshly prepared ABTS (2,2’-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) substrate with hydrogen peroxide (0.1%) and incubate at room temperature for 30 minutes.

xv) Measure absorbance at 405 nm. A test sample is considered to be positive if the absorbance of the test sample in wells containing virus antigen is at least twice the absorbance of negative control serum in wells containing virus antigen and at least twice the absorbance of the sample tested in parallel in wells containing control antigen.

b) Plaque reduction neutralisation (applicable to serum from any species)

The PRN test is performed in Vero cell cultures in either 25 cm² flasks or 6-well plates. The sera can be screened at a 1/10 and 1/100 final dilution or may be titrated to establish an endpoint. A description of the test as performed in 25 cm² flasks using 100 plaque-forming units (PFU) of virus is as follows.

Prior to testing, serum is heat inactivated at 56°C for 30 minutes and diluted (e.g. 1/5 and 1/50) in media. Virus (200 PFU per 0.1 ml) working dilution is prepared in media containing 10% guinea-pig complement. Equal volumes of virus and serum are mixed and incubated at 37°C for 75 minutes before inoculation of 0.1 ml on to confluent cell culture monolayers. The inoculum is adsorbed for 1 hour at 37°C, followed by the addition of 4.0 ml of primary overlay medium. The primary overlay medium consists of two solutions that are prepared separately. Solution I contains 2 × Earle’s Basic Salts Solution without phenol red, 4% fetal bovine serum, 100 µg/ml gentamicin and 0.45% sodium bicarbonate. Solution II consists of 2% Noble agar that is sterilised and maintained at 47°C. Equal volumes of solutions I and II are adjusted to 47°C and mixed together just before use. The test is incubated for 72 hours at 37°C. A second 4.0 ml overlay prepared as above, but also containing 0.003% neutral red is applied to each flask. Following a further overnight incubation at 37°C, the number of virus plaques per flask is assessed. Endpoint titres are based on 90% reduction compared with the virus control flasks, which should have about 100 plaques.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

In February 2003, the United States Department of Agriculture (USDA) issued a license for a formalin-inactivated WNV vaccine derived from tissue culture for use in horses. As recently as December 2003, the USDA licensed a live canarypoxvirus vectored WNV vaccine for use in horses. These vaccines have demonstrated sufficient efficacy and safety in adequately vaccinated horses. Vaccination may be helpful in preventing neurological signs associated with WN infection.

¹ Available from the Centers for Disease Control and Prevention, Biological Reference Reagents, 1600 Clifton Road NE, Mail Stop C21, Atlanta, Georgia, 30333, USA.
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 isolate of WNV used for vaccine production must be accompanied by documentation describing its origin and passage history. The isolate must be safe in host animals at the intended age of vaccination and provide protection after challenge.

   b) **Method of culture**

      The WNV should be propagated in cell lines known to support the growth of WNV. Cell lines should be free from extraneous viruses, bacteria, fungi, and mycoplasma. Viral propagation should not exceed five passages from the master seed virus (MSV), unless further passages prove to provide protection in the host animal.

   c) **Validation as a vaccine**

      The MSV should be free from bacteria, fungi and mycoplasma. The MSV must be tested for and be free of extraneous viruses, including equine herpesvirus, equine adenovirus, equine viral arteritis virus, bovine viral diarrhoea virus, reovirus, and rabies virus by the fluorescent antibody technique. The MSV must be free from extraneous virus by CPE and haemadsorption on the Vero cell line and an embryonic equine cell type.

      In an immunogenicity trial, the MSV at the highest passage level intended for production must protect susceptible horses against a virulent challenge strain. A statistically significant number of vaccinated horses must be protected from viraemia when compared with the controls. Field trial studies should be conducted to determine the safety of the vaccine.

2. **Method of manufacture**

   The susceptible cell line is seeded into suitable vessels. Minimal essential medium, supplemented with fetal bovine serum (FBS), is used as the medium for production. Incubation is at 37°C.

   Cell cultures are inoculated directly with WN working virus stock, which is generally from 1 to 4 passages from the MSV. Inoculated cultures are incubated for 1–8 days before harvesting the culture medium. During incubation, the cultures are observed daily for CPE and bacterial contamination.

   Killed virus vaccines are chemically inactivated with either formalin or binary ethylenimine and mixed with a suitable adjuvant.

3. **In-process control**

   Production lots of WN must be titrated in tissue culture for standardisation of the product. Low-titred lots may be concentrated or blended with higher-titred lots to achieve the correct titre.

4. **Batch control**

   Final container samples are tested for purity, safety and potency.

   a) **Purity**

      Samples are examined for bacterial and fungal contamination. To test for bacteria, ten vessels, each containing 120 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The ten vessels are incubated at 30–35°C for 14 days and observed for bacterial growth. To test for fungi, ten vessels, each containing 40 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The vessels are incubated at 20–25°C for 14 days and observed for fungal growth.

   b) **Safety**

      Safety tests can be conducted in a combination of guinea-pigs, mice or horses. Field safety studies should be conducted before the vaccine receives final approval. Generally, two serials should be used, in three
different geographical locations, and a minimum of 600 animals. About one-third of the animals should be at
the minimum age recommended for vaccination (correlated to efficacy). If the final product is a modified-live
vaccine, additional safety testing of the MSV is required to demonstrate a lack of virulence.

c) **Potency**

Killed virus vaccines may use host animal or laboratory animal vaccination-serology tests or
vaccination/challenge tests to determine potency of the final product. Parallel-line assays using ELISA
antigen-quantifying techniques to compare a standard with the final product are acceptable in determining
the relative potency of a product. The standard should be shown to be protective in the host animal (22).
Live viral products are titred in cell cultures to determine the potency of the final product. The final release
potency titre should include an additional 0.7 log\(_{10}\) for test variability and 0.5 log\(_{10}\) for end-of-dating stability
than the minimum protective dose established in the immunogenicity trial.

d) **Duration of immunity**

Duration of immunity studies are conducted before the vaccine receives final approval. The duration should
be for the length of the mosquito season in the infected areas.

e) **Stability**

All vaccines are initially given 24 months before expiry. Real-time stability studies are conducted to confirm
the appropriateness of all expiration dating.

f) **Preservatives**

Antibiotics are added during production, generally gentamicin sulphate or neomycin not to exceed 30 µg/ml.

g) **Precautions (hazards)**

Vaccination is only recommended for horses in WN-positive areas. Vaccinated horses may develop a
serological titre that may interfere with the ability to export the horse.

5. **Tests on the final product**

a) **Safety**

See Section C.4.b.

b) **Potency**

See Section C.4.c.

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NB: There is an OIE Reference Laboratory for West Nile 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 2.10.8.

CAMPYLOBACTER JEJUNI AND CAMPYLOBACTER COLI

SUMMARY

Campylobacter jejuni and C. coli are the most frequently isolated Campylobacter species in infected and diseased humans and can be transmitted from animals to humans directly from contact with animals or through consumption and handling of animal food products.

Both C. jejuni and C. coli commonly colonise the intestinal tract of most mammals and birds (especially chickens, turkeys and ducks). This colonisation is generally persistent, sometimes with intermittent shedding, and usually without clinical signs. The faecal contamination of meat (especially poultry meat) during processing is considered to be a major source of human food-borne disease. Campylobacter colonisation in young companion and food production animals (kittens, puppies, piglets, lambs and calves), in some small mammals used for experimental studies (ferrets and rats) and in some avian species may be associated with intestinal disease, but whether Campylobacter is the causative agent is not clear. In humans, extraintestinal infections, including bacteraemia, can occur and some sequelae of infection, such as polyneuropathies, though rare, can be serious. The prevalence of such complications in animals is unknown. Both C. jejuni and C. coli, like C. fetus, can also be isolated from bovine and ovine aborted fetuses, presumably as a consequence of translocation across the intestinal mucosa or by ascending infections.

Isolation of the agent: In mammals and birds, detection of intestinal colonisation is based on the isolation of the organism from faeces, rectal swabs and/or caecal contents. In the case of aborted fetuses, Campylobacter may be isolated from fetal stomach, placental and internal organs. Contamination of animal food products is detected by isolation of Campylobacter directly or after selective enrichment. Polymerase chain reaction based methods are described for the detection of Campylobacter in faecal/intestinal samples and from enriched meat samples.

Identification of the agent: Campylobacter jejuni and C. coli are thermophilic, Gram-negative, highly motile bacteria that, for optimal growth, require microaerobic conditions at 37–42°C. Agar media containing selective antibiotics are required to isolate these bacteria from faecal/intestinal samples and from enriched meat samples. Alternatively, their high motility can be exploited using filtration techniques for isolation. Enrichment techniques may be required for organisms that have been damaged by environmental stresses, such as temperature changes, dehydration, atmospheric oxygen, nutrient deprivation and osmotic shock, e.g. during sample transportation. Enrichment techniques can also be used to detect campylobacters when present in low numbers (e.g. on meat samples). Preliminary confirmation of isolates can be made by light microscopy. Characteristic organisms in the log growth phase are short S-shaped, Gram-negative bacteria. In older cultures, coccoid forms predominate. Under phase-contrast microscopy the organisms have a characteristic rapid corkscrew-like motility. Phenotypic identification is based on reactions under different growth conditions. Biochemical and molecular tests can be used to confirm various Campylobacter species. Generally, C. jejuni can be distinguished from all other campylobacters by the hydrolysis of hippurate.

There are several phenotypic methods for subtyping C. jejuni and C. coli including serotyping and phage typing, but the reagents required for these techniques have restricted availability. Recently, molecular-typing methods have been developed and are now extensively used for epidemiological purposes. There are no obvious relationships between subtypes and pathogenicity.
Serological tests: Colonised birds and mammals, as well as infected humans, develop circulating antibody responses to C. jejuni/coli. There are no validated serological tests for detection of Campylobacter infections, but simple preparations of complex antigens including acid-extractable surface proteins can be used in an enzyme-linked immunosorbent assay.

Requirements for vaccines and diagnostic biologicals: There are no effective vaccines available for the prevention of enteric Campylobacter infections in birds or mammals.

A. INTRODUCTION

The genus Campylobacter comprises a number of species (34). Members of the genus are typically Gram-negative, non-spore-forming, S-shaped or spiral shaped bacteria (0.2–0.8 μm wide and 0.5–5 μm long), with single polar flagella at one or both ends, conferring a characteristic corkscrew-like motility. These bacteria are microaerophilic, but some can also grow aerobically or anaerobically. They neither ferment nor oxidise carbohydrates. Some species, particularly C. jejuni, C. coli and C. lari, are thermophilic, growing optimally at 42°C. They can colonise mucosal surfaces, usually the intestinal tract, of most mammalian and avian species tested. The species C. jejuni comprises two subspecies (C. jejuni subsp. jejuni and C. jejuni subsp. doylei) that can be discriminated on the basis of nitrate reduction (subsp. doylei does not reduce nitrate). Subspecies jejuni is much more frequently isolated than subspecies doylei. Campylobacter jejuni and C. coli are major pathogenic species of interest because they are zoonotic agents. This chapter will, therefore, focus on these species unless otherwise stated. Campylobacter fetus, the causal agent of bovine genital campylobacteriosis, is reviewed in Chapter 2.3.2.

In susceptible humans, C. jejuni/coli infection is associated with acute enteritis and abdominal pain lasting for up to 7 days or more. Although such infections are generally self-limiting, complications can arise and may include bacteraemia, Guillain–Barré syndrome, reactive arthritis, and abortion (29).

Campylobacters are the main cause of human bacterial intestinal disease identified in many industrialised countries (32). Over 80% of cases are caused by C. jejuni and about 10% of cases are caused by C. coli. Other Campylobacter species, such as C. concisus, C. upsaliensis, C. lari, and C. fetus, may also be associated with human diarrhoea. The detection of such campylobacters is uncommon in the industrialised world but more common in the developing world (17). As the incidence of infection in humans in industrialised countries is estimated at about 1% of the population per annum, the social and economic burden of this disease is significant (12). The primarily source of C. jejuni/coli infections in human is believed to be the handling and/or consumption of contaminated meat, especially poultry meat. However, contact with pets and livestock, the consumption of contaminated water or raw milk and travelling in high prevalence areas are also considered risks factors in human disease (12). The control of Campylobacter in the food chain has now become a major target of agencies responsible for food safety world-wide.

Campylobacter jejuni and C. coli are generally considered commensals of livestock, domestic pet animals and birds. However, they have also been associated with disease in a range of hosts. To what extent Campylobacter is the causative agent of disease or whether it can colonise better under certain conditions, e.g. watery gut contents, is not clear. In cats and dogs, especially young animals or animals under stress, C. jejuni is associated with diarrhoea (11, 33), and this is a well-recognised source of human infection (32). Dogs and cats are also frequently colonised by C. upsaliensis (dogs) and C. helveticus (cats) (3). Outbreaks of Campylobacter-associated enteritis have been reported in some animals including breeding groups of non-human primates and even small laboratory-reared mammals. Large numbers of Campylobacter have been isolated from young livestock, including piglets, lambs and calves, with enteritis, but the organisms are also found in healthy animals. In birds, especially poultry, disease is rare, if it occurs at all, despite high levels of colonisation with C. jejuni or C. coli. Outbreaks of avian hepatitis have been reported, but the pathogenic role of Campylobacter spp. is uncertain. One possible exception is ostriches where Campylobacter-associated death and enteritis occurs in young birds. Campylobacter spp. are frequently found in wild birds (7, 36).

Campylobacter jejuni, C. coli, C. hyointestinalis and C. sputorum, as well as C. fetus (Chapter 2.3.2), may also be associated with infections of the reproductive tract (21). In cattle, all these strains can be associated with abortion. In sheep up to 20% of Campylobacter-associated abortions are due to C. jejuni or C. coli. Such infections are presumably a consequence of translocation from the gastrointestinal tract or via an ascending route.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The existing ISO (International Organization for Standardization) procedure for the horizontal method for detection of thermostolerant Campylobacter in food and animal feeding stuffs (13) is currently being revised (14).
An additional ISO procedure for the isolation of Campylobacter from water is under development (ISO/CD 17995:2002). However, neither of these standard methods may be optimal for the isolation of campylobacters from live animals.

Samples to be collected are faecal or intestinal (caecal) samples from poultry (chickens, turkeys, laying hens, etc.) and other food-producing mammals (cattle, sheep and pigs) and domestic pets (dogs and cats). In the slaughterhouse, skin or meat samples can be collected. In sporadic cases, e.g. in young ostriches, campylobacters have been associated with enteric disease and even death. In such cases, and in aborted fetuses, campylobacters can be recovered from the intestines, liver and spleen samples taken at post-mortem.

Campylobacters are notoriously fastidious organisms to culture. Recovery from faecal material or intestines may be accomplished by:

- exploiting the rapid motility and small size of campylobacters relative to other gut flora by allowing these organisms to translocate across membrane filters on to growth agar, or
- use of agar-containing selective antibiotics, usually including various combinations of cefoperazone, amphotericin-B, trimethoprim, vancomycin, etc., and
- the thermophilic C. jejuni and C. coli recovery using the above approaches can be further enhanced by selective growth at the optimum growth temperature of 42°C, which may inhibit growth of contaminating bacteria.

a) Collection of samples

i) Poultry

Poultry is found to be colonised primarily with C. jejuni (65–95%) less often with C. coli and rarely with other species (23). Colonisation rates in broiler chickens are age-related. Most flocks are negative until 2–3 weeks of age. Once Campylobacter colonisation occurs in a broiler flock, transmission, via coprophagy, is extremely rapid and up to 100% of birds within a flock can become colonised within 72 hours. Samples from live birds, destined for the food chain, should therefore be taken as close to slaughter as possible (23). The majority of birds shed large numbers of organisms (>10^6 colony-forming units/g faeces). Campylobacters can be isolated from fresh faeces/caecal droppings or cloacal swabs. For reliable detection of Campylobacter by culture, freshly voided faeces (preferably without traces of urine) should be collected. Such samples must be prevented from drying out before culture. When swabs are used, a transport medium (like Amies, Cary Blair or Stuart) must be used.

ii) Cattle, sheep and pigs

Campylobacters are frequent colonisers of the intestine of livestock such as cattle, sheep and pigs (2, 37, 38). Cattle and sheep are found to be colonised mainly with C. jejuni, C. coli, C. hyointestinalis, and C. fetus, whereas pigs are predominantly colonised by C. coli. In young animals, the numbers are higher than in older animals. In older animals, the organisms can be intermittently detected in faeces, probably due to low numbers or due to intermittent shedding. Fresh samples have to be taken (rectal samples if possible) and they should be prevented from drying out. When swabs are used, a transport medium (like Amies, Cary Blair or Stuart) must be used.

iii) Cats and dogs

Occasionally, C. jejuni in young pets is associated with clinical disease, particularly enteritis. In such cases the number of organisms shed may be high. Dogs and cats are also frequently colonised by C. upsaliensis (dogs) and C. helveticus (cats) (3). Faecal samples should be collected fresh and should be prevented from drying out. When swabs are used, a transport medium (like Amies, Cary Blair or Stuart) must be used.

iv) Internal organs (heart, spleen, liver or stomach contents)

At post-mortem, organs are removed under aseptic conditions and sent to the laboratory on the same day.

v) Slaughterhouse samples

In poultry, the caeca are usually used for the detection of Campylobacter. They can be cut with sterile scissors from the remaining part of the intestines and submitted intact to the laboratory in a plastic bag or Petri-dish. To ascertain the status of a flock at the end of the slaughter line, skin samples (neck skin or breast skin) can be collected or whole carcass rinses can be carried out.
Samples from cattle, sheep and pigs are collected from the intestines by aseptically opening the gut wall or by taking rectal swabs. Meat samples can be collected and transported to the laboratory in a sterile bag.

*Campylobacter* in the food supply has been reviewed by Jacobs-Reitsma (15).

**b) Transportation and treatment of samples**

i) **Transport**

Campylobacters are remarkably sensitive to environmental conditions, including dehydration, atmospheric oxygen, sunlight and elevated temperature. Transport to the laboratory and subsequent processing should therefore be as rapid as possible (preferably the same day, but within at least 2 days). Samples must not be transported on dry swabs, as the campylobacters will dry out and die off quickly. A suitable transport medium increases the likelihood of retaining culturable campylobacters in swab samples. The samples must be protected from light.

No recommendation on the ideal temperature for transportation can be made, but it is clear that freezing or high temperatures can reduce viability. High temperatures (>20°C), low temperatures (<0°C) and fluctuations in temperature must be avoided. During laboratory processing, samples can be kept at room temperature for short periods (to avoid unnecessary temperature shocks). When the time between sampling and processing is longer, storage at 4 (±2)°C is advised. The procedures for shipping, outlined in Chapter 1.1.1. Sampling methods, should be followed.

ii) **Transport media**

*Swabs:* The use of transport tubes, containing media and swabs, is recommended. These are available commercially. The medium in the tubes may be Amies, a plain agar or a charcoal-based medium. The function of the medium is not growth of *Campylobacter* but protection from drying out and from the toxic effects of oxygen.

When only small amounts of faecal/caecal samples can be collected and transport tubes are not available, shipment of the specimen in transport media is recommended. Several transport media have been described: Cary-Blair, modified Cary-Blair, modified Stuart medium, Campythioglycolate medium, alkaline peptone water and semisolid motility test medium. Good recovery results have been reported using Cary-Blair (18, 28).

iii) **Treatment of samples**

On arrival at the laboratory, samples should be processed as soon as possible, preferably on the day of arrival or at the least within 3 days. To avoid temperature variation, samples should only be refrigerated when they cannot be processed on the same day, otherwise they should be kept at room temperature. When samples are submitted or kept in the laboratory at 4°C, they should be allowed to equilibrate to room temperature before processing to avoid temperature shock.

- For faecal/caecal or intestinal samples, no treatment is needed and they can be plated directly on to selective media. When the filtration method is used, a suspension (usually 1 in 10) of the faeces is made in phosphate buffered saline (PBS) and diluted so that drops of the suspension can be put on the filter.

- Caeca are aseptically opened by cutting the end with sterile scissors and material is squeezed out to be processed.

- Fetal stomach contents are inoculated directly onto a suitable culture medium. Internal organs or pieces of organs are flamed after dipping in alcohol (70%) to sterilise the surface, and then homogenised. The homogenate is inoculated on to culture medium.

- Skin samples are pooled (usually to a total amount of 25 g) and transferred to an enrichment medium. Meat samples can be incubated in an enrichment medium or they can be washed and the washing medium then added to an enrichment medium. If large volumes are used, for example carcass washings, sterile saline can be used and added to an equal volume of double-strength enrichment medium.

**c) Isolation of Campylobacter**

Isolation of *Campylobacter* from faecal/caecal or intestinal samples is usually performed by direct plating on to selective medium or by using the filtration method on nonselective agar. Enrichment is recommended to enhance the culture sensitivity of potentially environmentally stressed organisms or in the case of low levels of organisms in faeces, for example from cattle, sheep or pigs. However, enrichment from the latter samples is not carried out routinely. Skin and meat products generally need enrichment for the culture of usually low numbers of (stressed) campylobacters. After selective enrichment, the samples are subcultured on to solid selective media.
Chapter 2.10.8. — Campylobacter jejuni and Campylobacter coli

Selective media for isolation

Many media are in current use for the bacteriological culture of Campylobacter spp. A detailed description of the developments in Campylobacter detection and the variety of existing media is given by Corry et al. (9, 10). The selective media can be divided into two main groups: blood-containing media and charcoal-containing media. Blood components and charcoal serve to remove toxic oxygen derivatives. Most media are commercially available. The selectivity of the media is determined by the antibiotics used. Cefalosporins (generally cefoperazone) are used, sometimes in combination with other antibiotics (e.g. vancomycin, trimethoprim). Cycloheximide (actidione) and more often recently amphotericin B are used to inhibit yeasts and moulds (20). The main difference between the media is the degree of inhibition of contaminating flora. All the selective agents allow the growth of both C. jejuni and C. coli. There is no medium available that allows growth of C. jejuni and inhibits C. coli or vice versa. To some extent, other Campylobacter species (e.g. C. lari, C. upsaliensis, C. helveticus, C. fetus and C. hyointestinalis) are also able to grow on most media, especially at the less selective temperature of 37°C. Where required, the species of the isolated Campylobacter should be determined.

Examples of selective enrichment broths:

- Bolton broth
- Preston broth
- Exeter broth
- Park and Sanders broth
- CCDB (charcoal cefoperazone deoxycholate broth)

Examples of selective blood-containing solid media:

- Preston agar
- Skirrow agar
- Butzler agar
- Campy-cefex

Examples of charcoal-based solid media:

- mCCDA (modified charcoal cefoperazone deoxycholate agar), slightly modified version of the originally described CCDA (5, 6)
- Karmali agar or CSM (charcoal-selective medium) (17)
- CAT agar (cefoperazone, amphotericin and teicoplanin), facilitating growth of C. upsaliensis (1).

Inoculation of media

For samples that do not need enrichment, a small quantity (ca. 0.1 g) is spread directly (using a loop) on to a solid selective medium to facilitate isolation of single colonies.

For samples that need enrichment (e.g. skin and meat samples), 25 g of material is diluted 1/10 in enrichment medium. Meat samples or complete chicken carcasses can be washed with saline or PBS, after which one volume of this washing fluid is added to nine volumes enrichment medium. Larger volumes of washing fluid can be added to an equal volume of double-strength enrichment broth. When smaller meat samples are used for analysis, they can be washed with enrichment fluid, which is subsequently incubated.

For research purposes faecal/caecal swabs can be enriched. They are placed into 10 ml enrichment broth, either individually or pooled, and incubated.

Passive filtration avoids the use of selective media and is, therefore, very useful for the isolation of the more antimicrobial-sensitive Campylobacter species. This method was developed by Steele & McDermott (30). For passive filtration, faeces are mixed with PBS (approximately 1/10 dilution) to produce a suspension. Approximately 100 µl of this suspension are then carefully layered on to a 0.45 or 0.65 µm filter, which has been previously placed on top of a nonselective blood agar plate. Care must be taken not to allow the inoculum to spill over the edge of the filter. The bacteria are allowed to migrate through the filter for 30–45 minutes at 37°C or room temperature. The filter is then removed, the fluid that has passed through the filter is spread with a sterile glass or plastic spreader, and the plate is incubated microaerobically at 42°C (or at 37°C to also isolate non-C. jejuni/C. coli species).

Incubation

- Atmosphere

Microaerobic atmospheres of 5–10% oxygen, 5–10% carbon dioxide (and preferably 5–9% hydrogen) are required for optimal growth (10, 34). 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 gases are used. Gas generator kits are available from commercial sources. Variable atmosphere incubators are more suitable if large numbers of cultures are undertaken.

For enrichment, no specific atmosphere is needed when a small head space (<2 cm) in the enrichment bottle is used, provided the lid is tightly sealed.

- **Temperature of incubation**
  Media may be incubated at 37°C or 42°C, but it is common practice to incubate at 42°C to minimise growth of contaminants and to select for optimal growth of *C. jejuni/C. coli*. The fungistatic agents cycloheximide or amphotericin are added in order to prevent growth of yeasts and mould at 37°C (6). In some laboratories, incubation takes place at 41.5°C to harmonise with *Salmonella* and *Escherichia coli* O157 isolation protocols (13, 14). For enrichment, specific protocols are sometimes used in which the temperature is increased over time of incubation in order to recover sublethally injured cells.

- **Time of incubation**
  Enrichment broth is incubated for 24–48 hours and streaked on to a solid selective medium.

  *Campylobacter jejuni* and *C. coli* usually show growth on solid media within 24–48 hours at 42°C. As the additional number of positive samples obtained by prolonged incubation is very low, 48 hours of incubation is recommended for routine diagnosis (6).

v) **Identification on solid medium**
  On Skirrow or other blood-containing agars, characteristic *Campylobacter* colonies are slightly pink, round, convex, smooth and shiny, with a regular edge. On charcoal-based media such as mCCDA, the characteristic colonies are greyish, flat and moistened, with a tendency to spread, and may have a metal sheen.

d) **Confirmation**
  A pure culture is required for confirmatory tests, but a preliminary confirmation can be obtained by direct microscopic examination of suspect colony material.

  The confirmatory tests for presence of thermophilic campylobacters and the interpretation (14) are given in Table 1. Confirm results of confirmation tests using positive and negative controls.

<table>
<thead>
<tr>
<th>Confirmatory test</th>
<th>Result for thermophilic <em>Campylobacter</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Small curved bacilli</td>
</tr>
<tr>
<td>Motility</td>
<td>Characteristic (highly motile and cork-screw like)</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
</tr>
<tr>
<td>Glucose (TSI)</td>
<td>–</td>
</tr>
<tr>
<td>Lactose (TSI)</td>
<td>–</td>
</tr>
<tr>
<td>Sucrose (TSI)</td>
<td>–</td>
</tr>
<tr>
<td>Gas (TSI)</td>
<td>–</td>
</tr>
<tr>
<td>H₂S production (TSI)</td>
<td>(traces of blackening may occur in the presence of <em>C. coli</em>)</td>
</tr>
<tr>
<td>Growth at 25°C</td>
<td>–</td>
</tr>
</tbody>
</table>

TSI = triple sugar iron agar.

i) **Microscopic examination of morphology and motility**: material from a suspect colony is suspended in saline and evaluated, preferably by a phase-contrast microscope, for characteristic, spiral or curved slender rods with a corkscrew-like motility. Older cultures show less motile coccioid forms.

ii) **Detection of oxidase**: take material from a suspect colony and place it on to a filter paper moistened with oxidase reagent. The appearance of a violet or deep blue colour within 10 seconds is a positive reaction. If a commercially available oxidase test kit is used, follow the manufacturer’s instructions.

iii) **Fermentation of sugars and production of hydrogen sulphide**: triple sugar iron agar (TSI) is inoculated with longitudinal streaks on the slant and stabbing into the base of the media (the butt). Incubate microaerobically at 42°C for 24–48 hours. Interpret the results as given in Table 2.
Chapter 2.10.8. – Campylobacter jejuni and Campylobacter coli

Table 2. Interpretation of results in TSI agar

<table>
<thead>
<tr>
<th>Appearance</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butt:</td>
<td></td>
</tr>
<tr>
<td>Yellow</td>
<td>Glucose-positive (fermentation of glucose)</td>
</tr>
<tr>
<td>Red or unchanged</td>
<td>Glucose-negative (no fermentation of glucose)</td>
</tr>
<tr>
<td>Black</td>
<td>Formation of hydrogen sulphide ($H_2S$)</td>
</tr>
<tr>
<td>Bubbles or cracks</td>
<td>Gas production from glucose</td>
</tr>
<tr>
<td>Slant surface:</td>
<td></td>
</tr>
<tr>
<td>Yellow</td>
<td>Lactose and/or sucrose positive (one or both sugars used)</td>
</tr>
<tr>
<td>Red or unchanged</td>
<td>Lactose and sucrose negative (no sugars used)</td>
</tr>
</tbody>
</table>

iv) **Growth at 25°C:** Inoculate the pure culture onto a non-selective blood agar plate and incubate at 25°C in a microaerobic atmosphere for 48 hours.

v) **Latex agglutination tests** for confirmation of pure cultures of *C. jejuni/C. coli* (often also including *C. lari*) are commercially available.

e) **Identification of Campylobacter to the species level**

Among the *Campylobacter* spp. growing at 42°C, the most frequently encountered species from samples of animal origin are *C. jejuni* and *C. coli*. However, low frequencies of other species have been described. Generally, *C. jejuni* can be differentiated from other *Campylobacter* species on the basis of the hydrolysis of hippurate as this is the only hippurate-positive species. The presence of hippurate-negative *C. jejuni* strains has been reported (31). Table 3 gives some basic classical phenotypic characteristics of the most important thermophilic *Campylobacter* species (14). Sensitivity to nalidixic acid used to be one of the most commonly tested characteristics, but nowadays may give difficulties in interpretation, both due to an increase in nalidixic acid-resistant strains of *C. jejuni* and *C. coli* and to the isolation of nalidixic acid-sensitive genogroups of *C. lari*. More extensive speciation schemes have been described in the literature (25, 34). Speciation results should be confirmed using defined positive and negative controls.

Biochemical speciation may be supplemented or even replaced with molecular methods. A variety of DNA probes and polymerase chain reaction (PCR)-based identification assays have been described for *Campylobacter* species (25, 34). On *et al.* (26) evaluated the specificity of 11 PCR-based identification assays for *C. jejuni* and *C. coli*.

Table 3. Basic phenotypic characteristics of selected thermophilic Campylobacter species

<table>
<thead>
<tr>
<th>Characteristics</th>
<th><em>C. jejuni</em></th>
<th><em>C. coli</em></th>
<th><em>C. lari</em></th>
<th><em>C. upsaliensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis of hippurate</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>– or slight</td>
</tr>
<tr>
<td>Indoxyl acetate</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
</tbody>
</table>

Key: + = positive; – = negative; S = sensitive; R = resistant

i) **Detection of hippurate hydrolysis:** Suspend a loopful of growth from a suspect colony in 400 µl of a 1% sodium hippurate solution (care should be taken not to incorporate agar). Incubate at 37°C for 2 hours, then slowly add 200 µl 3.5% ninhydrin solution to the side of the tube to form an overlay. Reincubate at 37°C for 10 minutes, and read the reaction. Positive reaction: dark purple/blue. Negative reaction: clear or grey. If commercially available hippurate hydrolysis test disks are used, follow the manufacturer’s instructions.

ii) **Detection of catalase activity:** Place a suspect colony on a glass slide. Put one drop of 3% $H_2O_2$ on the bacterial material. Examine immediately for production of gas, which indicates catalase activity. The test is positive if gas bubbles appear within 30 seconds.

iii) **Detection of indoxyl acetate hydrolysis:** Place a suspect colony on an indoxyl acetate disk and add a drop of sterile distilled water. If indoxyl acetate is hydrolysed a colour change to dark blue occurs.
within 5–10 minutes. No colour change indicates hydrolysis has not taken place. If commercially available indoxyl acetate hydrolysis test disks are used, follow the manufacturer’s instructions.

iv) **Test for sensitivity to cephalothin:** Sensitivity to cephalothin is tested by the disk diffusion technique as previously described (14, 27).

f) **Molecular detection of Campylobacter**

PCR-based methods for the detection of Campylobacter in animal faecal samples and enriched meat samples have been previously described in the literature (24, 25). One of these assays is in use in Denmark for routine screening of cloacal swabs from broilers at the slaughterhouse (4, 19). There is at least one PCR test commercially available for meat samples following enrichment.

g) **Antigen-capture-based tests**

Several commercial enzyme immunoassays are available for the detection of Campylobacter in human stool samples. One of these tests has been used for chicken caecal samples (n = 142) with a sensitivity of 91% and a specificity of 64% (35). For the detection of Campylobacter in enriched food samples, there are at least two commercial assays on the market.

2. **Serological tests**

Although intestinal colonisation, whether symptomatic or not, is associated with circulating and mucosal antibody responses, there are no validated serological tests developed for the identification of infected mammals or birds. However, simple preparations of complex antigens can be used in an enzyme-linked immunosorbent assay (ELISA). The most commonly used antigens to detect antibody responses are the acid extractable (AE) surface proteins (8). These primarily comprise flagellin and a series of peripheral surface proteins called the PEB proteins. It must be noted that Campylobacter flagella contain antigenically cross-reacting epitopes with other closely related organisms such as helicobacters.

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

There are no vaccines specifically developed for C. jejuni or C. coli in animals or birds. However, an oral, monovalent, killed whole cell C. jejuni (strain 81176) vaccine, combined with a modified E. coli heat-labile toxin adjuvant, is currently under development for use in humans and such an approach might be applicable to birds and mammals (www.armymedicine.army.mil/usammda/info196.pdf).

Antisera directed against campylobacters can be produced by hyperimmunisation of rabbits, goats etc. (22). Rabbits can be immunised intramuscularly with formalin-treated (0.3% formalin for 30 minutes then washed in PBS) bacterial suspension in an appropriate adjuvant. The rabbits are boosted at 14-day intervals with a series of subcutaneous injections of the same antigen. To obtain antisera cross-reactive for multiple serotypes of C. jejuni/coli, the animals should be sequentially immunised with different strains, preferably of different serotypes. It should be noted that antisera produced in this way will cross-react with most Campylobacter species, including C. hyointestinalis and C. fetus as well as Helicobacter spp.

**REFERENCES**


Chapter 2.10.8. — Campylobacter jejuni and Campylobacter coli


*  *  *
**CHAPTER 2.10.9.**

**CRYPTOSPORIDIOSIS**

**SUMMARY**

Cryptosporidiosis is caused by protozoan parasites of the genus Cryptosporidium, in which there are 13 'valid' species. In livestock, C. parvum, C. andersoni, C. baileyi and C. meleagridis have been reported to cause morbidity and outbreaks of disease. Laboratory identification is required to confirm diagnosis. Cryptosporidium parvum cryptosporidiosis causes scour in young, unweaned mammalian livestock, however, weaned and adult animals can also become infected. Signs range from a mild to apparent infection to severe scouring, and the young, old or immunocompromised are most susceptible. Death is infrequent. Infected, weaned and adult animals normally do not exhibit identifiable signs of disease, but will excrete oocysts that contaminate the environment. Cryptosporidium andersoni cryptosporidiosis affects the digestive glands of the abomasum of older calves and adult cattle. Some infected animals exhibit reduced weight gain, but do not develop diarrhoea. Cryptosporidium baileyi, C. meleagridis and C. galli cryptosporidiosis are diseases of birds. Cryptosporidium baileyi affects primarily the bursa of Fabricius and cloaca of gallinaceous birds, while C. meleagridis affects primarily the ileum of turkey poults.

**Identification of the agent:** There is no prescribed test for Cryptosporidium infection. The demonstration of Cryptosporidium species oocysts or Cryptosporidium antigen in a properly collected and submitted sample is sufficient for a positive diagnosis. Diagnosis is established microscopically, with the acid-fast Ziehl–Neelsen or auramine phenol methods using unconcentrated or concentrated faecal smears. Microscopy-based methods for detecting oocysts and enzyme-linked immunosorbent assays for detecting Cryptosporidium antigens are relatively insensitive, but are sufficiently sensitive for detecting clinical cases. Neither tinctorial- nor fluorescence-based stains can determine the species of Cryptosporidium present if the oocysts fall within the size range of 4–6 µm. These methods can detect oocysts in clinically ill animals, but sometimes are not sufficiently sensitive to detect infection in clinically normal animals. Nucleic acid detection tests have a greater sensitivity. The polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) and/or sequencing can be used to determine some or all Cryptosporidium species/genotypes or subtypes. Specimens for primary diagnosis should be collected during acute infection, and should be processed as soon as possible, ideally, within 24 hours. Transportation to the laboratory should be in accordance with the International Air Transport Association regulations, which are summarised in Chapter I.1.1.

Demonstration of Cryptosporidium oocysts or Cryptosporidium-specific antigen in faecal samples is the most appropriate test for most applications. Most infections that cause morbidity and/or mortality in mammalian livestock are likely to be due to C. parvum cryptosporidiosis. C. baileyi, C. meleagridis and C. galli cryptosporidiosis cause morbidity and/or mortality in avian livestock. The species of Cryptosporidium responsible can be determined by PCR-RFLP and/or sequencing of Cryptosporidium oocyst DNA. There are no international standards for the preparation of purified oocysts, antisera, antigens, monoclonal antibodies or hybridomas, although a variety of purified oocysts and coproantigen detection kits using monoclonal antibodies are available commercially.

**Serological tests:** Cryptosporidiosis is often a disease of the newborn and unless there has been exclusion of exposure to infectious oocysts, serological tests do not offer any benefit.

**Requirements for vaccines and diagnostic biologicals:** There is no control programme for cryptosporidiosis, neither is there a rigorously tested and accepted vaccine available.
A. INTRODUCTION

Originally described in 1907, Cryptosporidium spp. were regarded as commensals until their association with diarrhoea in young turkeys (C. meleagridis) in the 1950s, and with large outbreaks of diarrhoea in calves (C. parvum) in the 1970s. Cryptosporidium is an important pathogen of livestock and human beings, and since the 1980s, C. parvum cryptosporidiosis has been recognised as a common cause of acute self-limiting gastroenteritis in immunocompetent hosts. Fayer et al. (5) provide a good account of the biology of Cryptosporidium.

Cryptosporidiosis is caused by protozoan parasites of the genus Cryptosporidium (family Cryptosporidiidae, order Eucoccidiorida, subclass Coccidiasina, class Sporozoasida, phylum Apicomplexa). Although more than 20 ‘species’ of this coccidian parasite have been described on the basis of the animal hosts from which they were isolated, host specificity as a criterion for speciation appears to be ill-founded as some ‘species’ lack such specificity. Species definition and identification of this genus is constantly changing, with the addition of ‘new’ species based primarily on molecular criteria. Currently, there are 13 ‘valid’ species namely: C. parvum, C. meleagridis, C. hominis, C. baileyi, C. serpentis, C. andersoni, C. felis, C. canis, C. muris, C. baileyi, C. molnari, C. wrairi, C. canis, and C. nasorum. In human and non-human hosts, molecular methods including the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and DNA sequencing have demonstrated a broader range of Cryptosporidium species than previously thought. Cryptosporidium meleagridis has been described in immunocompetent human patients, as well as C. hominis and C. parvum. Cryptosporidium meleagridis oocysts purified from faeces are indistinguishable from C. parvum by conventional methods (described in this chapter), but show genetic identity to C. meleagridis at a variety of separate genetic loci. Cryptosporidium felis and C. muris infections have also been described in immunocompromised humans. For immunocompetent humans, the most significant zoonotic threat is from C. parvum and C. meleagridis.

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The discovery of sequence-based differences within the ribosomal RNA (rRNA) gene repeat unit between individual isolates within a ‘previously valid’ species has resulted in revision of the taxonomy of the genus. Currently, C. parvum consists of nearly 20 genotypes, some of which may represent different species. Included in this list are the pig (2 genotypes), sheep, horse, cattle, rabbit, marsupials, opossum (2 genotypes) monkey, ferret, fox, deer (2 genotypes), muskrat (2 genotypes), squirrel, bear and deer mouse genotypes (27). Again, the current classification of C. parvum genotypes is subject to change.

<table>
<thead>
<tr>
<th>Cryptosporidium species</th>
<th>Mean oocyst size (µm) and (size range)</th>
<th>Site of infection</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. parvum</td>
<td>5.5 × 4.5 (3.8–6.0 × 3.0–5.3)</td>
<td>Small intestine</td>
<td>Mammals</td>
</tr>
<tr>
<td>C. muris</td>
<td>7.5 × 5.0 (6.5–8.0 × 5.0–6.5)</td>
<td>Stomach</td>
<td>Mice</td>
</tr>
<tr>
<td>C. andersoni</td>
<td>7.4 × 5.6 (6.6–8.1 × 5.0–6.5)</td>
<td>Stomach</td>
<td>Cattle</td>
</tr>
<tr>
<td>C. felis</td>
<td>4.5 × 5.0</td>
<td>Small intestine</td>
<td>Cats</td>
</tr>
<tr>
<td>C. felis</td>
<td>4.6 × 4.0 (3.2–5.1 × 3.0–4.0)</td>
<td>Stomach</td>
<td>Cats</td>
</tr>
<tr>
<td>C. canis</td>
<td>5.0 × 4.7 (3.7–5.9 × 3.7–5.9)</td>
<td>Stomach</td>
<td>Dogs</td>
</tr>
<tr>
<td>C. wrairi</td>
<td>5.4 × 4.6 (4.8–5.6 × 4.0–5.0)</td>
<td>Small intestine</td>
<td>Guinea-pigs</td>
</tr>
<tr>
<td>C. baileyi</td>
<td>6.2 × 4.6 (5.6–6.3 × 4.5–4.8)</td>
<td>Trachea, bursa of Fabricius, cloaca</td>
<td>Gallinaceous birds</td>
</tr>
<tr>
<td>C. meleagridis</td>
<td>5.2 × 4.6 (4.5–6.0 × 4.2–5.3)</td>
<td>Intestine</td>
<td>Turkeys</td>
</tr>
<tr>
<td>C. serpentis</td>
<td>6.2 × 5.3 (5.6–6.6 × 4.8–5.6)</td>
<td>Stomach</td>
<td>Snakes</td>
</tr>
<tr>
<td>C. saurophilum</td>
<td>5.7 × 4.7 (5.3–5.7 × 4.2–5.7)</td>
<td>Intestinal and cloacal mucosa</td>
<td>Lizards</td>
</tr>
<tr>
<td>C. nasorum</td>
<td>3.6 × 3.6</td>
<td>Intestine</td>
<td>Fish</td>
</tr>
<tr>
<td>C. molnari</td>
<td>4.3 × 3.3 (3.5–4.7 × 2.5–4.0)</td>
<td>Intestine</td>
<td>Fish</td>
</tr>
<tr>
<td>Cryptosporidium sp.</td>
<td>4.5–6.0 × 3.6–5.6</td>
<td>Small intestine</td>
<td>Bobwhite quail</td>
</tr>
<tr>
<td>Cryptosporidium sp.</td>
<td>5.8–5.0 × 8.0–5.6</td>
<td>? Intestine</td>
<td>Snakes, reptiles</td>
</tr>
</tbody>
</table>
From a public and veterinary health perspective, it is important to be able to differentiate between genotypes so that risk factors and sources of infection can be evaluated. For many Cryptosporidium species in Table 1, oocyst size and shape are similar, making species identification based on morphometry at the light microscope level difficult if not impossible, due to size overlap.

1. Clinical signs

Cryptosporidium parvum cryptosporidiosis is a cause of scour in young, unweaned livestock. Endogenous stages infect enterocytes of the distal small intestine, caecum and colon. Villous atrophy, shortening of microvilli and sloughing of enterocytes are the major pathological changes associated with disease, and affected animals usually recover within 2 weeks of showing signs of illness. Scouring is most common in young animals however, weaned and adult animals can also become infected. Signs can range from a mild to apparently infection to severe scouring. Death is an infrequent consequence. As for other pathogens, the young, old and immunocompromised are most susceptible to disease. Infected weaned and adult animals normally exhibit no obvious, identifiable signs of disease, such as scouring, however, infected clinically normal animals excrete oocysts that can be transmitted to other susceptible hosts.

Cryptosporidium parvum infections of cattle can cause varying degrees of dullness, anorexia, fever and loss of condition. Rarely do they cause the acute dehydration, collapse and high mortality seen with enterotoxigenic Escherichia coli or rotavirus, which can occur at a similar time. Cryptosporidium parvum infections are usually mild (though occasionally they can be severe, but transient), resulting in varying degrees of morbidity, but generally low mortality. Oocysts can be detected in clinically normal and clinically ill hosts. Scouring calves and lambs can excrete between $10^6$ and $10^8$ oocysts per g of faeces. Infected adult cattle excrete far fewer oocysts, yet subclinical infections of adult cattle can generate similar numbers of oocysts over a 12-month period.

Cryptosporidium andersoni colonises the digestive glands of the abomasum of older calves and adult cattle. The microvilli of peptic glands are destroyed by endogenous stages, which may account for the elevated concentrations of plasma pepsinogen detected in infected hosts. Some infected animals exhibit reduced weight gain compared with uninfected controls. Infected cattle do not develop diarrhoea, but can excrete oocysts for several months.

Cryptosporidium is a primary pathogen in chicken, turkeys and quail, causing respiratory and/or intestinal disease, leading to morbidity and mortality. Currently, two named species infect chicken and turkeys (C. bailey and C. meleagridis), and a third, unnamed species infects quail (Cryptosporidium sp.). Cryptosporidium spp. are common intestinal infections in broiler chicken in the USA and Japan. Cryptosporidium baileyi cryptosporidiosis is a disease of the epithelial lining of the bursa of Fabricius and cloaca of chicken, although the trachea and the conjunctiva are lesser sites of infection. Cryptosporidium baileyi intestinal cryptosporidiosis of chicken does not normally result in gross lesions or result in overt signs of disease. Villous atrophy, shortening of microvilli and enterocyte detachment are the major pathological changes associated with disease. Cryptosporidium baileyi respiratory cryptosporidiosis of chicken can result in severe morbidity and, on occasion, mortality. Initially, severe disease is accompanied by sneezing and coughing, followed by head extension to facilitate breathing. Epithelial cell deciliation and hyperplasia, mucosal thickening and discharge of mucocellular exudate into the airways are major pathological changes associated with disease in young broilers. Severe signs of respiratory disease can last up to 4 weeks post-infection (4). Cryptosporidium baileyi cryptosporidiosis of turkeys is similar to that of chicken. Chicken isolates of C. baileyi cause infection in other birds.

Cryptosporidium meleagridis cryptosporidiosis is a disease of the ileum of turkey and other poults and human beings. Cryptosporidium meleagridis cryptosporidiosis can cause severe diarrhoea in turkey poults. Villous atrophy, crypt hyperplasia and shortening of microvilli are major pathological changes associated with disease (4). Transmission of a turkey isolate of C. meleagridis to chicken and domestic ducks has been reported.

Cryptosporidium galli cryptosporidiosis is a disease of chicken and finches. Unlike the life cycle stages of either C. meleagridis or C. baileyi, the life cycle stages of C. galli develop in the epithelial cells of the proventriculus. Cryptosporidium galli oocysts (8.0–8.5 x 6.2–6.4 µm) are larger than those of C. baileyi.

Respiratory and intestinal cryptosporidiosis have been reported in commercially grown quail caused by Cryptosporidium of an inadequately described species (Cryptosporidium sp.) whose oocysts are smaller than those of C. baileyi and are not infectious to chicken or turkeys. Pathological changes are similar to those described for C. baileyi respiratory and intestinal cryptosporidiosis of chicken (4).

2. Infectious dose

The infectious dose for C. parvum varies from isolate to isolate and from host species to host species. For mouse (CD-1 strain) neonates the ID$_{50}$ (median infectious dose) is between 87 and 60 oocysts (11). Ten oocysts produced infection in two out of two primates tested, and five oocysts produced clinical disease in gnotobiotic
lambs. The infectious dose for cattle is not known, but is thought to be small. Whether C. parvum isolates vary in their ability to colonise different host species is unknown. In healthy adult human volunteers, the ID_{50} is also dependent on both the isolate and the host immune status. In human volunteer infectivity studies, C. parvum isolates differ in their ID_{50}, their attack rate, and the duration of diarrhoea they induce. The ID_{50} of the C. parvum TAMU isolate (equine derived, from Texas A & M University, USA) is 9 oocysts; the C. parvum IOWA isolate (bovine derived, from Ames, Iowa, USA) is 132 oocysts; and the C. parvum TAMU isolate (equine derived, from Texas A & M University, USA) is 9 oocysts (15). Oral infection with 100 C. baileyi oocysts can result in intestinal cryptosporidiosis (4).

3. Transmission

Transmission can occur via any route by which material contaminated with viable oocysts excreted by infected individuals can be ingested. Practices likely to enhance the spread of cryptosporidiosis include indoor calving and lambing and the communal feeding and husbandry of neonates, where young susceptible animals are in close contact with each other and the faeces of infected animals. Similarly, the disposal of faeces, farmyard manure or other contaminated waste in land-based dumps, when followed by periods of heavy rainfall or melting snow can lead to C. parvum oocyst contamination of water courses. These courses may be used as a source of drinking water for other animals and for potable water for human consumption. Contaminated waste includes both the liquid and solid by-products of animal husbandry.

4. Maintenance of infection

A variety of mammals act as hosts to C. parvum (21, 27, 28), but little is known of the importance of their involvement in transmitting infection to, or maintaining infection, in domesticated species in farmyard environments. Their role in ‘on farm’ epidemiology in domesticated species is also uncertain. The methods used for diagnosing infection in small mammals and wildlife are the same as those described for farm animals. Oocysts are environmentally robust and can survive for long time periods (>6 months) in moist, cool microclimates. Evidence exists for transmission of cryptosporidiosis from clinically normal dams to suckling neonates, but, in general, the duration of the carrier state remains unknown. A variety of bird species act as hosts to C. baileyi.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

There is no prescribed test for diagnosing Cryptosporidium infection. The demonstration of Cryptosporidium species oocysts or Cryptosporidium antigen in a properly collected and handled sample is sufficient for a positive diagnosis, and the methods of choice for collection of the samples are non-invasive. There are no reproducible in-vitro culture techniques available to amplify parasite numbers prior to identification, therefore the detection of the oocyst (the transmissive stage), Cryptosporidium antigen and/or DNA from faeces, or other suitable body fluids are the methods of choice. In addition to these tests, haematoxylin and eosin can be used for histological confirmation of the diagnosis on post-mortem. Haematoxylin and eosin histology is useful for confirming diagnosis, is commonplace world-wide, and will not be described in this chapter.

Further analyses including species and/or C. parvum genotype identity, can be performed on Cryptosporidium DNA using molecular techniques, such as the PCR-RFLP and/or sequencing of products amplified from defined genetic loci. This not only confirms diagnosis, but also provides discrimination beyond that possible with morphology and morphometry using light microscopy.

For Cryptosporidium species that infect the gastrointestinal tract (Table 1), primary diagnosis is based on the demonstration of oocysts in faeces by conventional tinctorial stains, fluorescent/immunofluorescent stains or Cryptosporidium antigen(s) in faeces by enzyme-linked immunosorbent assay (ELISA). The majority of diagnostic methods have been developed using C. parvum because of its commercial importance and availability. There is anecdotal evidence indicating that in a minority of samples, the methods described below may not detect all isolates. The methods described below are expected to detect most C. parvum infections, but their usefulness for detecting non-parvum species from clinical material is less well understood.

Demonstration of Cryptosporidium oocysts or Cryptosporidium-specific antigen in faecal samples is the most appropriate test for most applications. Most infections that cause morbidity and/or mortality are likely to be due to C. parvum cryptosporidiosis. The species of Cryptosporidium responsible can be determined by PCR-RFLP or sequencing of Cryptosporidium oocyst DNA, later. There are no international standards for the preparation of purified oocysts, antisera, antigens, monoclonal antibodies (MAbs) or hybridomas, although a variety of purified oocysts and coproantigen detection kits using MAbs are available commercially.
a) **Laboratory diagnosis**

The diagnostic features of *C. parvum* oocysts viewed in suspension using Nomarski Differential Interference Contrast (DIC) microscopy are as follows. Oocysts are smooth, thick walled, colourless, have spherical or slightly ovoid bodies containing, when fully developed (sporulated), four elongated, naked (i.e. not within a sporocyst) sporozoites and a cytoplasmic residual body. The modal size measurement of *C. parvum* oocysts is 4.5 × 5.0 µm (range 4–6 µm).

Diagnosis is normally established by conventional microscopic methods, and the modified Ziehl–Neelsen (mZN) or auramine phenol (AP) methods using unconcentrated faecal smears are frequently used (2, 3, 17). Where low oocyst numbers are expected in samples, or purified oocysts are required for molecular investigations, concentrating oocysts in faecal samples can increase the sensitivity of detection. Sugar (e.g. Sheather), salt, zinc sulphate or formalin-ether (formalin-ethyl acetate) solutions are the best options for concentrating oocysts from faeces (17, 18).

b) **Demonstration in faeces**

Stool samples from most clinically ill cases will contain large numbers of thick-walled oocysts and sufficient *Cryptosporidium* antigen, therefore, the use of standard staining and immunological techniques should result in a positive diagnosis. The numbers of clinically normal excretors is not known, and, given the insensitivity of conventional methods, low oocyst excretors may not be diagnosed using conventional techniques, as oocyst numbers may be below the limit of detection of these methods (23, 24). In clinically ill animals, oocysts can normally be demonstrated in unconcentrated stool smears. The use of an oocyst concentration method can enhance the detection rate. Both flotation and sedimentation methods are suitable for concentrating *Cryptosporidium* spp. oocysts, and oocyst antigens can be still be sought in faeces following these oocyst concentration procedures. Nucleic acid detection tests such as the PCR are being used increasingly as they offer both improved sensitivity and species/genotype/subtype identity.

*Cryptosporidium* spp. positive faecal samples should be available to personnel who are familiarising themselves with staining and concentration techniques, and smears from positive faecal samples should be included each time a test is performed. Stool samples containing *C. parvum* oocysts can be stored at 4°C in either 2.5% K₂Cr₂O₇ or 10% formalin for reference purposes. Similarly, oocyst-positive faecal smears, air dried and fixed in absolute methanol, can be prepared in advance from previous positive samples for use as positive controls. Where bronchio-pulmonary involvement is suspected, similar tests can be performed on bronchial and pleural exudates or lavages.

Note that 2.5% K₂Cr₂O₇ can be inhibitory to PCR. Oocyst positive stool samples or partially purified oocysts stored in 2.5% K₂Cr₂O₇ and intended for nucleic acid amplification by PCR should be washed in deionised water to remove residual 2.5% K₂Cr₂O₇ prior to DNA extraction. A series of three washes each followed by centrifugation (3000 g for 10 minutes), removal of the supernatant and resuspension of the pellet in deionised water should minimise PCR inhibition by 2.5% K₂Cr₂O₇.

c) **Laboratory staff and operator safety**

*Cryptosporidium* is included in laboratory risk Hazard Group 2 and all laboratory procedures that can give rise to infectious aerosols must be conducted in a biosafety cabinet. Specimens for *Cryptosporidium* analyses can contain other pathogenic organisms and should be processed accordingly. In order to safeguard the health of laboratory workers, the safety procedures outlined in Chapter I.1.6. Human safety in the veterinary microbiology laboratory must be followed.

The laboratory should have an internal and external quality assurance programme in place as outlined in Chapter I.1.2. Quality management in veterinary testing laboratories.

d) **Collections and submission of samples**

Where possible, specimens for primary diagnosis should be collected during acute infection, and should be processed as soon as possible. Ideally, transportation systems should be selected to ensure that specimens arrive at the laboratory within 24 hours. If prompt examination for *Cryptosporidium* cannot be carried out, the deterioration of protozoan morphology and their overgrowth by other microorganisms, particularly yeasts, can be reduced by the addition of 10% aqueous (v/v) formalin, although 10% formalin can interfere with PCR tests. Both oocyst morphology for microscopic identification and sporozoite DNA for PCR testing can usually be preserved for long periods at 4°C without formalinisation.

The procedures used for collection and transport of specimens are critically important for successful laboratory analyses. Specimens should be collected in a suitable leak-proof sample container and should be enclosed in secure primary and secondary packaging. Procedures for packaging and shipping of
specimens must be as outlined in the International Air Transport Association’s Dangerous Goods Regulations (8). These regulations are summarised in Chapter I.1.1. Sampling methods.

e) Threshold of detection in faeces

Most tinctorial and fluorescence methods for detecting oocysts, and ELISAs for detecting Cryptosporidium antigens, are relatively insensitive. These methods can detect oocysts in clinically ill animals, but may not be sufficiently sensitive to detect infection in clinically normal animals. Anusz et al. (1) reported a detection limit of $10^6$ oocysts per ml of faeces using the Kinyoun modification of mZN on unconcentrated faecal smears. Concentrating oocysts in the sample can increase the sensitivity of detection. In oocyst-positive human stool samples, between $1 \times 10^4$ and $5 \times 10^4$ oocysts per g of unconcentrated stool are necessary to obtain a 100% detection efficiency using the Kinyoun mZN staining method (23). Variations in faecal consistency influence the ease of detection, with oocysts being more easily detected in concentrates made from watery, diarrhoeal stools than from formed stool specimens (23). In addition to microscopic techniques, a number of antigen-capture ELISAs have been reported in the literature with detection limits in the region of $3 \times 10^5$–$10^6$ oocysts per ml (1, 16), which indicates that they do not appear to offer increased sensitivity over microscopic methods.

In bovine faecal samples, oocysts were not detected in samples seeded with 10,000 C. parvum oocysts per g following formol–ether sedimentation and examination using AP or immunofluorescence (IF) staining. When oocysts were concentrated using sucrose flotation, the threshold of detection was 4000 oocysts per g for both staining methods. After salt flotation, 4000 oocysts per g could be reliably detected by AP staining, but the detection limit was increased to 6000 oocysts per g using IF staining (23). Webster et al. (24) also compared microscopy with PCR and found that PCR coupled with immunomagnetic particle separation (IMS) of oocysts from faecal samples detected five oocysts per ml of diluted faeces, which corresponds to 80–90 oocysts per g. Even allowing for the dilution of formed faecal samples required for IMS, this represented an increase in sensitivity of several orders of magnitude over the conventional coprodiagnostic methods. Currently, a variety of sensitive, PCR-based tests are available (see Section B.1. Nucleic acid recognition methods).

- Preparation of unconcentrated faecal (or appropriate body fluid) smears (include a positive control slide each time this procedure is performed)
  - Test procedure
    i) Wear protective clothing and disposable gloves. Score the reference number of the specimen on a microscope slide with a diamond marker\(^1\), and use separate microscope slides for each specimen. Place 1 drop of saline (about 50 µl) in the centre of the slide.
    ii) Remove a small sample of faeces (about 2 mg) with the tip of a clean applicator stick\(^2\) (or pipette after mixing thoroughly, if liquid) and emulsify the sample in saline by thorough mixing. For liquid stools (or other appropriate body fluid) dispense one drop directly on to the slide. In liquid stools, mucus strands and exudates or pus can be mixed with saline on the microscope slide. Liquid stools can be diluted with a drop of 150 mM saline solution.
    iii) Prepare a medium to thick smear with areas of varying thickness. Ensure that the smear is of the correct transparency\(^3\).
    iv) Air dry the smear at room temperature.
    v) Fix the smear\(^4\) in methanol for 3 minutes.

- Preparation of faecal (or appropriate body fluid) smears following concentration by flotation or sedimentation

No flotation or sedimentation method is specific for Cryptosporidium spp. oocysts. Flotation fluids are denser than the parasites to be concentrated and are formulated to a defined specific gravity using a suitable hydrometer available from most large laboratory suppliers. Parasites concentrated by flotation or sedimentation methods can be identified by all the methods described in this chapter. Flotation/sedimentation fluids can sometimes interfere with diagnostic tests. Excess sucrose can reduce both oocyst attachment to glass slides and subsequent antibody binding, prolonged exposure to NaCl can distort morphology and morphometry, and formalin can reduce the sensitivity of PCR reactions. When

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\(^1\) Alternatively, a lead pencil can be used to mark the etched (frosted) portion of a frosted glass microscope slide.

\(^2\) For formed stools, the sample should include portions from the surface and from within the stool.

\(^3\) Moderately thick smears are recommended for this procedure. If the smear is too thin or thick, oocysts will be missed. An acceptable thickness can be achieved when either the hands of your watch or the print on this page can just be read when viewed through the preparation.

\(^4\) Air-dried, methanol-fixed smears can be kept at room temperature for >6 months before staining.
oocysts are concentrated, excess flotation/sedimentation fluid can be removed by washing the concentrate in water and re-centrifuging. The supernatant is then aspirated and discarded, care being taken not to disturb the pellet. These concentration methods are suitable for any appropriate body fluid that could contain oocysts.

1. **Flotation**

   The flotation principle uses a liquid suspending medium, which is denser than the oocysts to be concentrated. Therefore, when mixed with flotation fluid, the oocysts rise to the surface and can be skimmed out of the surface film and detected using the chosen method. For a flotation fluid to be useful in diagnostics, when morphology and morphometry are the critical factors, the suspending medium must not only be heavier than the object to be floated but must not produce shrinkage sufficient to render the object undiagnosable (18). Sucrose flotation, zinc sulphate flotation and saturated salt flotation methods are all suitable for concentrating *Cryptosporidium* oocysts. The following is a description of the methods used to prepare flotation solutions and to concentrate oocysts.

- **Sucrose flotation**

   Sucrose solution (specific gravity 1.18) is prepared in a glass beaker by adding 256 g of sucrose to 300 ml of deionised water. The solution is gently heated (<60°C) and continuously stirred with the aid of a magnetic stirrer on a hot plate stirrer, until the sucrose has dissolved completely. The sucrose solution is either placed on ice or in a refrigerator until its temperature has adjusted to 4°C. The cold sucrose solution is transferred to a 500 ml measuring cylinder and its specific gravity is adjusted to 1.18 by adding sufficient cold, deionised water (4°C). The sucrose solution is transferred to a screw-cap glass bottle, labelled, dated, initialled and stored at 4°C until used.

- **Zinc sulphate flotation**

   Zinc sulphate solution (specific gravity 1.18) is prepared in a glass beaker by adding 100 g of zinc sulphate to 300 ml of deionised water. The solution is gently heated (<60°C) and continuously stirred with the aid of a magnetic stirrer on a hot plate stirrer, until the zinc sulphate has dissolved completely. The zinc sulphate solution is transferred to a 500 ml measuring cylinder and its specific gravity is adjusted to 1.18 by adding sufficient cold, deionised water (4°C). The zinc sulphate solution is transferred to a screw-cap glass bottle, labelled, dated, initialled and stored at 4°C until used.

- **Salt flotation**

   Saturated salt solution (specific gravity 1.2) is prepared by adding approximately 200 g of sodium chloride to 200 ml of deionised water. The solution is gently heated (<60°C) and continuously stirred with the aid of a magnetic stirrer on a hot plate stirrer. Small amounts of sodium chloride (approximately 10 g) are added at 10-minute intervals until the solution becomes saturated. The saturated salt solution is then decanted into a clean glass bottle and either placed on ice or in a refrigerator until its temperature had adjusted to 4°C. The cold saturated salt solution is then transferred into a 500 ml measuring cylinder and its specific gravity is adjusted to 1.2 by adding cold, deionised water (4°C). The saturated salt solution is transferred to a screw-cap glass bottle and labelled, dated, initialled and stored at 4°C until used.

Brine is a concentrated aqueous NaCl solution, which has a specific gravity between 1.120 and 1.200 depending on the impurity of the salt used. While suitable for concentrating *Cryptosporidium* spp. oocysts, some protozoan cysts can become badly shrivelled or open up in this flotation fluid. The optimal time to examine specimens obtained from brine flotation is between 5 and 20 minutes after their recovery following flotation.

Centrifugal flotation has also been used to recover *Cryptosporidium* oocysts (and a various other parasite cysts and ova) from faeces. Most centrifugal flotation methods are based on modifications of the Clayton-Lane technique, whereby oocysts are concentrated by flotation and collected as a hanging drop on the underside of a glass cover-slip placed onto the positive meniscus of the flotation fluid. Centrifugation is used to separate particles which are denser than the flotation fluid from oocysts and particulates which will float on the surface of the flotation fluid. The inclusion of a centrifugation step speeds up separation of oocysts from other particulates (and hence, the concentration of oocysts) and minimises the risk of the flotation fluid adversely affecting the morphometry or morphology of the oocysts. The operator should note the health and safety issues, including lacerations and puncture wounds, associated with handling cover-slips.
Concentration of *Cryptosporidium* spp. oocysts by flotation

- **Test procedure**
  
i) Wear protective clothing and disposable gloves. Transfer approximately 1–2 g of faeces\(^5\) with an applicator stick to 3 ml of flotation fluid in a 12 ml test tube and mix thoroughly. If the stool is liquid, mix thoroughly, and dispense approx. 1–2 ml fluid into the test tube.
  
ii) Add, with gentle stirring, sufficient flotation fluid to form a positive meniscus at the rim of the test tube. Remove any large particles from the surface and if necessary, add more flotation fluid to maintain this positive meniscus.
  
iii) Leave for 20 minutes, then, taking great care not to disturb the positive meniscus gently remove the meniscus with a disposable pipette and dispense gently on to a microscope slide\(^6\).
  
iv) Air dry the smear at room temperature.
  
vi) Fix the smear\(^7\) in methanol for 3 minutes.

Concentration of *Cryptosporidium* spp. oocysts by centrifugal flotation

- **Test procedure**
  
i) Wear protective clothing and disposable gloves. Transfer approximately 1–2 g of faeces\(^8\) with an applicator stick to 3 ml of flotation fluid in a 12 ml centrifuge tube and mix thoroughly. If the stool is liquid, mix thoroughly, and dispense approx. 1–2 ml fluid into the centrifuge tube.
  
ii) Add, with gentle stirring, sufficient flotation fluid to form a positive meniscus at the rim of the centrifuge tube. Remove any large particles from the surface and if necessary, add more flotation fluid to maintain this positive meniscus.
  
iii) Place the centrifuge tube in a bench top centrifuge with swing out buckets, and place a 22 mm glass cover-slip onto the rim of the centrifuge tube, so that it flattens the positive meniscus. Add a balance tube if necessary, and centrifuge at 1100 \(g\)\(^9\) for 5 minutes.
  
iv) Once the centrifuge stops, pick up the glass cover-slip between index finger and thumb at opposing corners of the cover-slip. A hanging drop will be present on the underside of the cover-slip. Carefully place the cover-slip, with the hanging drop lowermost, onto a glass microscope slide.

2. **Centrifugal sedimentation**

Parasites will settle more rapidly if the stool suspension is subjected to centrifugation, however, food particles will also sediment more rapidly and can mask the presence of parasites in the film examined. To overcome this potential problem, larger food particles can be removed prior to centrifugation by filtering the emulsified stool through a sieve with an aperture size large enough for parasites to pass through, but which retains the larger food particles. As this process is more efficient than sedimentation by gravity, a smaller faecal sample (500 mg–1 g: the size of a pea) is sufficient for examination. Although centrifugation concentrates the material more quickly, faecal debris, which can obscure parasites, remains present. The efficiency of detection is increased by adding formalin for fixation and preservation of parasites, and ether to remove fats and oils. Both 10% formalin and ether are bactericidal. After centrifugation, a fatty plug, which may adhere to the inner walls of the tube, can be seen at the interface of the two liquids. The ether layer, the fatty plug and the formalin below it are discarded and the whole pellet is retained for examination.

Many modifications to this procedure have been advocated, and the following protocol is typical of the method used in diagnostic laboratories. Less distortion of protozoan cysts occurs with this method than with zinc sulphate flotation. This method achieves a concentration of 15–50-fold, dependent on the parasite sought, and provides a good concentrate of protozoan cysts and helminth eggs, which are diagnostically satisfactory. All steps that can generate aerosols (excluding centrifugation) should be performed in an operator protection safety cabinet.

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\(^5\) For formed stools, the sample should include portions from the surface and from within the stool.

\(^6\) Moderately thick smears are recommended for this procedure. If the smear is too thin or thick, oocysts will be missed. An acceptable thickness can be achieved when either the hands of your watch or the print on this page can just be read when viewed through the preparation. Score the reference number of the specimen on a microscope slide with a diamond marker, and use separate microscope slides for each specimen. Alternatively, a lead pencil can be used to mark the etched (frosted) portion of a frosted glass microscope slide.

\(^7\) Air-dried, methanol-fixed smears can be kept at room temperature for >6 months before staining.

\(^8\) For formed stools, the sample should include portions from the surface and from within the stool.

\(^9\) Centrifugation at speeds higher than 1100 \(g\) for longer (>5 minutes) periods of time is not advised as some parasites may deform and/or rupture and collapse.
Concentration of Cryptosporidium spp. oocysts by (formol/ether) centrifugal sedimentation

- **Test procedure**
  
  i) Wear protective clothing and disposable gloves. Sample approximately 500 mg–1 g faeces with an applicator stick and place in a clean 12–15 ml centrifuge tube containing 7 ml of 10% formalin. If the stool is liquid, dispense about 750 µl into the centrifuge tube.
  
  ii) Break up the sample thoroughly and emulsify using the applicator stick.
  
  iii) Filter the resulting suspension through a sieve into a beaker, then pour the filtrate back into the same centrifuge tube.
  
  iv) Add 3 ml of diethyl ether (or ethyl acetate) to the formalinised solution, seal the neck of the tube with a rubber bung (or a gloved thumb over the top of the tube) and shake the mixture vigorously for 30 seconds. Invert the tube a few times during this procedure and release the pressure developed gently by removing the rubber bung (or your thumb) slowly.
  
  v) Centrifuge the tube at 1100 g for 2 minutes.
  
  vi) Loosen the fatty plug with a wooden stick by passing the stick between the inner wall of the tube and the plug. Discard the plug and the fluid both above and below it by inverting the tube, allowing only the last one or two drops to fall back into the tube. Discard this fluid, containing diethyl ether and formalin, into a marked re-sealable liquid waste container.
  
  vii) Re-suspend the pellet by agitation. Pour the whole, or the majority of the re-suspended pellet on to a microscope slide, or transfer the re-suspended contents on to a microscope slide with a disposable pipette, and air dry.

A commercial device for concentrating helminth ova, larvae and protozoan cysts and oocysts using the formalin-ether method is available. Sold as the Fecal Parasite Concentrator (FPC, Evergreen Scientific, Los Angeles, California 90058, USA) it is an enclosed system, and consists of two polypropylene tubes, a flat-bottomed tube used for emulsifying the stool, and a conical tube used for centrifugation, with an interconnecting sieve. The comprehensive method states that both fresh and preserved (10% formalin, merthiolate-iodine-formalin, polyvinyl alcohol, and sodium acetate-formalin) stool specimens may be used.

g) **Conventional staining methods**

Both mZN and AP are effective for detecting Cryptosporidium oocysts in faeces (2, 3, 17, 18, 23, 24). mZN-stained slides should be screened under the x40 objective lens and putative oocysts confirmed and measured under the x100 objective lens (morphology and morphometry) using a bright-field microscope with a x10 eyepiece. AP-stained slides require to be read using an epifluorescence microscope equipped with a fluorescein isothiocyanate (FITC) filter set (excitation 490 nm; emission 510 nm). A UV filter set (excitation 355 nm, emission 450 nm) can assist in visualising AP-stained sporozoites. AP-stained slides can be screened under the x20 objective lens and oocysts with typical morphology can be confirmed under the x40 objective lens. The x100 objective lens must be used for all morphometric (size) measurements. AP-stained oocysts visualised under either the FITC or UV filters can be measured by slowly increasing the voltage (light intensity) of the bright-field light source so that both fluorescent and bright-field images can be seen concurrently. The object can then be measured with the eye-piece graticule.

- **Calibration of size using the eye-piece and stage micrometers**

Diagnosis of intact organisms often necessitates the measurement of the size and shape (morphometry) of the organism in question, in order to ensure that it falls within the accepted range of standard parameters (e.g. size and shape) for the species in question. At the light microscope level, measurement of objects <1 mm is achieved by means of a stage micrometer used in conjunction with an eye-piece micrometer.

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10 This is the size of a pea.
11 The sample should include portions from the surface and from within a formed stool.
12 425 µm aperture size, 38 mm diameter is equivalent to 36 mesh British Standard (BS 410-86) or 40 mesh American Standard (ASTM E11-81). The skirt of the sieve should fit neatly into the rim of the beaker. Debris trapped on the sieve is discarded by inverting the sieve and passing a stream of tap water through the mesh. Both the sieve and the beaker should be washed thoroughly in running tap water between each sample.
13 Ethyl acetate, although less flammable than diethyl ether is nevertheless flammable, therefore the procedure should be performed in well ventilated areas, ensuring that they contain no naked flames. Avoid prolonged breathing or skin contact.
14 Centrifugation at speeds higher than 1100 g for longer (>5 minutes) periods of time is not advised as some parasites may deform and/or rupture and collapse.
15 Too large a pellet is indicative of one or more of the following: centrifuging above the recommended speed and/or time, insufficient shaking (step iv), taking too large a faecal sample.
Objects are measured in Systeme International (SI) units, and the standard unit of measurement for conventional microscopy is the micron ($\mu = 0.001 \text{ mm}$).

The stage micrometer consists of a 76 × 26 mm glass slide that has a millimetre scale, graduated in microns permanently mounted on it. The eye-piece micrometer is a disc of transparent glass or plastic bearing a graduated scale, which is placed in one of the eye-pieces of a binocular microscope. The scale is usually 1 cm in length and is subdivided into millimetre intervals. When the microscope is focused on the object to be measured, both the scale on the eye-piece micrometer and the image of the object are seen simultaneously in focus. The standard scale on the stage micrometer is usually 1 or 2 mm.

When measurements are to be made, the appropriate objective lens, which is dependent on the magnification required, is chosen, and the number of divisions corresponding to the length or breadth of the image of the object are read on the scale of the eye-piece micrometer. The observed measurement is translated into real length (which corresponds to the number of eye-piece micrometer divisions representing the chosen parameter to be measured) by substituting the stage micrometer for the object and determining the number of divisions on the eye-piece micrometer corresponding to a definite number of divisions of the millimetre scale on the stage micrometer, under the same magnification.

Remember that your calculation, in real length, of the value of the division on the eye-piece micrometer scale will only be valid for the magnification of the objective chosen. You will have to recalculate the value of a division on the eye-piece micrometer for each objective of differing magnification on the microscope.

Because morphometry is a significant component of diagnostic parasitology, repetitive measurements of similar objects present in a single sample, or of various objects of varying sizes in sequential samples, necessitating the use of a variety of magnifications, will have to be undertaken. By determining the micrometer value of the eye-piece scale for each objective used, the constant interchange of objects and stage micrometer can be overcome. This enables rapid calculation of morphometry, in millimetres, or fractions thereof, to be undertaken with any of the objective lenses available.

The graticule is placed in the eye-piece by unscrewing the lower component of the eye-piece and placing it into the open tube. It must be seated correctly before the lower component is screwed back on to the eye-piece. Ensure that the diameter of the graticule is similar to the internal diameter of the lower lens tube. Do not touch the surface of the graticule – hold it by its edges. Make sure it is dust and grease free. The eye lens is focused on to the graticule by adjusting it until the scale on the graticule is critically sharp.

The determination is carried out as follows:

i) Insert the eye-piece micrometer, with its scale already in focus, into the microscope, making sure that the graticule scale is the right way up.

ii) Select the lowest power objective lens (e.g. ×10 objective) and focus the microscope on the stage micrometer, rotating the eye-piece and positioning the stage micrometer until the scales of the eye-piece micrometer and the stage micrometer lie parallel and close to or overlapping each other.

iii) Count the number of intervals on the stage micrometer that correspond exactly to a whole number of divisions on the eye-piece micrometer.

iv) Divide the value observed on the stage micrometer by the number of divisions counted on the eye-piece micrometer scale to determine the value of each division on the eye-piece micrometer scale.

v) Repeat the above for each objective lens.$^{16}$

vi) Keep a permanent record of the calculation of the value for each of the divisions on the eye-piece micrometer for each objective lens in close proximity to or attached on to the body of the microscope (e.g. a piece of cardboard stuck to the front of the microscope).

**Example**

For each objective lens:

\[ \frac{x \text{ eye-piece divisions}}{1 \text{ eye-piece division}} = y \mu\text{m (on the stage micrometer)} \]

\[ y = \frac{y}{x} \mu\text{m} \]

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$^{16}$ Notice that the value calculated in millimetres for each of the divisions on the eye-piece micrometer will be different for objectives of different magnifications, with values calculated for real length being smaller for each of the divisions of the eye-piece micrometer with increasing magnification.
• Microscopical examination of a sample

The sample must be examined in a systematic manner. Observation should commence using the lowest suitable objective, ensuring that the entire sample is viewed. A suggested scheme is as follows: commence in the upper left-hand corner of the sample, working across the slide from left to right, one field width at a time, until the upper right-hand edge of the sample is reached. Move down one field height and continue working across the slide from right to left, field by field, until the lower right-hand edge of the sample is reached. Continue in this manner until the end of the sample (lower right-hand corner) is reached. During this period of observation, the fine focus should be adjusted continuously so that the depth of the sample is also scanned. When a suspicious object is located, it is inspected under high power magnification and either verified or disregarded. If the magnification of the image of the object is insufficient to be able to visualise definitive morphological characteristics under the high (dry) objective, the immersion lens (×100) must be used. Wet mounts can be sealed with nail varnish or a proprietary permanent sealant.

Neither tinctorial- nor fluorescence-based stains can determine the species of Cryptosporidium present if the oocysts fall within the size range of 4–6 µm (see Table 1). For mammalian livestock, the consensus opinion is that the majority of infections are likely to be due to C. parvum, therefore, a preliminary diagnosis of C. parvum cryptosporidiosis can be made. However, the presence of oocysts of the size range 4–6 µm does not necessarily indicate that the infectious species is C. parvum. Similarly, for birds, a preliminary diagnosis of C. baileyi or C. meleagridis cryptosporidiosis can be made depending on infection site and oocyst size. Molecular identification of species/genotype/subtype can be performed later.

• Reporting results of microscopical examination

Negative specimens should be reported as ‘NO Cryptosporidium oocysts seen’. Positive specimens should be reported as ‘Cryptosporidium oocysts seen’.

A scoring system for positive samples can be used, based on the number of oocysts observed under the ×40 objective lens. However, microscopic examination cannot be considered as a quantitative determination as oocyst numbers vary considerably during the course of infection.

+ = less than 5 oocysts per slide
++ = 1 to 10 oocysts per field of view
+++ = 11 or more oocysts per field of view

• Modified Ziehl–Neelsen (mZN)

Strong carbol fuchsin: Dissolve 20 g basic fuchsin in 200 ml absolute methanol and mix on a magnetic stirrer until dissolved. Add 125 ml liquid phenol (GPR [80% w/w in distilled water]) carefully until well mixed, and make up to the final volume with 1675 ml deionised water. Mix thoroughly. Filter through Whatman No.1 filter paper to remove debris and store in a stock reagent bottle. Label, date and initial. Store the stock reagent in a dark cupboard at room temperature.

Commercial supplies are also available. Often the concentration of basic fuchsin can vary within the acceptable range of 1–3%.

1% acid methanol: Carefully add 20 ml hydrochloric acid (GPR/SLR) to 1980 ml of absolute methanol and mix. Transfer to a stock reagent bottle, and label, date and initial.

0.4% malachite green: Add 2 g malachite green to 480 ml deionised water and mix on a magnetic stirrer. Filter through Whatman No.1 filter paper into a stock reagent bottle, label, date and initial.

• Test procedure

Include a positive control slide each time you perform this procedure.

i) Wear protective clothing and disposable gloves. Fix the air-dried smear or concentrate17 in methanol for 3 minutes.

ii) Immerse the slide in cold strong carbol-fuchsin and stain for 15 minutes.

iii) Rinse the slide thoroughly in tap water.

iv) Decolourise in 1% acid methanol for 10–15 seconds18.

v) Rinse the slide in tap water.

17 Moderately thick smears are recommended for this procedure.
18 Over-destaining must be avoided.
vi) Counterstain with 0.4% malachite green for 30 seconds.

vii) Rinse the slide in tap water.

viii) Air-dry the slide. (The smear can be examined with or without a cover-slip. A little immersion oil is spread over the smear which is then viewed with either dry or oil immersion lenses, without the addition of a cover-slip. An alternative method is to add a cover-slip and mounting medium and then examine the smear.)

ix) Examine for the presence of oocysts by scanning the slide using the ×40 objective lens of a bright-field microscope. Confirm the presence of oocysts under the oil immersion objective lens.

x) Measure the size and shape of the red-stained bodies.

**Diagnostic features of Cryptosporidium spp. oocysts stained with mZN**

*Cryptosporidium* spp. oocysts stain red on a pale green background. The degree and proportion of staining varies with individual oocysts. In addition, the internal structures take up the stain to varying degrees. Some may appear amorphous while others may contain the characteristic crescentic forms of the sporozoites. *Cryptosporidium parvum* oocysts appear as discs, 4–6 µm in diameter. Yeasts and faecal debris stain a dull red. Some bacterial spores may also stain red, but these are too small to cause confusion.

**Auramine-phenol**

*Auramine phenol (AP):* Dissolve 3 g phenol in 100 ml deionised water and slowly add 0.3 g Auramine O. Filter through Whatman No. 1 filter paper into a stock reagent bottle. Label, date and initial the stock reagent. Store at room temperature in a light-proof glass bottle with an airtight stopper. Commercially available stains, such as Lempert’s reagent, are also acceptable.

3% Acid methanol: Carefully add 60 ml hydrochloric acid (GPR/SLR) to 1940 ml absolute methanol and mix. Transfer to a stock reagent bottle, and label, date and initial.

0.1% potassium permanganate: Add 0.5 g potassium permanganate to the 499.5 ml deionised water and mix using a magnetic stirrer. Filter through Whatman No. 1 filter paper into a stock reagent bottle, and label, date and initial.

**Test procedure**

Include a positive control slide each time you perform this procedure.

i) Wear protective clothing and disposable gloves. Fix air-dried smears or concentrate in absolute methanol for 3 minutes.

ii) Immerse the slides in AP stain for 10 minutes.

iii) Rinse in tap water to remove excess stain.

iv) Decolourise with 3% acid alcohol for 5 minutes.

v) Counterstain in 0.1% potassium permanganate for 30 seconds.

vi) Air dry slide at room temperature (see Modified Ziehl–Neelsen (mZN) step viii, above).

vii) Examine for the presence of oocysts, using an epifluorescence microscope equipped with FITC filters, by scanning the slide under the ×20 objective lens. Confirm the presence of oocysts under the ×40 objective lens.

viii) Measure the size and shape of the fluorescent bodies.

**Diagnostic features of Cryptosporidium spp. oocysts stained with AP**

*Cryptosporidium* spp. oocysts appear ring or ovoid shaped and exhibit a characteristically bright apple-green fluorescence against a dark background. *Cryptosporidium parvum* oocysts are ring or doughnut-shaped.
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shaped, measuring 4–6 µm in diameter. If available, view the preparation under a UV filter (excitation 355 nm, emission 450 nm), as sporozoites are more readily seen under the UV rather than the FITC filter set. Under the UV filter, oocysts appear light green and sporozoites appear yellow green.

• **Culture**

There is no reproducible method for culturing *Cryptosporidium* from body fluids. *In-vitro* cell culture systems have been described for semi-purified, infectious oocysts, but they have not been tested in sufficient depth with enough isolates or inhibitory materials to be recommended for routine purposes.

• **Immunological methods**

Two approaches to the immunological detection of *Cryptosporidium* oocysts have proven useful, and a variety of commercial kits are available. Each has a similar level of sensitivity, and either unconcentrated or concentrated stool samples can be used depending on the likely number of oocysts in the sample. Compared with conventional tinctorial stains, antibody-based detection kits (immunofluorescence and ELISA) appear to be expensive, considering that they seem to have a similar detection threshold.

a) **Direct immunofluorescence**

In direct immunofluorescence, a FITC-labelled MAb reactive with genus-specific, surface-exposed epitopes on *Cryptosporidium* oocysts binds to oocysts present in the sample. UV excitation using a FITC filter system (maximum excitation wavelength 490 nm, mean emission wavelength 530 nm) causes the labelled oocysts to exhibit a bright apple-green fluorescence. Materials provided with commercial kits vary but *C. parvum* oocyst positive and negative controls, FITC-labelled anti-*Cryptosporidium* MAb (provided at the working dilution), and glycerol-based mounting medium containing a photo-bleaching inhibitor are normally included. Known negative and positive samples must always be included in each test.

Air-dried faecal smears or faecal concentrates are fixed in absolute methanol (or acetone, depending on the manufacturers’ instructions) and air dried. Often the slides provided in the kit are welled slides into which both sample and kit reagents are dispensed and retained. The manufacturers’ instructions must be followed. It is false economy to dilute kit reagents to increase the testing volume. The FITC-labelled anti-*Cryptosporidium* genus-specific MAb (*Cryptosporidium* mAb), at the predetermined working dilution, is applied over the fixed, air-dried specimen(s) and the slide(s) are incubated horizontally in the dark in a humidified chamber. Excess antibody is removed by gentle rinsing, and the excess moisture drained. Mounting medium is placed over the specimen and a cover-slip is applied to the sample, ensuring that no air bubbles are trapped over the specimen. If mounting medium is not supplied, a mixture of 50% non-fluorescent glycerol: 50% phosphate buffered saline (PBS) (V/V) is suitable. Samples are scanned using ×20 objective lens, oocysts are confirmed using the ×40 objective lens, and the number of oocysts present is determined. Numbers can be recorded as identified previously. In the absence of a manufacturer’s method, the following method will produce satisfactory results.

The nuclear fluorogen, 4'6-diamidino-2-phenyl indole (DAPI; \([C_{16}H_{15}N_5.2HCl, FW 350.2]\)), can be used to highlight sporozoite nuclei within fluorescent oocysts providing further confirmatory morphological information (6, 19). DAPI is a non-specific DNA intercalator, therefore DNA of other cellular interferents including bacteria and yeasts will also be stained. DAPI at a working strength of 0.4 µg/ml is particularly useful when oocysts are sought in non-faecal samples (e.g. water and foodstuffs). DAPI intercalates with the nuclei of the sporozoites within viable or non-viable oocysts and causes them to fluoresce sky blue.

A blue filter block (excitation 490 nm; emission 510 nm) is used to visualise FITC-C-mAb localisation and an ultra-violet (UV) excitation (excitation 355 nm, emission 450 nm) is used to determine the presence of DAPI stained sporozoite nuclei.

• **Test procedure**

Include a positive and negative control slide each time you perform this procedure.

i) Wear protective clothing and disposable gloves. Fix air-dried faecal smears or faecal concentrates in absolute methanol for 5 minutes.23

ii) Dispense 50 µl of anti-*Cryptosporidium* MAb at its working dilution on to the well of each slide. Ensure complete coverage of the well.24

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23 Alternatively, allow the methanol to evaporate to dryness at room temperature.

24 Volumes, times, etc. can vary according to manufacturers’ instructions. Always follow the manufacturers’ instructions.
iii) Place the prepared slide(s) in a humidity chamber with the slide(s) elevated above the absorbent material used to generate humidity. Ensure that the absorbent material is moist.

iv) Place the humidity chamber in an incubator at approximately 37°C for the period of time prescribed by the manufacturer (normally 30–60 minutes).22

v) Using a Büchner-type aspirator gently aspirate excess MAb from each well. Tilt the slide to an angle of about 45°C from the horizontal towards the operator, and aspirate the fluid that collects at the bottom of the well(s) by placing the tip of an aspirator close to, but not touching, the fluid. The suction at the aspirator tip will draw the fluid to waste. Repeat this procedure for each slide well containing a sample.

vi) Dispense 50 µl of PBS to each well and allow to stand for 2 minutes at room temperature.22

vii) Gently aspirate the PBS from each well as described in step v. Apply a further 50 µl of PBS to each well and allow to stand for a further 2 minutes, before gently aspirating the PBS as described.22

viii) Apply 50 µl of a 1/5000 DAPI in PBS solution to each well and allow to stand for 2 minutes at room temperature. The working solution of DAPI is prepared by diluting a 2 mg/ml DAPI stock solution by 1/5000 in PBS (150 mM, pH 7.2). The working solution should be prepared for each day it is required. A stock solution of DAPI (2 mg/ml in methanol) can be stored at 4°C in the dark, indefinitely.

ix) Gently aspirate off the DAPI solution from each well as described in step v.

x) Apply 50 µl of deionised water to each well and leave to stand for 1–3 seconds at room temperature, then gently aspirate the deionised water from each well as described in step v.

xi) Dispense 50 µl of mounting medium to the centre of each well of each slide, then gently apply a coverslip on to the microscope slide.26

xii) Allow the coverslip to settle into place before scanning the slide.25

xiii) Scan the preparation for oocysts under the ×20 and confirm under the ×40 objective of an epifluorescence microscope equipped with an FITC filter set. Measure oocysts under the ×100 objective. If necessary, slides can be stored at room temperature, in the dark, until read.

• Diagnostic features of Cryptosporidium spp. oocysts stained by FITC-labelled anti-Cryptosporidium MAb

Cryptosporidium spp. oocysts are round or slightly ovoid objects that exhibit a bright apple-green fluorescence under the FITC filter set. Their measurements (measured length × breadth) are presented in Table 1. Often the fluorescence has an increased intensity around the entire circumference of the oocyst, with no visible breaks in oocyst wall staining. If Evans’ blue, which reduces nonspecific fluorescence, is included in the kit, the background fluorescence will be red. Nonspecific fluorescence is yellow. Always refer to the positive control to ensure that the size, shape and colour of the putative oocyst is consistent with those of the positive control. DAPI intercalates with the nuclei of the sporozoites within viable or non-viable oocysts and causes them to fluoresce sky blue. Under the ×100 oil immersion lens, a sporozoite nucleus is spherical to subspherical, measuring approximately 1 micron in diameter. In the event of an oocyst being distorted, the demonstration of up to four fluorescent nuclei in an object of a comparable size to an oocyst will assist in its identification (6, 19).

b) Detection of Cryptosporidium antigens by enzyme linked immunosorbent assay

In the ELISA, the presence of Cryptosporidium antigens in faeces (coproantigen) is sought. Depending on the commercial kit, Cryptosporidium coproantigens are captured and developed using a mixture of monoclonal and polyclonal antibodies. With the exception of increased throughput and automation, coproantigen detection kits do not offer increased sensitivity beyond the methods described.

Commercially available sandwich ELISA antigen detection kits contain anti-Cryptosporidium-coated well strips for capturing Cryptosporidium coproantigens, anti-Cryptosporidium antibodies for developing the reaction that is conjugated to an enzyme (frequently horseradish peroxidase), substrate, chromogen/substrate development system and stopping solution (which inhibits further enzyme catalysis when added to the reaction mixture). These have been developed to detect C. parvum antigens in stool samples, but

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25 These temporary mounts can be made semi-permanent by sealing around the edges of the cover-slip with clear nail varnish. Allow the cover-slip to settle into place for approximately 30 minutes to 1 hour before sealing with nail varnish. Using the brush supplied with the nail varnish, carefully apply the nail varnish around the perimeter of the settled coverslip, using the width of the brush as the guide to the width of nail varnish applied. Ensure even coverage around the cover-slip perimeter. Do not leave any gaps. Allow the nail varnish to dry at room temperature before labelling the slide(s) appropriately with their unique identifier number. If required, use a scalpel to carefully shave/scrape any excess dried and hardened nail varnish.

26 Putative oocysts are measured by slowly increasing the voltage (light intensity) of the bright-field light source so that both fluorescent and bright-field images can be seen concurrently. Objects can then be measured with the eye-piece graticule.
they may also be capable of detecting common epitopes from non-\textit{C. parvum} infections. Known negative and positive samples are included in commercial kits. Commercial kits normally contain all the necessary reagents to perform the analysis and the manufacturers’ instructions must be followed. It is false economy to dilute kit reagents to increase testing capacity. A comprehensive method and a formula for calculating the cut-off value and assigning positive or negative status to samples are usually included. Kit reagents are normally stored at 4°C when not in use. All reagents should reach room temperature before being used. Because of the variation in the methods described for different commercial kits, no method for coproantigen detection is included in this chapter.

- Nucleic acid recognition methods

PCR is more sensitive than conventional and immunological assays for detecting oocysts in faeces, although the sensitivity of published methods can range between 1 and 10^6 oocysts. These techniques are often restricted to specialist laboratories. Care is necessary when choosing primers, as some primers amplify genus-specific products (amplicons) whereas others amplify species-specific amplicons. Prior to routine adoption in clinical laboratories, both the variability between methods and the recognised difficulties in amplifying nucleic acids from faecal specimens by PCR must be overcome.

Faecal samples can contain many PCR inhibitors. In addition to bilirubin and bile salts, complex polysaccharides are also significant inhibitors. For \textit{Cryptosporidium}, boiling faecal samples in 10% polyvinylpyrrolidone (PVVP) before extraction can reduce inhibition.

The most robust information regarding species/genotype/subtype information has been derived from the study of three genetic loci (two 18S rRNA [9, 13, 25, 26] and the \textit{Cryptosporidium} Oocyst Wall Protein [COWP] [8, 20]) gene fragments by PCR-RFLP and/or sequencing amplicons. PCR amplification of \textit{Cryptosporidium} DNA using the 18S rRNA primers (CPB-DIAGF/R) of Johnston et al. (9) yields products that vary in length from 428 bp to 455 bp. The Johnson et al. primers (9) are included because they have been evaluated for cross reactions against a total of 23 microorganisms and the primers have been shown to work in a variety of matrices. The Ward et al. (22) modification of the Johnson et al. (9) reverse primer (substitution of CPB-DIAGR with PW99R [TAA-GGA-ACA-ACC-TCC-AAT-CTC], which produces an amplicon of approximately 420 bp) was shown by the authors to be more sensitive that CPB-DIAGF/R (9) in both direct and nested PCR assays. Further corroboration is required in different matrices before PW99R (22) can be fully recommended as a replacement for CPB-DIAGR. The nested (Nichols–Johnson; 13) 18S rRNA assay has also been shown to be sensitive.

Currently, there is no ‘standard’ genetic locus recommended for species identity, but RFLP or sequencing of 18S gene loci provide information about more species than the COWP gene locus. For detecting small numbers of oocysts (<100) consistently, a nested PCR is required. Two 18S rRNA PCRs have been tested extensively in the author’s laboratory (13, 25, 26) and the Nichols–Johnson primers (13) appear to be more sensitive, particularly with small numbers (<10) of \textit{C. parvum}, \textit{C. hominis}, \textit{C. felis}, and \textit{C. muris} oocysts. Although less sensitive, the Xiao et al. assay (25, 26) has the benefit of being able to detect more species than the Nichols-Johnson assay (13) by RFLP.

Table 2 contains the \textit{Cryptosporidium} spp. and genotypes determined by RFLP of the amplicon defined by the CPB-DIAGR/F primers following digestion with restriction enzymes \textit{VspI}, \textit{Dral} and \textit{Ddel}.

Not all \textit{Cryptosporidium} species/genotypes can be identified by PCR-RFLP of the 18S rRNA loci, however most of the species that are currently known to be commercially important for livestock can be identified by PCR-RFLP. Sequencing of the amplicon can offer better information than PCR-RFLP, but sequencing is more expensive and takes longer than PCR-RFLP. Currently, sequencing availability varies in different parts of the world and, for the more common species that infect livestock, PCR-RFLP has an important role to play.

A single-tube nested PCR-RFLP assay (8) amplifying a fragment of the gene coding for COWP distinguishes between \textit{C. hominis} and \textit{C. parvum}. This assay is recommended over and above that in reference 20 as it is more sensitive and offers a solution to the contamination frequently experienced in nested PCR products due to re-amplification of PCR products. In this single-tube nested PCR, the inner and outer primers are added to the initial reaction mixture. Optimisation of primer set concentrations and annealing temperatures result in the preferential amplification of one product size only, defined by the inner primers.

No recommended method for extracting \textit{Cryptosporidium} DNA from oocysts exists, and the sensitivity of most methods described has not been addressed fully. \textit{Cryptosporidium} DNA can be extracted either following partial purification of oocysts using one of the flotation/sedimentation techniques described above, or from oocysts in faeces following zirconia bead extraction (12). If concentration by formol–ether sedimentation is the routine laboratory test, oocyst concentrates suitable for lysis and amplification by PCR can be made by substituting deionised water for the 10% formalin used in the method described. DNA loss can be a consequence of subsequent DNA purification using commercial purification columns, but normally there should be an adequate
number of oocysts present in the sample to extract sufficient Cryptosporidium DNA for PCR-RFLP/sequencing analysis.

Table 2. Cryptosporidium spp. and genotypes determined by RFLP of the amplicons defined by the CPB-DIAGR/F primers following digestion with enzymes VspI, Drai and DdeI

<table>
<thead>
<tr>
<th>Cryptosporidium species and genotypes (Amplicon length in bp)</th>
<th>VspI</th>
<th>Drai</th>
<th>DdeI</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. parvum 1 (438)</td>
<td>222,104,112</td>
<td>None</td>
<td>204,68,166</td>
</tr>
<tr>
<td>C. parvum 2 (435)</td>
<td>219,104,112</td>
<td>None</td>
<td>204,68,166</td>
</tr>
<tr>
<td>C. muris (432)</td>
<td>320,112</td>
<td>None</td>
<td>42,224,166</td>
</tr>
<tr>
<td>C. andersoni (431)</td>
<td>319,112</td>
<td>None</td>
<td>265,166</td>
</tr>
<tr>
<td>C. felis (455)</td>
<td>239,104,112</td>
<td>50,405</td>
<td>221,68,166</td>
</tr>
<tr>
<td>C. baileyi (428)</td>
<td>212,104,112</td>
<td>84,344</td>
<td>262,166</td>
</tr>
<tr>
<td>C. meleagridis (434)</td>
<td>47,171,104,112</td>
<td>None</td>
<td>200,68,166</td>
</tr>
<tr>
<td>C. serpentis (430)</td>
<td>318,112</td>
<td>None</td>
<td>264,166</td>
</tr>
<tr>
<td>C. wrairi</td>
<td>219,104,112</td>
<td>None</td>
<td>201,68,166</td>
</tr>
<tr>
<td>Cryptosporidium pig (435)</td>
<td>219,104,112</td>
<td>None</td>
<td>204,68,166</td>
</tr>
<tr>
<td>Cryptosporidium desert monitor (432)</td>
<td>216,108,112</td>
<td>None</td>
<td>198,68,166</td>
</tr>
<tr>
<td>Cryptosporidium mouse (439)</td>
<td>48,175,104,112</td>
<td>None</td>
<td>205,68,166</td>
</tr>
<tr>
<td>Cryptosporidium ferret (438)</td>
<td>48,174,103,113</td>
<td>None</td>
<td>204,68,166</td>
</tr>
<tr>
<td>Cryptosporidium dog (429)</td>
<td>213,104,112</td>
<td>None</td>
<td>195,68,166</td>
</tr>
<tr>
<td>Cryptosporidium koala (436)</td>
<td>220,104,112</td>
<td>None</td>
<td>202,68,166</td>
</tr>
<tr>
<td>Cryptosporidium kangaroo (436)</td>
<td>220,104,112</td>
<td>None</td>
<td>202,68,166</td>
</tr>
<tr>
<td>Cryptosporidium monkey (436)</td>
<td>220,104,112</td>
<td>None</td>
<td>202,68,166</td>
</tr>
<tr>
<td>Cryptosporidium bear (432)</td>
<td>216,104,112</td>
<td>None</td>
<td>87,111,68,166</td>
</tr>
</tbody>
</table>

The following method is effective in extracting DNA from small numbers (~10+) of partially purified oocysts, and is used in the author's laboratory (14). Partially purified oocysts are suspended in 100 µl of lysis buffer (50 mM Tris/HCl, pH 8.5, 1 mM ethylene diamine tetra-acetic acid, pH 8, 0.5% sodium dodecyl sulphate [SDS], Sigma-Aldrich) and subjected to 15 freeze–thaw cycles (1 minute in liquid nitrogen; 1 minute 65°C). Samples are then transferred to a 55°C water bath, proteinase K (at a final concentration of 200 µg/ml) is added, and the samples are incubated for 3 hours. Proteinase K is heat denatured (90°C, 20 minutes), samples are chilled on ice for 1 minute, centrifuged (16,000 g, 5 minutes) then 70 µl of supernatant is removed for PCR amplification. SDS is inhibitory to Taq polymerase at concentrations as low as 0.01%, therefore, neutralisation of SDS in the extracted DNA is necessary. The addition of 2% Tween 20 will neutralise up to 0.05% SDS.

Reagents for PCR reactions are dispensed in 0.5 ml thin-walled tubes. Each tube contains 90 µl of pre-mixed reagents (200 µM each of the four dNTPs, 200 nM each of primers CPB-DIAGR and CPB-DIAGF, bovine serum albumin at a final concentration of 400 µg/ml, MgCl2 at 3.5 mM, 2.5 U of Taq polymerase in PCR buffer and Tween 20 at a final concentration of 2% to inactivate 0.05% SDS). Finally, 10 µl of DNA template is introduced below approximately 40 µl of mineral oil. Samples are subjected to 39 amplification cycles, and products are visualised following ethidium bromide staining of 1.4% agarose gels (9).

The primers and step cycle protocols for amplifying either 18S rRNA gene fragment (9, 25, 26) or the COWP gene fragment (8) are given in Table 3.

- **Reporting results of PCR-RFLP/sequencing examination**

  Negative specimens should be reported as 'NO Cryptosporidium DNA detected'.

  Positive specimens should be reported as 'Cryptosporidium DNA detected' inserting the species/genotype(s)/subtype(s) identified (see Table 2 and references 6 and 20) after identifying the respective species from the RFLP profiles presented in Table 2.
Table 3. Step cycle PCR protocols for 18S rRNA (refs 9, 25 & 26) and single-tube nested COWP (ref. 8) PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Step cycle protocol</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPB-DIAGF</td>
<td>80°C, 5 minutes; 98°C, 30 seconds</td>
<td>1 cycle 9</td>
</tr>
<tr>
<td>AAG-CTC-GTA-GTT-GGA-TTT-CTG</td>
<td>55°C, 30 seconds; 72°C, 1 minute; 94°C, 30 seconds</td>
<td>38 cycles</td>
</tr>
<tr>
<td>CPB-DIAGR</td>
<td>72°C, 10 minutes</td>
<td>1 cycle soak</td>
</tr>
<tr>
<td>TAA-GGT-GCT-GAA-GGA-GTA-AGG</td>
<td>4°C</td>
<td>9</td>
</tr>
<tr>
<td>XF1 (outer)</td>
<td>94°C, 3 minutes</td>
<td>1 cycle 25, 26</td>
</tr>
<tr>
<td>TTC-TAG-AGC-TAA-TAC-ATG-CG</td>
<td>94°C, 35 seconds; 55°C, 45 seconds; 72°C, 1 minute</td>
<td>35 cycles</td>
</tr>
<tr>
<td>XR1 (outer)</td>
<td>72°C, 7 minutes</td>
<td>1 cycle soak</td>
</tr>
<tr>
<td>CCC-ATT-TCC-TTC-GAA-ACA-GGA</td>
<td>94°C, 3 minutes</td>
<td>1 cycle 8</td>
</tr>
<tr>
<td>XF2 (inner)</td>
<td>94°C, 1 minute; 67°C, 1 minute; 72°C, 1 minute</td>
<td>20 cycles</td>
</tr>
<tr>
<td>GGA-AGG-GTT-GTA-ATT-AGA-TAA-AG</td>
<td>94°C, 1 minute; 54°C, 1 minute; 72°C, 1 minute</td>
<td>35 cycles</td>
</tr>
<tr>
<td>XR2 (inner)</td>
<td>72°C, 10 minutes</td>
<td>1 cycle soak</td>
</tr>
<tr>
<td>AAG-GAG-CAA-GGA-ACT-TCC-A</td>
<td>4°C</td>
<td>8</td>
</tr>
</tbody>
</table>

2. Serological tests (and/or tests for cellular immunity where relevant)

Cryptosporidiosis is often a disease of the newborn and unless there is sufficient evidence to exclude exposure to infectious oocysts, serological tests do not offer any benefit. Serological tests can be used for seroepidemiological surveys of exposure: most are ELISA based, using various aqueous extracts of *C. parvum* oocysts (e.g. ref. 7). Tests for cellular immunity do not appear to offer specific benefit, and are not available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There is no control programme for cryptosporidiosis, neither is there a rigorously tested and accepted vaccine available.

REFERENCES


28. **WWW.KSU.EDU/PARASITOLOGY/ Click on ‘Host checklist for C. parvum’**

* * *
HENDRA AND NIPAH VIRUS DISEASES

SUMMARY

Hendra virus (HeV) and Nipah virus (NiV) emerged in the last decade of the twentieth century as the causes of outbreaks of respiratory and neurological disease that infected a number of animal species. NiV has claimed the lives of over 100 people and HeV has caused the death of two people. In 1994, HeV caused severe respiratory disease and the death of 13 horses and a horse trainer at a stable in Brisbane, Australia. Between September 1998 and April 1999, after spreading unrecognised as a respiratory infection in Malaysian pigs, NiV appeared in the human population as the cause of fatal encephalitis. Over one million pigs were culled to stop spread of the disease. Fruit bats (flying foxes) in the genus Pteropus are natural hosts of both viruses.

HeV infection of horses is characterised progressively by high fevers, facial swelling, severe respiratory difficulty and, terminally, copious frothy nasal discharge. Some horses display neurological signs. The most common post-mortem observations are dilated pulmonary lymphatics, severe pulmonary oedema and congestion. The underlying lesion is generalised degeneration of small blood vessels in a range of organs. Syncytial endothelial cells containing viral antigen are common in capillaries and arterioles. HeV infection of horses is not uniformly fatal and some horses manifesting clinical signs survive infection. Laboratory transmission experiments have shown that HeV is not readily transmitted between horses, a finding consistent with the observation that in the original outbreak, the infection did not spread widely to horses in adjacent properties.

NiV infection of pigs is highly contagious, but it was not initially identified as a new disease because morbidity and mortality were not marked and clinical signs were not significantly different from other known pig diseases. Observations made during the outbreak investigation and during experimental infections confirmed that NiV infection of pigs is characterised by fever with respiratory involvement and occasional nervous signs. Some infected animals display an unusual barking cough. Abortion is reported in sows. Immunohistochemical lesions are found in either or both the respiratory system (tracheitis and bronchial and interstitial pneumonia) and the brain (meningitis) of infected animals. Syncytial cells containing viral antigen are seen in small blood vessels, lymphatic vessels and the respiratory epithelium.

Both viruses affect companion animals. HeV causes pulmonary disease in cats similar to that observed in horses. Natural infection of dogs with NiV causes a distemper-like syndrome with a high mortality rate. Experimentally NiV causes a similar disease to HeV in cats. Syncytial endothelial cells containing viral antigen were demonstrated in both HeV and NiV infections in cats and in NiV infection in dogs.

There appears to have been no human-to-human transmission. The spread to humans apparently is from animal contact: NiV from swine and HeV from horses.

HeV and NiV are closely related members of the family Paramyxoviridae. Differences between them and other family members have led to their classification in a new genus, Henipavirus, in the subfamily Paramyxovirinae. HeV and NiV are biosafety level 4 agents and it is vital that samples from suspect animals be transported to authorised laboratories only under biologically secure conditions and according to international regulations.

**Identification of the agent:** Both HeV and NiV may be propagated in a range of cultured cells. Virus isolation from unfixed field samples should be attempted, but only in situations where operator safety can be assured. Identification procedures following virus isolation include immunostaining of infected cells and neutralisation with specific antisera.
Viral antigen is present in vascular endothelium, and in the case of NiV in pigs, the respiratory epithelium. A wide range of formalin-fixed tissues can be examined to detect HeV and NiV antigens. Submissions for immunohistochemistry should include samples of brain at various levels, lung, spleen and kidney. In pregnant animals or in cases of abortion, uterus, placenta and fetal tissues should be included as appropriate.

**Serological tests:** Virus neutralisation tests (VNT) and enzyme-linked immunosorbent assay (ELISA) are available. VNT is currently accepted as the reference procedure. The ability of antisera to HeV and NiV to cross-neutralise to a limited degree means that a single VNT using either virus does not provide definitive identification of antibody specificity. Neutralising antibodies to HeV and NiV can be differentiated by the greater capacity to neutralise the homologous compared with the heterologous virus. This may not be a major impediment in outbreak situations where the causative agent is known, but serum samples from suspect cases or from areas of the world other than Australia and Malaysia should be subjected to VNT analyses with both HeV and NiV. The serological relationship between HeV and NiV ensures that ELISAs using HeV or NiV antigen can be used to detect antibodies to both viruses. Nonspecific reactions will occur in the ELISA and ELISA reactors should be confirmed by VNT before being considered indicative of NiV or HeV infection.

**Requirements for vaccines and diagnostic biologicals:** There are no vaccines currently available for either HeV or NiV.

## A. INTRODUCTION

Hendra virus (HeV) and Nipah virus (NiV) are viruses of fruit bats commonly known as ‘flying foxes’, members of the genus *Pteropus*. Antibodies to HeV are found in approximately 50% of the four Australian *Pteropus* species (25), and in one small serological survey antibodies to NiV were readily detected in a number of Malaysian bats, including two Pteropid species (14). HeV has been in the Australian flying fox population for at least 20 years (15). HeV and NiV have been isolated from Australian and Malaysian flying foxes, respectively (1, 7).

HeV emerged in Brisbane, Australia, in September 1994 as the cause of an outbreak of acute respiratory disease that killed 13 horses and a horse trainer (16). The virus was initially called equine morbillivirus, but subsequent genetic analyses indicated that it did not resemble morbilliviruses closely enough to merit inclusion in that genus. There have been two other instances of fatal HeV infection of horses in northern Queensland. Two horses developed an acute disease and died almost 1 month before the Brisbane outbreak, but HeV was determined to be the cause of death only after the horse owner, who probably acquired HeV during necropsy of the horses, died 13 months later with HeV-mediated encephalitis (20). A third horse died in January 1999 with no associated human disease (6).

Retrospective studies of archival histological specimens indicate that NiV has caused low mortality in Malaysian pigs since 1996, but remained unknown until 1998 when it emerged as the causative agent of an outbreak of encephalitis in humans (2). Unlike respiratory disease caused by HeV in horses, which was frequently fatal but characterised by poor transmissibility (24), respiratory disease caused by NiV in pigs was often subclinical and highly contagious, properties that led to rapid virus dispersal through the Malaysian pig population and forced authorities to choose culling as the only means to control spread (17). Over one million pigs were destroyed; 106 of 267 infected humans, mostly pig farmers in Malaysia and abattoir workers in Singapore who had direct contact with live pigs, died of encephalitis (1, 19).

Diagnosis of disease caused by Henipavirus is by virus isolation or demonstration of viral antigen in tissue samples. Detection of specific antibody can also be useful particularly in pigs where NiV infection may go unnoticed. Identification of HeV antibody in horses is less useful because of the high case fatality rate of infection in that species. Demonstration of specific antibody to HeV or NiV is of diagnostic significance because of the rarity of infection and the serious zoonotic implication of transmission of infection.

## B. DIAGNOSTIC TECHNIQUES

1. **Identification of the agent**

   a) **Virus isolation and characterisation**

   HeV and NiV are classified as biosafety level 4 (BSL4) agents, as there is a high risk of infection of laboratory personnel and appropriate precautions must be taken (21). The OIE biosafety guidelines in
Chapter I.1.6. Human safety in the veterinary microbiology laboratory, provide additional information. However, due to the high risk of human infection in the laboratory, BSL4 requirements surpass the OIE containment level 4 requirements. Virus isolation greatly facilitates identification procedures and definitive diagnosis should be undertaken where operator safety can be guaranteed. Isolation is especially relevant in any new case or outbreak, particularly in countries or geographical areas where infection by HeV or NiV has not been previously documented. Proof that wildlife species act as natural hosts of the viruses requires virus isolation from wild-caught animals.

i) Sampling and submission of samples
Diagnostic samples should be submitted to designated laboratories in specially designed containers. The International Air Transport Association (IATA), Dangerous Goods Regulations (DGR) for shipping specimens from a suspected zoonotic disease must be followed (13). The requirements are summarised in Chapter I.1.1. Sampling methods.

The range of tissues yielding virus in natural and experimental cases has been summarised (5). Brain, lung, kidney and spleen should always be submitted. Samples should be transported on ice or at 4°C and if this is not possible, on dry ice. They should not be held at –20°C for long periods.

ii) Isolation in cultured cells
Virus propagation should be conducted under BSL4 conditions. Strict adherence to this guideline would limit the handling of diagnostic specimens where the presence of HeV or NiV may be suspected but not confirmed to laboratories with BSL4 facilities. Primary virus isolation from suspect samples may of necessity be conducted under BSL3 conditions. However, if this is to be attempted, stringent local guidelines must be developed to ensure operator safety and applied if a ‘paramyxovirus-like’ cytopathic effect (CPE) develops in infected cultures. Such guidelines will emphasise good laboratory practice, the use of class II cabinets and may require methanol fixation of infected cells, to destroy infectious virus, followed by immunofluorescent detection of Henipavirus antigen. The culture medium from Henipavirus-positive cells should be transferred to a BSL4 laboratory.

At the recipient laboratory tissues are handled under sterile conditions, and 10% (w/v) suspensions are generated by grinding the tissues in a closed homogenisation system, e.g. stomacher/bag mixer using plastic bag or mixer mills using autoclavable steel balls in closed metal cylinders. Following clarification of the homogenate by centrifugation at 300 g, the supernatant is added to cultured cell monolayers. Virus isolation is aided by the fact that HeV and NiV grow rapidly to high titre in many cultured cells. African green monkey kidney (Vero) and rabbit kidney (RK-13) cells have been found to be particularly susceptible. HeV also replicates in suckling mouse brain and in embryonated hens eggs, and although the former may represent a viable method of primary isolation, there are no data on the relative susceptibility of in-vivo systems such as these compared with the more convenient cell culture systems. A CPE usually develops within 3 days, but two 5-day passages are recommended before judging the attempt unsuccessful. After low multiplicity of infection, the CPE is characterised by formation of syncytia that may, after 24–48 hours, contain over 60 or more nuclei. Syncytia formed by NiV in Vero cell monolayers are significantly larger than those created by HeV in the same time period. Although the distribution of nuclei in NiV-induced syncytia early in infection resembles that induced by HeV, with nuclei aggregated in the middle of the syncytia, nuclei in mature NiV-induced syncytia are distributed around the outside of the giant cell (12).

iii) Methods of identification
• Immunostaining of fixed cells
The speed with which HeV and NiV replicate and the high levels of viral antigen generated in infected cells make immunofluorescence a useful method to rapidly identify the presence of Henipaviruses using either anti-NiV or anti-HeV antiserum. At present the Henipavirus genus consists of HeV and NiV and there are no known antigenically related viruses.

The serological cross reactivity between HeV and NiV means that polyclonal antiserum to either virus or mono-specific antisera to individual proteins of either virus, will fail to differentiate between HeV and NiV. Monoclonal antibodies (MAbs) are currently being generated and tested to fulfil this function both in primary identification of the virus upon isolation and for use in immunohistochemical examination of tissues from suspect cases.

• Test procedure
Under BSL4, monolayers of Vero or RK-13 cells grown on glass cover-slips or chamber slides are infected with the isolated virus, and the monolayers are examined for the presence of syncytia after incubation for 24–48 hours at 37°C. It is recommended that a range of virus dilutions (undiluted, 1/10, 1/100) be tested because syncytia are more readily observed after infection at low multiplicity. Once visible syncytia are detected, infected cells are fixed by immersion in a vessel filled completely with methanol. The vessel is sealed and surface sterilised prior to removal to a less secure laboratory environment, for example BSL2, where the slides are air-dried. Viral antigen is detected using anti-
HeV or anti-NiV antiserum and standard immunofluorescent procedures. A characteristic feature of Henipavirus-induced syncytia is the presence of large polygonal structures containing viral antigen. These are observed most readily with monospecific and MAbs to the nucleocapsid protein N and phosphoprotein P.

- **Immunoelectron microscopy**
  The high titres generated by HeV and NiV in cells in vitro permits their visualisation in the culture medium by negative-contrast electron microscopy without a centrifugal concentration step. Detection of virus–antibody interactions by immunoelectron microscopy provides valuable information on virus structure and antigenic reactivity, even during primary isolation of the virus. Other ultrastructural techniques, such as grid cell culture (11), in which cells are grown, infected and visualised on electron microscope grids, and identification of replicating viruses and inclusion bodies in thin sections of fixed, embedded cell cultures and infected tissues complement the diagnostic effort. The details of these techniques and their application to the detection and analysis of HeV and NiV have been described (12).

**b) Virus neutralisation: differentiation of HeV and NiV**
Neutralisation tests rely on quantification methods and three procedures are available to titre HeV and NiV. In the traditional plaque and microtitre assay procedures, the titre is calculated as plaque forming units (PFU) or the tissue culture infectious dose capable of causing CPE in 50% of replicate wells (TCID\textsubscript{50}), respectively. In an alternative procedure, the viruses are titrated on Vero cell monolayers in 96-well plates and after 18–24 hours, foci of infection are detected immunologically in methanol-fixed cells using anti-viral antiserum (3). The virus titre is expressed as focus forming units (FFU)/ml.

Neutralisation assays using these three methods are described below. A virus isolate that reacts with anti-HeV or anti-NiV antiserum in an immunofluorescence assay is considered to be serologically identical to either HeV or NiV if it displays the same sensitivity to neutralisation by anti-HeV and anti-NiV antisera as HeV or NiV. Anti-HeV antiserum neutralises HeV at an approximately four-fold greater dilution than that which neutralises NiV to the same extent. Conversely, anti-NiV antiserum neutralises NiV approximately four times more efficiently than HeV (2). Virus quantification procedures should be conducted at BSL4.

i) **Plaque reduction**
Stock HeV and NiV and the unidentified Henipavirus are diluted in media and replicates of each virus containing approximately 100 PFU in 50–100 µl are mixed with an equal volume of either Eagle's minimal essential media (EMEM) or a range of dilutions of anti-HeV or anti-NiV antiserum in EMEM. The virus–antiserum mixtures are incubated at 37°C for 45 minutes, adsorbed to monolayers of Vero cells at 37°C for 45 minutes and the number of plaques determined by traditional plaque assay procedures after incubation at 37°C for 3 days.

ii) **Microtitre neutralisation**
Stock HeV and NiV and the unidentified Henipavirus are diluted and replicates of each virus containing approximately 100 TCID\textsubscript{50} in 50 µl are added to the test wells of a flat bottom 96-well microtitre plate. The viruses are mixed with an equal volume of either EMEM or a range of dilutions of anti-HeV or anti-NiV antiserum in EMEM. The mixtures are incubated at 37°C for 45 minutes and approximately 2.4 × 10^4 cells are added to each well to a final volume of approximately 200 µl. After 3 days at 37°C, the test is read using an inverted microscope and wells are scored for the degree of CPE observed. Those that contain cells only or cells and antiserum should show no CPE. In contrast, wells containing cells and virus should show syncytia and cell destruction. A positive well is one where all or a proportion of cells in the monolayer form large syncytia typical of henipavirus infection.

iii) **Immune plaque assay**
Vero cells (2 × 10^4 in 200 µl medium/well) are added to flat-bottom microtitre plates and grown overnight at 37°C. Stock HeV and NiV and the unidentified Henipavirus are diluted and replicates containing about 60 FFU/50 µl are mixed with an equal volume of either EMEM or a range of dilutions of anti-HeV and anti-NiV antisera diluted in EMEM. Virus–antiserum mixtures are incubated for 45 minutes at 37°C and adsorbed to Vero cell monolayers for 45 minutes at 37°C. Virus–antisera mixtures are removed, 200 µl EMEM is added to each well and incubation is continued at 37°C. After 18–24 hours the culture medium is discarded and plates are immersed in cold, absolute methanol for 10 minutes and then placed in plastic bags, which are filled with methanol, heat-sealed and surface sterilised with 4% (v/v) lysol during removal from the BSL4 laboratory. Gluteraldehyde can also be used for sterilisation at concentrations as low as 0.1% for 24 hours. It is recommended that each laboratory determine the concentration of gluteraldehyde required for sterilisation within the time frame required. Methanol-fixed plates are air-dried, the wells are blocked with phosphate buffered saline (PBS) containing 0.05% Tween 20 and 2% skim milk powder, and incubated for 30 minutes at 37°C with antiserum to either HeV or NiV or a monospecific antiserum to a virus protein. Anti-viral antibody
binding to syncytia can be detected using alkaline phosphatase-conjugated species-specific antibody and the substrate 5-bromo-4-chloro-3-indolyl phosphate and p-nitro blue tetrazolium substrate (NBT/BCIP; Promega, Catalog number S3771). When purple plaques appear against a clear background (10–30 minutes), the substrate is removed and the plates are rinsed with distilled water and air-dried. Plaques are counted using a magnifying glass.

c) Nucleic acid based recognition methods

The genomes of both HeV and NiV have been entirely sequenced (22), and polymerase chain reaction-based methods have been used to detect virus and are being validated in a number of laboratories (10).

d) Henipavirus antigen detection in fixed tissue - immunohistochemistry

Immunohistochemistry has proven to be one of the most useful tests in HeV and NiV detection. Performed on formalin-fixed tissues or formalin-fixed cells, it is safe and has allowed retrospective investigations on archival material (9). As virus replication and the primary pathology occur in the vascular endothelium (8), there is a wide range of tissues in which HeV and NiV antigen can be detected (5). It is thought that HeV antigens may be cleared from lung tissue early in the course of infection and so the sample submitted should include a range of tissues, not just lung. HeV antigen has been detected in the kidney of a horse 21 days post-infection (24) and so this organ should always be submitted. Ideally a submission for immunohistochemistry would include samples of the brain at various levels, lung, mediastinal lymph nodes, spleen and kidney. In pregnant animals the uterus, placenta and fetal tissues should be included.

A range of antisera to HeV and NiV may be used in immunohistochemical investigations of HeV- and NiV-infected tissues, but rabbit antisera to plaque-purified HeV and NiV have been found to be particularly useful. Some MAbs are also available. Although a biotin–streptavidin peroxidase-linked detection system has been used successfully in the past (8), the preferred detection system is the anti-rabbit/anti-mouse dextran polymer-linked reagent conjugated with alkaline phosphatase.

- Test procedure
  i) Dewax slides containing formalin-fixed, paraffin-embedded test material and positive and negative control tissue sections by immersion three times in xylene for 1 minute. Hydrate sections through two changes of 98–100% ethanol, one change of 70% ethanol and running tap water to remove residual alcohol.
  ii) Rinse slides in distilled water, immerse in 0.01 M CaCl₂ (adjusted to pH 7.8 with 0.1 M sodium hydroxide) containing 0.1% (w/v) trypsin (Difco Trypsin 250) for 20 minutes at 37°C and wash in distilled water.
  iii) Lay slides flat in a humid chamber and rinse with PBS for 5 minutes. Add 200 µl 3% aqueous H₂O₂ to each slide for 20 minutes at room temperature to block endogenous peroxidase. Rinse slides in PBS for 5 minutes.
  iv) Add 200 µl of an appropriate dilution of rabbit anti-Nipah or anti-Hendra antibody in PBS containing 0.1% (w/v) skim milk powder to test tissue slides and positive and negative control slides. To a duplicate set of test and positive and negative control slides add rabbit antibody to an unrelated pathogen. Cover the slides and incubate at 37°C for 1 hour.
  v) Rinse slides in PBS for 5 minutes and apply 2–3 drops of Envision™ solution (anti-rabbit Ig conjugated to peroxidase-labelled dextran polymer [DAKO Corporation, 6392 Via Real, Carpinteria, CA 03013]). Incubate at 37°C for 20 minutes.
  vi) Prepare the substrate by dissolving 2 mg 3-aminono-9-ethylcarbazole (AEC) in 200 µl dimethyl formamide (Merck) and add to 10 ml 0.02 M acetate buffer, pH 5.0. Add 5 µl H₂O₂ (30% w/v) and mix. Check the positive control slide for sufficient staining, usually 2–5 minutes, and stop the reaction by rinsing in distilled water. The substrate solution should be made fresh prior to use.
  vii) Counterstain the slides in haematoxylin for 1–3 minutes, rinse in tap water, add Scott's solution (0.04 M sodium bicarbonate, 0.3 M magnesium sulphate), and wash well in running tap water. Rinse the slides in distilled water and mount with a cover-slip using aqueous mounting medium.
  viii) Read the slides for cytoplasmic deposition of the chromogen indicating the presence of viral antigen. Brown/red granular staining will be seen in the cytoplasm of positive cells. The cell nuclei are blue and this facilitates identification of tissue morphology and assists in the location of viral antigen within the tissue.

2. Serological tests

In laboratories doing serological testing, particularly in outbreak situations, several strategies have been adopted to reduce the risk of exposure of laboratory personnel to HeV and NiV. Sera may be gamma-irradiated
(6 kiloGreys) or diluted 1/5 in PBS containing 0.5% Tween 20 and 0.5% Triton-X100 and heat-inactivated at 56°C for 30 minutes.

a) Virus neutralisation tests

Henipaviruses can be quantified by plaque, microtitre or immune plaque assays and these assays can be modified to detect anti-virus antibody (see above). The virus neutralisation test (VNT) is accepted as the reference standard. In the most commonly used microtitre assay, which is performed under BSL4 conditions, sera are incubated with virus in the wells of 96-well microtitre plates prior to the addition of Vero cells. Sera are screened starting at a 1/2 dilution although this may lead to problems with serum-induced cytotoxicity. Where sample quality is poor or sample volumes are small, as may be the case with flying fox or microbat sera, an initial dilution of 1/5 may be used. Cultures are read at 3 days, and those sera that completely block development of CPE are designated as positive. If cytotoxicity is a problem the immune plaque assay described above has merit because the virus/serum mixtures are removed from the Vero cell monolayers after the adsorption period, thereby limiting their CPE.

b) Enzyme-linked immunosorbent assay

Henipavirus antigens for use in the enzyme-linked immunosorbent assay (ELISA) are irradiated with 6 kiloGreys prior to use, a treatment that has negligible effect on antigen titre. In the indirect ELISA developed in response to the initial outbreak at Hendra in 1994, antigen was derived from HeV-infected cells subjected to several cycles of freezing and thawing and treatment with 0.1% (w/v) sodium dodecyl sulphate (P. Selleck, unpublished data). In the national swine surveillance programme in Malaysia in 1999 (4) a similar indirect ELISA format was used in which antigen was derived by non-ionic detergent treatment of NiV-infected cells. Subsequently, to control for high levels of nonspecific binding activity in some porcine antisera, a modified ELISA was developed based on the relative reactivity of sera with NiV antigen and a control antigen derived from uninfected Vero cells. At the Centers for Disease Control, Atlanta, USA, the approach has been to not only have an indirect ELISA for detection of IgG but also to use a capture ELISA for detection of IgM.

The specificity of the indirect NiV ELISA (98.4%) (18) means that in surveillance programmes, the test will yield false positives. This may not be a significant problem in the face of a NiV outbreak where a high proportion of pigs are infected and the purpose of the surveillance is to detect infected farms. However, this level of test specificity creates a problem in the absence of an outbreak or if the number of samples to be tested is limited. If a positive ELISA result was indicative of a bona fide infection, failure to respond may lead to virus spread and human fatalities. In contrast, initiating control measures in response to a false-positive ELISA result would be wasteful of resources (5). The current approach is to test all ELISA reactive sera by VNT, with sera reacting in the VNT considered to be positive. Confirmatory VNT should be done under BSL4 conditions and this may entail sending the samples to an internationally recognised laboratory.

The following procedure for the NiV ELISA has been developed at Australian Animal Health Laboratory (AAHL) for porcine sera and standardised after collaborative studies in the Veterinary Research Institute, Ipoh, Malaysia.

- Test procedure

  Preparation of NiV antigens

  i) Grow Vero cells until confluent in roller bottles in EMEM containing 10% (v/v) fetal calf serum (FCS). To infect with virus, pour off all but 5 ml of the medium from each roller bottle and, in a BSL4 laboratory, add low passage, plaque-purified NiV to a multiplicity of infection of 0.1 TCID50/cell.

  ii) Rotate roller bottles for 30 minutes at 33°C to adsorb virus, add 60 ml EMEM containing 10% FCS to each bottle and roll for a further 48 hours at 33°C. The multiplicity of infection, incubation time and temperature are chosen so that although the majority of cells become infected and are incorporated into syncytia within 48 hours, few cells detach into the culture medium. The culture medium of cells infected under these conditions is an excellent source of virus for further purification (23).

  iii) Wash monolayers of virus-infected cells once with cold 0.01 M PBS and, using a large scraper, scrape cells from each roller bottle into 5–10 ml ice-cold PBS.

  iv) Pool scraped cells into 50 ml tubes kept in ice and pellet the cells at 300 g for 5 minutes at 4°C. Pour off PBS and resuspend cells in ice cold TNM (10 mM Tris, 10 mM NaCl, 1.5 mM MgCl2, pH 7.2), approximately 0.5 ml TNM per roller bottle.

  v) Add NP40 (non-ionic detergent, Nonidet P40) to 1% (by addition of 1/10 volume of 10% [v/v] NP-40 in water) and lyse cells using 5–10 strokes of a Dounce homogeniser. This also releases from the cytoskeleton viral antigen that would otherwise be removed by centrifugation (step vi).
vi) Pellet the nuclei at 600 \( g \) for 10 minutes at 4°C. The nuclei will not lyse under these conditions and should form a tight white pellet.

vii) Gently remove the supernatant cytoplasmic extract into a clean tube and add ethylene diamine tetraacetic acid to 1.5 mM. Make up to 10 ml with TNE, aliquot in small amounts, freeze at –80°C and gamma-irradiate with 6 kiloGreys. Store aliquots at –80°C.

**Preparation of control, uninfected Vero cell antigen**

viii) Grow Vero cells in roller bottles in EMEM containing 10% FCS. When confluent, wash monolayers once with cold PBS and scrape the cells from each roller bottle into 5–10 ml ice-cold PBS. Proceed as described for virus-infected cells in steps iv–vii above.

**Preparation of test sera**

ix) In a biological class II safety cabinet, dilute test serum 1/5 in PBS containing 0.5% (v/v) Triton X-100 and 0.5% (v/v) Tween 20 in the wells of a 96-well microtitre plate. Seal the microtitre plate. Laboratory personnel should wear gowns and gloves and spray both their hands and the sealed microtitre plate with 1% Virkon before removing the microtitre plate from the biosafety cabinet to heat at 56°C for 30 minutes.

x) Mix 22.5 \( \mu l \) heat-inactivated serum with an equal volume of uninfected Vero cell antigen diluted 1/100 in PBS. Mix thoroughly and incubate at 18–22°C for 30 minutes.

xi) Add 405 \( \mu l \) blocking solution (PBS containing 5% chicken serum and 5% skim milk powder) to give a final serum dilution of 1/100 and incubate at 18–22°C for 30 minutes. Aliquots of 100 \( \mu l \) are added to two wells containing NiV antigen and two wells containing uninfected Vero cell control antigen as described in step xiv.

**ELISA procedure**

xii) Dilute Vero cell control and NiV antigens in PBS to ensure that control and virus antigen wells are coated with a similar concentration of protein. Antigen is usually diluted 1/1000 to 1/4000, but a specific dilution factor must be determined for each batch of antigen. Add 50 \( \mu l \) virus and cell control antigen to the wells of a Nunc Maxisorp 96-well microtitre plate as follows: virus antigen in columns 1, 3, 5, 7, 9 and 11 and cell control antigen in columns 2, 4, 6, 8, 10 and 12 (Fig. 1). Incubate at 37°C for 1 hour with shaking. Plates can be also incubated at 4°C overnight.

xiii) Wash ELISA plates three times with PBS containing 0.5% Tween 20 (PBST) (250 \( \mu l/well \)) and block with PBS containing 5% chicken serum and 5% skim milk powder (100 \( \mu l/well \)) for 30 minutes at 37°C on a shaker.

xiv) Wash plates three times with PBST and add 100 \( \mu l \) of inactivated, absorbed sera from step xi to each well as indicated in the format below. Add 100 \( \mu l \) PBS containing 5% chicken serum and 5% skim milk powder to conjugate and substrate control wells. Incubate the plates without shaking for 1 hour at 37°C and wash three times with PBST.

xv) Dilute protein A-horseradish peroxidase conjugate (ICN Pharmaceuticals; catalogue number 622811) in PBST containing 1% (v/v) skim milk powder. The dilution factor is approximately 1/3000. Mix well and add 100 \( \mu l \) protein A-conjugate to all wells except the substrate control wells. Add 100 \( \mu l \) PBST containing 1% skim milk powder to the substrate control wells. Incubate the plates for 1 hour at 37°C without shaking and wash three times with PBST.

xvi) Prepare the substrate (3,3’,5,5’-tetramethylbenzidine; TMB; Sigma, catalogue number T 3405) by dissolving one tablet (1 mg) in 10 ml of 0.05 M phosphate citrate buffer, pH 5.0, and add 2 \( \mu l \) of fresh 30% (v/v) \( H_2O_2 \). Add 100 \( \mu l \) of the TMB substrate to each well. Incubate for 10 minutes at 18–22°C and stop the test by adding 100 \( \mu l \) 1 M sulphuric acid to each well.

xvii) Read plates after blanking on a substrate control well. The optical density (OD) at 450 nm on NiV antigen and control Vero cell antigen are used to calculate an OD ratio for each serum (OD on NiV antigen/OD on Vero control antigen).

**Interpretation of results**

xviii) An OD ratio >2.0 with an OD on NiV antigen >0.20 is considered positive.

xix) An OD ratio >2.0 with an OD on NiV antigen <0.20 is considered negative.

xx) Sera displaying an OD ratio between 2.0 and 2.2 should be considered doubtful.

xxi) Doubtful and positive sera should be tested by VNT.
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**Fig. 1.** ELISA plate format.

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NIV: Antigen from NIV-infected cells; Con: Antigen from uninfected Vero cells; H: High positive porcine serum; N: Negative porcine serum; L: Low positive porcine serum; Conj: Conjugate control; Subs: Substrate control.

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

There are no vaccines currently available for either HeV or NIV.

**ACKNOWLEDGEMENTS**

Many individuals at the Australian Animal Health Laboratory contributed to the development of the tests described here, specifically Peter Hooper, Gail Russell and Megan Braun (immunohistochemistry), Paul Selleck, Chris Morrissy, Brenda van der Heide, Greer Meehan and John White (ELISA) and Gary Crameri (immune plaque assay).

**REFERENCES**


Chapter 2.10.10. — Hendra and Nipah virus diseases


13. INTERNATIONAL AIR TRANSPORT ASSOCIATION (2002). Dangerous Goods Regulations, 43rd edition. International Air Transport Association (IATA), 800 Place Victoria, P.O. Box 113, Montreal, Quebec H4Z 1M1, Canada.


\* \* \*

\textbf{NB:} There is an OIE Reference Laboratory for Hendra and Nipah virus diseases (see Table in Part 3 of this \textit{Terrestrial Manual} or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.10.11.

SWINE INFLUENZA

SUMMARY

Swine influenza is a highly contagious viral infection of pigs. Swine influenza virus (SIV) infections cause respiratory disease characterised by coughing, sneezing, nasal discharge, elevated rectal temperatures, lethargy, difficulty breathing, and depressed appetite. In some instances, SIV infections are associated with reproductive disorders such as abortion. Clinical signs and nasal shedding of virus can occur within 24 hours of infection. Morbidity rates can reach 100% with SIV infections, while mortality rates are generally low. Secondary bacterial infections can exacerbate the clinical signs following infection with SIV. Transmission is through contact with SIV-containing secretions such as aerosols created by coughing or sneezing, and nasal discharges.

Identification of the agent: Virus identification is best accomplished by collection of samples within 24–48 hours after development of clinical signs. The pig of choice is an untreated, acutely ill pig with an elevated rectal temperature. Virus can readily be detected in lung tissue and nasal swabs. Virus isolation can be conducted in embryonated chicken eggs and on continuous cell lines. Isolated viruses can be subtyped using the haemagglutination inhibition (HI) and the neuraminidase inhibition tests. Immunohistochemistry can be conducted on formalin-fixed tissue and a fluorescent antibody test can be conducted on fresh tissue. Polymerase chain reaction tests are also available. Enzyme-linked immunosorbent assays (ELISA) are commercially available for detection of type A influenza viruses.

Serological tests: The primary serological test for detection of SIV antibodies is the HI test conducted on paired sera. The HI test is subtype specific. The sera are generally collected 10–21 days apart. A four-fold or greater increase in titre between the first and second sample is suggestive of a recent SIV infection. Additional serological tests that have been described are the agar gel immunodiffusion test, indirect fluorescent antibody test, virus neutralisation and ELISA.

Requirements for vaccines and diagnostic biologicals: Inactivated, adjuvanted SIV vaccines are commercially available. Vaccines may be in the form of a single SIV subtype or may contain multiple SIV subtypes. Vaccines should reflect the current antigenic profile of field viruses containing subtypes and strains that are changed as needed, to assure protection. The finished vaccine must be shown to be pure, safe, potent, and efficacious.

A. INTRODUCTION

Swine influenza is a highly contagious viral infection of pigs that can have significant economic impact on an affected herd (5, 16). The swine influenza virus (SIV) is a type A orthomyxovirus with a segmented RNA genome. The type A swine influenza viruses are further subdivided based on their haemagglutinin and neuraminidase proteins. Subtypes of SIV that are most frequently identified in pigs include H1N1, H1N2, and H3N2. Other subtypes that have been identified in pigs include H1N7, H3N1, H4N6, and H9N2. The H1N1, H1N2 and H3N2 viruses found in Europe are antigenetically and genetically different than those found in the United States of America (1, 2, 4, 8, 12, 15, 20). Pigs have receptors in their respiratory tract, which will bind swine, human, and avian influenza viruses. Consequently, pigs have been called 'mixing vessels' for the development of new influenza viruses when swine, avian, and/or human influenza viruses undergo recombination in pigs (13). SIV infections are described as causing respiratory disease characterised by coughing, sneezing, nasal discharge, elevated rectal temperatures, lethargy, difficulty breathing and depressed appetite. Agents that may cause respiratory disease in pigs include porcine reproductive and respiratory syndrome virus, Aujeszky's disease (pseudorabies) virus, porcine respiratory corona virus, Actinobacillus pleuropneumoniae and Mycoplasma hyopneumoniae, however, most of these have other signs that do not resemble swine influenza (11). Only Actinobacillus pleuropneumoniae, in the acute form of the infection, has clinical signs similar to swine influenza, such as dyspnoea, tachypnoea, abdominal breathing, coughing, fever, depression and anorexia. Clinical signs
and nasal shedding of virus can occur within 24 hours of infection. Two forms of the disease occur in swine, epidemic or endemic. In the epidemic form, the virus quickly moves through all phases of a swine unit with rapid recovery provided there are not complicating factors such as secondary bacterial infections. In the endemic form, clinical signs may be less obvious and not all pigs may demonstrate traditional clinical signs of infection. Morbidity rates can reach 100% with SIV infections, while mortality rates are generally low. The primary economic impact is related to weight loss resulting in an increase in the number of days to reach market weight. Transmission is through contact with SIV containing secretions such as aerosols created by coughing or sneezing, and nasal discharges. Human infections with SIV can occur and a limited number of deaths have been reported. Precautions should be taken to prevent human infection as described in Chapter I.1.6 Human safety in the veterinary microbiology laboratory.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Because SIV is a potential human pathogen, all work with infectious tissues, swabs, embryonated eggs, and cell cultures should be done in a class II biological safety cabinet.

a) Culture

- **Sample processing**
  
  Lung tissue can be processed for virus isolation in a variety of ways, for example with a mortar and pestle, stomacher, homogeniser, or mincing with a scalpel blade or scissors. Processing of the tissue is done in cell culture medium with antibiotic supplement (e.g. \( 10 \times \) working strength), at a final concentration of 10% weight to volume. The suspension can be incubated for 30 minutes in the dark at 20–22°C. Nasal swabs should be collected in cell culture medium or phosphate buffered saline (PBS). Upon receipt at the laboratory, the nasal swabs are vigorously agitated by hand or on a vortex mixer. The nasal swab and lung materials are centrifuged at 1500–1900 \( g \) for 15–30 minutes at 4°C. The supernatant is collected and maintained at 4°C until inoculated. If supernatant is to be held for longer than 24 hours before inoculation, it should be stored at –70°C. Lung supernatant is inoculated without further dilution. Nasal swab supernatant can also be inoculated without dilution or diluted 1/3 in cell culture medium. Antibiotics are added to the cell culture medium used for processing and/or the supernatant can be filtered to reduce bacterial contamination, but this may decrease virus titre.

- **Cell culture virus isolation**
  
  i) Virus isolation can be conducted in cell lines susceptible to SIV infection. Madin–Darby canine kidney (MDCK) is the preferred cell line, but primary swine kidney, swine testicle or swine lung epithelial cell lines can be used.
  
  ii) Wash confluent cell monolayers (48–72 hours post-seeding) three times with cell culture medium containing a final concentration of 2 µg/ml of trypsin; the concentration will depend on the type of trypsin and up to 10 µg/ml may be used. The cell culture medium can be supplemented with antibiotics, but is not supplemented with fetal bovine serum.
  
  iii) Inoculate cell cultures with an appropriate amount of tissue suspension or swab supernatant. Note: The volume of inoculum will vary with the size of the cell culture container. In general, 100–200 µl are inoculated in each well of a 24-well culture plate, 1 ml in each Leighton tube, and 1–2 ml into a 25 cm² flask.
  
  iv) Incubate inoculated cell cultures for 1–2 hours at 37°C. When using cell culture containers that are open to the environment, such as culture plates, incubation should be done in a humidified incubator with 5% CO₂.
  
  v) Remove the inoculum and wash the cell monolayer three times with the cell culture medium containing trypsin.
  
  vi) Add an appropriate volume of the cell culture maintenance medium to all containers and incubate at 37°C for 7 days with periodic examination for cytopathic effect (CPE). If CPE is not observed at the end of the incubation period, the cell culture contains can be frozen at –70°C, thawed, and blind passaged as described above (step iii). If CPE is observed, an aliquot of the cell culture medium can be tested for haemagglutinating viruses and can be collected and used as inoculum for confirmation by the fluorescent antibody technique (see Section B.1.e. below). Cover-slips (Leighton tube, 24-well cell culture plate) or chamber slides with MDCK (or other appropriate cell line) monolayer can be inoculated for this purpose. The isolation procedure is as described above (step iii). In some instances, it may be necessary to make tenfold dilutions of the cell culture virus in order to have appropriate CPE on the cover-slip. Influenza subtypes can be determined by the haemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests.
- Egg inoculation (14)
  
i) Use 10–11-day-old embryonated chicken eggs.
  
ii) Inoculate 0.1–0.3 ml of inoculum into the allantoic cavity and amniotic sac; many laboratories only inoculate via the allantoic route with similar sensitivity. Generally, four eggs are inoculated per sample.
  
iii) Incubate eggs at 35–37°C for 3–4 days and candle daily. Eggs with embryos that have died within 24 hours of inoculation are discarded.
  
v) Centrifuge fluids at 1500–1900 g for 10–20 minutes at 4°C. Transfer the supernatant to another tube for testing.

- Haemagglutination test
  
i) Prepare a 0.5% erythrocyte suspension from male turkey or chicken blood; male turkey blood is used in some US laboratories, but is not recommended for identification of European isolates. Washed erythrocytes and 0.5% suspensions of erythrocytes can be stored at 4°C for up to 1 week. Discard if haemolysis is observed.
  
ii) Dispense 50 µl PBS in a row of 12 wells on a 96-well V- or U-bottom microtitre plate for each unknown virus. One additional row of wells should be included for a positive control.
  
iii) Add 50 µl of undiluted isolate to the first well of each corresponding row.
  
iv) Serially dilute the isolate with a micropipette set to deliver 50 µl. The resulting dilutions will range from 1/2 (well 1) to 1/2048 (well 11). Well 12 contains PBS only and serves as a cell control.
  
v) Add 50 µl of 0.5% erythrocyte suspension to each well and agitate the plate to mix thoroughly. Note: keep erythrocytes thoroughly suspended during the dispensing process.
  
vi) Cover the plate with sealing tape and incubate at room temperature until a distinct button has formed (30–60 minutes) in the control well.
  
- Typing SIV isolates

b) Haemagglutination inhibition test
  
i) Dilute reference HA antigens (H1, H3, etc.) to a concentration of 8 HA units (HAU) per 50 µl (4 HAU/25 µl) in 0.01 M PBS, pH 7.
  
ii) Standardise unknown influenza A viruses to contain 8 HAU in 50 µl.
  
iii) Conduct a back titration (HA test) for all unknown isolates and the H subtype antigens to assure that the correct HAUs are present. The back titration is performed as described in the HA procedures except that six well dilutions are used instead of eleven.
  
iv) Treat the serum with RDE; add 50 µl serum to 200 µl RDE (receptor-destroying enzyme; 1/10 dilution in calcium saline solution equalling 100 units per ml). Incubate overnight (12–18 hours) in a 37°C water bath. Add 150 µl 2.5% sodium citrate solution and heat inactivate at 56°C for 30 minutes. Combine 200 µl treated sample and 25 µl PBS to make a 1/10 dilution of the serum. Note: RDE treatment is recommended as it will remove nonspecific haemagglutination and will enhance the identification of H1N2 and H3N2 isolates.
  
v) Remove natural serum agglutinins from the sera by treating diluted serum with 0.1 ml packed, washed erythrocytes per 1 ml diluted serum. Incubate for 30 minutes at room temperature with occasional
mixing to keep the erythrocytes suspended. Centrifuge the treated serum at 800 g for 10 minutes and then retain the serum.

vi) Dispense 25 µl of standardised antigen (unknown isolate or positive control antigen) into three wells of a 96-well V- or U-bottom microtitre plate. Add 50 µl of PBS to several wells to serve as an erythrocyte cell control. Note: 25 µl of PBS can be used in place of the 25 µl of standardised antigen.

vii) Add 25 µl of the appropriate standardised antiserum to the first well of the H subtype being evaluated. Serially dilute the antiserum in 25 µl volumes in the antigen wells with a pipette set to deliver 25 µl. Repeat this procedure for each H subtype being evaluated. Note: If 25 µl of PBS was used in place of the 25 µl of standardised antigen in step vi, add 25 µl of standardised antigen to each well containing the standardised antiserum.

viii) Cover plate(s) and incubate at room temperature for 20–60 minutes.

ix) Add 50 µl 0.5% erythrocyte suspension to each well and shake/agitate the plate(s) to mix thoroughly. Keep the erythrocytes thoroughly suspended during the dispensing process.

x) Cover the plate(s) with sealing tape and incubate at room temperature until a distinct button has formed in the positive control wells (usually 30–60 minutes). Observe the plates after about 20 minutes’ incubation for evidence of haemagglutination as some isolates may begin to elute (detach from erythrocytes) in 30 minutes.

xi) Read test results as described above for the HA test. A sample is considered positive for a specific H subtype if haemagglutination is inhibited. The test is considered valid if the positive reference antigen and its homologous antiserum demonstrate the expected HI titre and the back titration of each antigen (unknown and positive control) is 8 HAUs. If these conditions are not met, the test should be repeated.

xii) If erythrocytes in the cell control wells do not settle into a well-defined button, check the following as possible causes: incorrect formulation of PBS, excessive evaporation from the plates, erythrocytes too old, or incorrect concentration of erythrocytes.

- Neuraminidase inhibition test

Subtype identification based on the NI test is beyond the scope of many laboratories. Reference laboratories should be consulted for N typing of isolates.

c) Fluorescent antibody test

i) This technique can be used for tissue sections or cover-slips/slides of infected cell monolayers. Positive and negative controls should be included with all staining procedures.

ii) Inoculated cells are incubated for an appropriate length of time to allow 10–25% of the cells to become virus infected. Rinse the cover-slip or slide once in PBS, place in 100% acetone for 5–10 minutes and air-dry. Acetone should be used in a vented hood.

iii) Prepare frozen tissue sections on glass slides. Fix the glass slides in acetone for 5–10 minutes and air-dry.

iv) Apply conjugate (fluorescein-labelled swine influenza antibody) and incubate in a humid chamber at 37°C for 30 minutes.

v) Rinse in PBS, pH 7.2, soak for 5–10 minutes in fresh PBS, rinse in distilled water, and air-dry.

vi) Place cover-slips on glass slides, cell side down, with mounting fluid. Remove the rubber gasket from chamber slides and add mounting fluid followed by a glass cover-slip. Mounting fluid followed by a glass cover-slip is also placed over tissue sections on the slide.

vii) Observe stained slides in a darkened room with the use of an ultraviolet microscope. Cells infected with SIV are identified by the presence of bright apple-green fluorescence. It is recommended that the person examining the slides receive training in reading fluorescein-labelled slides as they can be difficult to interpret.

d) Immunohistochemistry (19)

i) Slice formalin-fixed, paraffin-embedded lung in 4-µm thick sections and place on poly-L-lysine-coated slides. Positive and negative control tissues should be included with all tests.

ii) Heat slides at 60°C for 15 minutes, deparaffinise, and rehydrate through immersions in decreasing concentrations of ethanol and then in distilled water.

iii) Treat samples with 3% hydrogen peroxide for 10 minutes and rinse twice in distilled water.

iv) Digest samples with 0.05% protease for 2 minutes and rinse twice for 2 minutes in 0.1 M Tris/PBS buffer, pH 7.2, at room temperature.
v) Apply primary mouse anti-SIV monoclonal antibody (directed against the viral nucleoprotein) to each slide and incubate at room temperature for 1 hour or overnight at 4°C. Rinse slides with Tris/PBS buffer.

vi) Apply secondary antibody (biotinylated goat anti-mouse antibody) for 10 minutes at room temperature. Rinse with Tris/PBS buffer.

vii) Apply tertiary antibody (peroxidase-conjugated streptavidin) for 10 minutes at room temperature. Rinse twice in distilled water.

viii) Apply diaminobenzidine tetrahydrochloride solution for 5 minutes at room temperature. Rinse twice in distilled water.

ix) Counterstain slides in Gill’s haematoxylin for 10–30 seconds, wash in water for 2 minutes, dehydrate, clear, and add cover-slips.

x) SIV-infected tissues are identified by the presence of brown staining in bronchiolar epithelium and pneumocytes.

e) Antigen-capture enzyme-linked immunosorbent assays

Type A antigen-capture enzyme-linked immunosorbent assays (ELISAs) are commercially available for detection of human influenza viruses. These types of assays have been used for detection of SIV in lung tissue and nasal swabs (10, 17). The assays are generally available through human health care companies.

f) Polymerase chain reaction

Polymerase chain reaction tests have been developed for the diagnosis of swine influenza (6). Extensive information on the validation of these tests is not currently available.

2. Serological tests

The primary serological test for detection of SIV antibodies is the HI test and it is subtype specific. It should be conducted on paired sera collected 10–21 days apart. A four-fold or greater increase in titre between the first and second sample is suggestive of a recent SIV infection. Additional serological tests that have been described but not commonly used are the virus neutralisation, agar gel immunodiffusion test and indirect fluorescent antibody test. ELISA technology for detection of SIV antibodies has been described in the literature and at least one commercial kit has been marketed. The validation of the EILISA kit(s) is ongoing.

- Haemagglutination inhibition test
  i) Dilute reference HA antigens (H1, H3, etc.) to a concentration of 4–8 HAU/25 µl in 0.01 M PBS, pH 7.2.
  ii) H1N1 test: Heat inactivate sera for 30 minutes at 56°C. Dilute 1/10 in PBS. Add 0.1 ml packed, washed male chicken erythrocytes to 1 ml of heat-inactivated, diluted serum and mix. Incubate at room temperature for 30 minutes with periodic shaking every 10–15 minutes. Centrifuge at 800 g for 10 minutes at 4°C. Note: Sera can be treated with RDE and chicken erythrocytes as described below in step iii as an alternative to heat inactivation and treating with packed chicken erythrocytes.
  iii) H1N2 and H3N2 test: Add 50 µl serum to 200 µl RDE (receptor-destroying enzyme; 1/10 dilution in calcium saline solution equaling 100 units per ml). Incubate overnight (12–18 hours) in a 37°C water bath. Add 150 µl 2.5% sodium citrate solution and heat inactivate at 56°C for 30 minutes. Combine 200 µl treated sample and 25 µl PBS. Add 50 µl of 50% male chicken or turkey erythrocytes for a final serum dilution of 1/10. Shake and incubate for 30 minutes at room temperature or overnight at 4°C. Centrifuge at 800 g for 10 minutes at 4°C. Note: Chicken erythrocytes are preferred for all European isolates.
  iv) Dispense 50 µl treated serum into two wells of a V- or U-bottom 96-well plate. Dispense 25 µl of treated serum into two wells to be used as a serum control. Positive and negative control sera are treated in the same way as the unknown sera.
  v) Dispense 25 µl PBS in the serum control wells and all empty wells except two wells identified as the cell control wells. Add 50 µl PBS in the cell control wells.
  vi) Make serial twofold dilutions of the serum in 25 µl volumes in the plate and then add 25 µl of appropriate antigen to all test wells except the serum control wells and the cell control wells.
  vii) Incubate covered plates at room temperature for 30–60 minutes.
  viii) Add 50 µl of 0.5% erythrocyte (chicken for H1N1 and turkey for H3N2) suspension to each well, shake, and incubate at room temperature for 20–30 minutes until a distinct button forms at the bottom of the cell control wells. Keep erythrocytes thoroughly suspended during the dispensing process.
ix) Conduct a HA test using the HI test antigens prior to and simultaneously to conducting the HI test to verify that antigen concentrations are appropriate.

x) For the test to be valid, there should be no haemagglutination in the serum control well, no inhibition of haemagglutination with the negative serum, the positive serum should have its anticipated HI titre and the HA back titration should indicate 4–8 HAU per 25 µl.

b) Enzyme-linked immunosorbent assay (9)

ELISA technology for detection of SIV antibodies has been described in the literature. At least one ELISA test is available as a commercially produced kit.

C. REQUIREMENT FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

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

Identity of the seed should be well documented, including the source and passage history of the organism. All defining characteristics such as haemagglutinin and neuraminidase subtype should be established. Haemagglutination inhibition and neuraminidase inhibition by subtype-specific antisera can be used to establish the H and N subtypes. Also, aliquots of the master seed virus (MSV) can be neutralised with specific antisera, e.g. antisera produced against H1N1 or H3N2 SIV, then inoculated into the allantoic sac of 10-day old embryonated chicken eggs or on to susceptible cell lines such as the MDCK cell line. Allantoic fluid or cell culture supernatant is harvested 72–96 hours post-inoculation and tested for HA activity. Identity is demonstrated by the lack of HA activity in the neutralised seed, and the presence of HA activity in the non-neutralised seed. Significant antigenic differences present in a given strain that set it apart from other members of its subtype, and that purportedly have a beneficial impact on its use as a vaccine, should be confirmed through sequencing and genetic analysis.

b) Method of culture

SIV seed can be grown in eggs or in cell culture. Selection of a culture method is dependent on the degree of virus adaptation, growth in medium, rate of mutation, and viral yield in the specific culture system. SIV vaccine products should be limited to five passages from the MSV to avoid antigenic variation.

c) Validation of culture

The purity of the seed and cells to be used for vaccine production must be demonstrated. The MSV should be free from adventitious agents, bacteria, or mycoplasma, using tests known to be sensitive for detection of these microorganisms. The test aliquot should be representative of a titre adequate for vaccine production, but not such a high titre that hyperimmune antisera are unable to neutralise seed virus during purity testing. Seed virus is neutralised with monospecific antiserum or monoclonal antibody against SIV and the virus/antibody mixture is cultured on several types of cell line monolayers. Cultures are subpassaged at 7-day intervals for a total of at least 14 days, then tested for cytopathogenic and haemadsorbing agents. Cells are also examined for adventitious viruses that may have infected the cells or seed during previous passages. Potential contaminants include bovine viral diarrhoea virus, reovirus, rabies virus, Aujeszky’s disease (pseudorabies) virus, transmissible gastroenteritis virus, porcine respiratory coronavirus, porcine parovirus, porcine adenovirus, haemagglutinating encephalomyelitis virus, porcine rotavirus, porcine circovirus, and porcine reproductive and respiratory syndrome virus. Cell lines on which the seed is tested include: an African green monkey kidney (Vero) cell line (rabies and reoviruses), a porcine cell line, a cell line of the species of cells used to propagate the seed, if not of porcine origin, and cell lines for any other species through which the seed has been passaged. Additionally, a cell line highly permissive for bovine viral diarrhoea virus, types 1 and 2, is recommended. Bovine viral diarrhoea virus is a potential contaminant introduced through the use of fetal bovine serum in cell culture systems.

Factors that may contribute to instability during production, such as replication on an unusual cell line, should be investigated. If production is approved for five passages from the master seed, then sequencing of the genes for H and N at the maximum passage may be warranted to confirm the stability of the viral seed.

d) Validation as a vaccine

Vaccine candidates should be shown to be pure, safe, potent, and efficacious.
Strains used in vaccine production should be antigenically relevant to SIV strains circulating in the field (3, 7, 18). Haemagglutination inhibition and neutralisation tests demonstrating cross-reactivity between antisera from animals vaccinated with the candidate vaccine strain and current field isolates can be used for the selection. A vaccination/challenge study in swine, using homologous and heterologous challenge strains, will indicate the degree of protection afforded by the vaccine. Swine used in vaccination/challenge studies should be free of antibodies against SIV. Vaccination/challenge studies should be conducted using virus produced by the intended production method, at the maximum viral passage permitted, and using swine of the minimum recommended age listed on the label. Initially, lots are formulated to contain varying amounts of viral antigen. The test lot containing the least amount of antigen that demonstrates protection becomes the standard against which future production lots are measured. The most valuable criterion for blind trial evaluations of treatment groups is a significant reduction of virus (titres and duration of shedding) in the respiratory tract of vaccinated pigs. Differences in clinical observations and lung lesions are also among the criteria used in evaluation of a successful trial. If in-vivo or in-vitro test methods are to be used to determine the potency of each production lot of vaccine, those assays should be conducted concurrent with the minimum antigen studies in order to establish the release criteria. Combination vaccines containing more than one strain of SIV are available in some countries. The efficacy of the different components of these vaccines must each be established independently and then as a combination in case interference between different antigens exists.

2. Method of manufacture

Once the vaccine is shown to be efficacious, and the proposed conditions for production are acceptable to regulatory authorities, approval may be granted to manufacture vaccine. Generally, large-scale monolayer or suspension cell systems are operated under strict temperature-controlled, aseptic conditions and defined production methods, to assure lot-to-lot consistency. When the virus has reached its maximum titre, as determined by HA, CPE, fluorescent antibody assay, or other approved technique, the virus is clarified, filtered, and inactivated. Several inactivating agents have been used successfully, including formalin or binary ethylenimine. An inactivation kinetics study should be conducted using the approved inactivating agent on a viral lot with a titre greater than the maximum production titre and grown using the approved production method. This study should demonstrate that the inactivation method is adequate to assure complete inactivation of virus. Samples taken at regular timed intervals during inactivation, then inoculated on to a susceptible cell line or into the allantoic sac of embryonated eggs, should indicate a linear and complete loss of titre by the end of the inactivation process. This is represented as less than one infectious particle per $10^4$ litres of fluids following inactivation. Typically, adjuvant is added to enhance the immune response. Often, the adjuvant is a mineral oil formulation although other adjuvants may be equally effective.

3. In-process control

Cell cultures should be checked macroscopically for abnormalities or signs of contamination and discarded if unsatisfactory. A lot is ready to harvest when viral CPE has reached 80–100%. Virus concentration can be assessed using antigenic mass or infectivity assays.

4. Batch control

a) Sterility

During production, tests for bacteria, mycoplasma, and fungal contamination should be conducted on both inactivated and live vaccine harvest lots and confirmed on the completed product (see Chapter I.1.5.).

b) Safety

Mice or guinea-pigs can be used to evaluate the safety of an inactivated product. In one model, eight mice are inoculated intraperitoneally or subcutaneously with 0.5 ml and observed for 7 days. The mouse safety test may not be applicable when certain adjuvants are used, especially saponin-based products. In the other model, two guinea-pigs are each injected with a 2-ml dose either intramuscularly or subcutaneously and observed for 7 days. Adverse clinical signs or mortality attributable to the vaccine is indicative of a lot that is not acceptable for use. The completeness of viral inactivation in a killed product can be determined by multiple passes in cell culture or eggs of the post-inactivation, pre-adjuvant production fluids, followed by HA testing for the presence of virus.

Final product may be evaluated in the host animal using two animals of the minimum age recommended for use, according to the instructions given on the label; the animals are observed for 21 days. Field safety studies conducted on vaccinates, in at least three divergent geographical areas, with at least 300 animals per area, are also recommended. If the vaccine is to be used in swine destined for market and intended for human consumption, a withdrawal time consistent with the adjuvant used (generally 21 days) should be
established by such means as histopathological examination submitted to the appropriate food safety regulatory authorities.

c) Potency

During production, antigen content is measured to establish that minimum bulk titres have been achieved. Antigen content is generally measured before inactivation and prior to further processing. Relative potency ELISA, HA, and HI are among the assays that can be used to determine antigen content in final product. It is necessary to confirm the sensitivity, specificity, reproducibility, and ruggedness of such assays.

d) Duration of immunity

The duration of immunity and recommended frequency of vaccination of a vaccine should be determined before a product is approved. Initially, such information is acquired directly using host animal vaccination/challenge studies. The period of demonstrated protection, as measured by the ability of vaccinates to withstand challenge in a valid test, can be incorporated into claims found on the vaccine label. Once a suitable potency assay has been identified, should antigenic drift require replacement of strains within the vaccine, strains of the same subtype can be evaluated in either the host animal or a correlated laboratory animal model. However, circulating strains may show significant antigenic differences from the vaccine strain, but the vaccine strain may still provide protection. Also, the vaccine may not protect against a new strain that appears to be antigenically similar to the vaccine. Consequently, it would appear that the efficacy of vaccine strains will always have to be evaluated in swine.

e) Stability

Vaccines should be stored at 4°C ± 2°C, with minimal exposure to light. The shelf life should be determined by use of the approved potency test (Section C.5.b.) over the proposed period of viability.

f) Preservatives

The most common preservative is thimerosol, at a final concentration not to exceed 0.01% (1/10,000). Addition of thimerosol or other mercury-based compounds should be avoided if possible. Additionally, residual antibiotics from cell culture media may be present in the final product in restricted amounts. For example, residual gentamicin is not to exceed 30 mcg per ml of vaccine.

g) Precautions

Inactivated SIV vaccines present no special danger to the user, although accidental inoculation may result in an adverse reaction due to the adjuvant and secondary components of the vaccine. Generally, healthy pigs of weaning age or older and pregnant sows at any stage of gestation may be safely vaccinated with inactivated SIV vaccines.

5. Tests on the final product

a) Safety

Final container samples of completed product from inactivated vaccines should be tested in young mice as described above in Section C.4.b.

b) Potency

The potency assay established at the time of the minimum antigen protection study should be used to evaluate new lots for release. The assay needs to be specific and reproducible. It must reliably detect vaccines that are not sufficiently potent. If laboratory animal serology is used instead of swine serology, it should first be demonstrated that vaccination of the laboratory animal induces a specific, sensitive, dose-dependent response as measured in the potency assay and is correlated to protection in swine (Section C.1.d.).

REFERENCES


* * *
CHAPTER 2.10.12.

TOXOPLASMOsis

SUMMARY

Definition and description of disease: Toxoplasmosis is a zoonotic infection of animals caused by the protozoan parasite Toxoplasma gondii. In sheep and goats toxoplasmosis causes abortion or the birth of weak lambs/kids, which may be accompanied by a mummified fetus. Characteristically the placental intercotyledonary membranes are normal, but white foci of necrosis, approximately 2–3 mm in diameter, may be visible in the cotyledons. Microscopically, these foci appear as areas of coagulative necrosis that are relatively free of inflammation. Inflammation, when present, is nonsuppurative. Toxoplasma tachyzoites are seen only rarely in association with these foci, usually at the periphery of the lesion. Examination of the brain may reveal focal microgliosis. The lesions often have a small central focus of necrosis that might be mineralised. Focal leukomalacia in cerebral white matter, due to anoxia arising from placental pathology, is often present. Focal microgliosis is more specific as leukomalacia reflects placental damage, but may occur in other conditions such as border disease or rarely ovine chlamydiosis. Infection in pigs may cause severe fetal losses in pregnant sows, but more usually is mild and unnoticed.

Identification of the agent: Toxoplasma gondii is often difficult to find in tissue sections, but is more likely to be present in sections of brain and placenta. Its identity can be confirmed by immunohistochemistry, while the polymerase chain reaction may be used to identify parasite DNA in tissues. Isolation of T. gondii from samples is expensive and slow, but, if required, is best achieved by inoculation of mice with tissue homogenate derived from fetal brain or placenta.

Serological tests: The dye test is the longest established serological method, and in many ways represents the ‘gold standard’, at least in humans. The dye test uses live, virulent Toxoplasma tachyzoites, a complement-like ‘accessory-factor’ and test serum. When specific antibody acts on the tachyzoites, the latter do not stain uniformly with alkaline methylene blue. The test has proven unreliable in some species. In addition, as live Toxoplasma is used, the test carries a potential risk of human infection as well as being expensive to conduct. The indirect fluorescent antibody (IFA) test gives titres comparable with the dye test, but is safer as it uses killed tachyzoites. The IFA test can be used to differentiate IgM and IgG antibodies. The direct agglutination test and the latex agglutination test are both relatively rapid and neither requires complex laboratory facilities. The enzyme-linked immunosorbent assay requires more sophisticated laboratory equipment but can handle large numbers of samples and does not rely on human interpretation for the result.

Requirements for vaccines and diagnostic biologicals: A vaccine composed of live T. gondii tachyzoites is available commercially for use in sheep in certain European countries and New Zealand. The vaccine is supplied as a concentrated suspension of tachyzoites with an approved diluent and delivery system. The vaccine must be maintained and handled strictly according to the manufacturers’ instructions as it has a very short shelf life.

A. INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite that may cause abortion in sheep, goats and pigs. The parasite also has zoonotic potential. In a typical case of abortion, a ewe or doe infected in midgestation produces a stillborn lamb/kid a few days earlier than the predicted end of pregnancy. The aborted fetus is often accompanied by either a weak sibling or a ‘mummified’ fetus (3). The ewe/doe remains clinically normal. In such cases, placental cotyledons are typically speckled with white foci around 2–3 mm in diameter while the intercotyledonary membranes appear normal. Infection in early pregnancy, when the fetus has only a
rudimentary immune system, results in fetal death and resorption. In this case the mother may present as barren, which in turn can mimic a flock/herd infertility problem. Mothers that become infected in late pregnancy would be expected to produce infected but clinically normal offspring. Following an infection, either during or outside of pregnancy, the parasite would not be expected to cause abortion in any subsequent pregnancy. Infection in pigs may cause severe fetal losses in pregnant sows, but under modern intensive farming conditions infection is more usually mild and unnoticed (7).

The life cycle of *T. gondii* has asexual and sexual components. The asexual phase takes place in the intermediate host, which consists of most, if not all, warm-blooded animals. The sexual phase occurs in enteropithelial cells of the feline definitive host and results in the production of oocysts. Following a primary infection of a cat, oocysts may be shed in the faeces for several days. The oocysts sporulate in the environment over the next 1–5 days (depending on aeration and temperature), at which time the oocysts become infective. They are very resistant and may remain infective in the environment for a year or more. Sporulated oocysts are $11 \times 13 \mu m$ in diameter and each contains four sporozoites in each of two sporocysts (5). When a susceptible animal ingests sporulated oocysts, the sporozoites penetrate the intestinal lining, become tachyzoites and establish an infection.

In the asexual cycle there are two developmental stages: the rapidly multiplying tachyzoite and the slowly multiplying bradyzoite. Tachyzoites actively penetrate host cells where they multiply causing the cell to rupture and release organisms locally and into the bloodstream. As the host develops immunity, the parasite retains its overall size and shape but transforms into the bradyzoite stage and multiplies more slowly within tissue cysts to establish a persistent infection. These microscopic tissue cysts are present most frequently in brain and skeletal muscle and represent the quiescent stage of the parasite within the host. In sheep, goats, pigs, horses and humans, cysts may remain for the rest of the life of the individual (5). *Toxoplasma* species do not usually cause clinical illness in cattle, camels or deer, but can cause fatal disease in New World monkeys, Australian marsupials, hares (brown and mountain) and some birds.

Abortion in sheep and goats due to *T. gondii* must be differentiated from that caused by other infectious agents, including infections with *Chlamydomphila abortus* (see Chapter 2.4.7. Enzootic abortion of ewes), *Coxiella burnetii* (see Chapter 2.2.10. Q fever), *Brucella melitensis* (see Chapter 2.4.2. Caprine and ovine brucellosis [excluding *Brucella ovis* infection]), *Campylobacter fetus fetus* (see Chapter 2.3.2. Bovine genital campylobacteriosis), *Salmonella* spp. (see Chapter 2.10.3.), border disease (see Chapter 2.10.5.), and the viruses that cause bluetongue, Wesselsbron’s disease and Akabane disease. In pigs, *Brucella suis* (see Chapter 2.6.2.) may also cause fetal death, mummification and abortion.

**Human health risks**

*Toxoplasma gondii* readily infects human beings and while infection is relatively common (approximately 30% of the population depending on age and environment), clinical illness is relatively uncommon. Those particularly at risk of developing clinical illness include pregnant women, as the parasite can pose a serious threat to the unborn child if the mother becomes infected for the first time while pregnant, and individuals who are immunosuppressed, such as tissue transplant patients, AIDS patients, patients with certain types of cancer and those undergoing certain forms of cancer therapy; these individuals are at risk of developing acute lethal infection if left untreated. The very young and very old may also be more susceptible. On occasions people with no apparent immune deficiency may develop an illness characterised by general malaise, fever and lymphadenopathy. The two most likely sources of human infection are ingestion of raw or lightly cooked meat containing live *T. gondii* tissue cysts, or exposure to oocysts derived from cat faeces, such as may be encountered in gardens and children’s sand pits.

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

   a) **Isolation**

   Isolation of *T. gondii* from aborted ovine and caprine fetuses and fetal membranes is best made by inoculation of laboratory mice. The best tissues for inoculation are fetal brain and placental cotyledons, and optimum results are obtained with fresh samples free from contamination. Samples must not be frozen at any stage, as this kills the parasite.

   i) With aseptic precautions, remove 2–5 g of placental cotyledon or brain tissue from the aborted fetus.

   ii) Homogenise the tissue in an equal volume of 0.3 M sterile phosphate buffered saline (PBS), pH 7.4, with added antibiotics (100 International Units [IU]/ml penicillin and 745 IU/ml streptomycin) in a
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‘stomacher’ (Seward Laboratory, London) or other suitable macerating equipment. Brain tissue may be effectively homogenised by passing it through a 16-gauge needle ten times by means of a syringe.

iii) Inoculate each of three *Toxoplasma*-free mice intraperitoneally with 0.5 ml of the homogenate.

iv) Kill the mice 6–8 weeks after inoculation and remove the brains. Blood should also be recovered from the mice at this stage and the serum separated and stored at –20°C. Brains from mice that die before 6–8 weeks should also be harvested.

v) Homogenise each mouse brain with an equal volume of sterile PBS by passing through a 16-gauge needle ten times by means of a syringe.

vi) Spread one drop (5 µl) of a given suspension on each of five slides.

vii) Dry and stain with Giemsa, dehydrate and mount under a cover-slip.

viii) Examine slides under a microscope. Tissue cysts appear as circular structures measuring 5–50 µm filled with blue-staining, crescent-shaped bradyzoites.

An alternative method for examining the mouse brain is to take a small portion of forebrain (approximately match-head size) squashed flat with a cover-slip. Tissue cysts should be easily detected.

If the tissues inoculated are heavily infected with *T. gondii*, mice may die at 1–2 weeks.

Failure to demonstrate tissue cysts does not rule out a positive diagnosis. Serum from the mice may be analysed for the presence of antibodies to *Toxoplasma* (e.g. using an indirect fluorescent antibody [IFA] test); if this analysis is also negative, infection with *Toxoplasma* is unlikely.

b) Tissue sections

In affected placental cotyledons from sheep and goats there are typically large foci of coagulative necrosis, which may have become mineralised with time. Associated inflammation is characteristically slight and nonsuppurative. Well preserved samples of placental cotyledons may show moderate oedema of the mesenchyme of the fetal villi with a diffuse hypercellularity due to the presence of large mononuclear cells. Sometimes small numbers of intracellular and extracellular toxoplasms are visible, usually on the periphery of a necrotic area or in a villus that is in the early stages of infection. The *Toxoplasma* tachyzoites appear ovoid, 2–6 µm long, with nuclei that are moderately basophilic and located centrally or towards the posterior end.

In the fetal brain primary and secondary lesions may develop. Microglial foci, typically with a necrotic and sometimes mineralised centre and often associated with a mild focal lymphoid meninitis, represent a fetal immune response following direct damage by local parasite multiplication. Toxoplasmas are only rarely found, usually at the periphery of these lesions. Focal leukomalacia is also common and is considered to be due to fetal anoxia in late gestation caused by advanced lesions in the placenta preventing sufficient oxygen transfer from mother to fetus. These foci occur most commonly in the cerebral white matter cores, but sometimes also in the cerebellar white matter. These neuropathological changes, when seen together, are typical of *Toxoplasma* infection. However confirmation of the identity of *T. gondii*-like structures in tissue sections of, for example, brain or placenta may be achieved by immunohistochemistry that labels intact *T. gondii* or antigenic debris. The method is both convenient and sensitive and is used with fixed tissues (including archive tissues) that may also exhibit a degree of decomposition, where isolation would not be appropriate or possible. The ABC indirect immunoperoxidase method and the peroxidase–antiperoxidase (PAP) technique (10) are equally good.

c) Nucleic acid recognition methods

Several polymerase chain reaction (PCR)-based assays have been developed for detection of DNA from *T. gondii*. The main target regions are the B1 repetitive sequence, the P30 (SAG1) gene or ribosomal RNA (rRNA). The B1 and P30 PCR are both widely used techniques and good diagnostic aids, but are better used in conjunction with another test, as they are insufficiently robust when used alone. A PCR assay based on the detection of 18S rRNA has been investigated and may prove to be more sensitive, however, further studies are required (6). The sensitivity of the PCR is dependent on the copy number of the target sequence (B1: 35 copies; P30: 1 copy; rRNA: 110 repeat units). Customised synthetic DNA oligonucleotides are commercially available (e.g. www.sigma-genosys.co.uk).

The method described is a nested form of the PCR, amplifying the B1 repetitive sequence of DNA (12). Parasite DNA can be extracted and purified from several tissues, including placenta, the central nervous system, heart and skeletal muscle.
Contaminating red blood cells in tissues are removed by washing in 10 mM Tris/HCl lysis buffer, pH 7.6, followed by centrifugation at 2000 g for 15 minutes. DNA is then extracted from the resultant pellet and resuspended in 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂ containing proteinase K 100 µg/ml and 0.5% Tween 20.

Samples are incubated at 55°C for at least 1 hour, then the proteinase K is inactivated by boiling. The PCR procedure is performed in 50 µl volumes. Amplification of the B1 gene is performed by modifying the procedure described in ref. 1. The reaction mixture contains 10 mM Tris, pH 8.3, 2.5 mM MgCl₂, 40 mM KCl, 0.01% gelatin, 0.1 mM dNTPs, 0.2 µM of each primer (oligonucleotide primers are those described in ref. 1), two sense primers P1 and P2 and two antisense primers P3 and P4) and 2.5 units of Taq polymerase.

Primary amplification is performed with primers 1 and 4 to give a 193 bp product over 25 cycles of 93°C for 1 minute, 50°C for 1.5 minutes and 72°C for 3 minutes. The amplified product is then diluted 1/20 in distilled water to reduce amplification of nonspecific products.

Secondary amplification using nested primers 2 and 3 and the same reaction conditions, is carried out over 15 cycles to give a 94 bp product. The final product is then visualised on 1% agarose gels. Southern blotting, using a labelled probe, can be used to confirm the identity of the B1 PCR products and to distinguish them from nonspecific products.

2. Serological tests

There are several serological tests available for the detection of *T. gondii* antibodies. In one type of test the observer judges the given colour of tachyzoites under a microscope, such as with the dye test (DT) and IFA test. Another depends on the principle of agglutination of *Toxoplasma* tachyzoites, red blood cells or latex particles, such as with the direct agglutination test (DAT) and indirect haemagglutination test (IHA) and latex agglutination (LA) test, respectively. With the enzyme-linked immunosorbent assay (ELISA), the degree of colour change defines the quantity of specific antibody in a given solution. The DT, IFA test, DAT and ELISA are outlined below and the IFA test is given in more detail.

The DT (9) is the so-called 'gold standard' serological test for *Toxoplasma* antibody in humans. Live *Toxoplasma* tachyzoites are incubated with a complement-like accessory factor and the test serum at 37°C for 1 hour before methylene blue is added. Specific antibody induces membrane permeability in the parasite so that the cytoplasm is able to leak out and the tachyzoite does not incorporate the dye and so appears colourless. Tachyzoites not exposed to specific antibody (i.e. a negative serum sample) take up the dye and appear blue. The DT is both specific and sensitive in humans, but may be unreliable in other species. In addition, it is potentially hazardous as live parasite is used. It is expensive and requires a high degree of technical expertise.

The IFA test (8) is a simple and widely used method. Whole, killed *Toxoplasma* tachyzoites are incubated with diluted test serum, the appropriate fluorescent antispecies serum is added, and the result is then viewed with a fluorescence microscope. Fluorescent-labelled antibodies are available commercially for a variety of animal species, the method is relatively inexpensive and kits are commercially available. However, the method requires a fluorescence microscope and the results are read by eye, so individual variation may occur. It may be difficult to find some species-specific conjugates and there is a risk of possible cross-reactivity with rheumatoid factor and anti-nuclear antibodies.

The DAT (4) is both sensitive and specific. Formalinised *Toxoplasma* tachyzoites are added to U-shaped well microtitre plates and dilutions of test sera are then applied. Positive samples will produce agglutination that can be graded, while negative samples will produce a 'button' of precipitated tachyzoites at the bottom of the well. The test is simple and easy to perform although relatively large amounts of antigen are required. Kits are commercially available. The method of growth and harvesting of parasites is given below.

The original ELISA (11) uses a soluble antigen preparation made from *Toxoplasma* RH strain tachyzoites (as described below) and layered into wells in a microtitre plate. Test sera (e.g. ovine in origin) are added, followed by an anti-species enzyme-labelled conjugate such as horseradish peroxidase-labelled anti-ovine-IgG. Any attached conjugate causes a colour change in the substrate that is directly related to the amount of bound antibody, and which can be read with a spectrophotometer at the absorbance specific to the substrate used. The assay is simple, can readily test a large number of samples and is easy to perform with the chosen anti-species conjugate. Defined anti-species conjugates, substrates and whole kits are commercially available. However, the assay does require a spectrophotometer. The ELISA is well suited to laboratories required to analyse large numbers of samples.

- **Preparation of antisera and antigens**

Antiserum to *T. gondii* and conjugated antisera for use in the IFA test and ELISA, to allow screening of a variety of animal species, may be obtained commercially. International standards for animal sera are not available.
Below are protocols for the preparation of tachyzoite antigen for use in the IFA test and ELISA. Tachyzoites may be grown in mice or in tissue culture and retained as whole parasites for the IFA test, or prepared as soluble antigen for the ELISA.

**Production of Toxoplasma tachyzoites in mice**

1. Inject each of six Toxoplasma-free mice intraperitoneally using a 1-ml syringe and a 23-gauge needle, with 0.2 ml of $1 \times 10^7$/ml *T. gondii* tachyzoites of the RH strain, collected fresh from a previous mouse passage or from tissue culture. (For optimum recovery of tachyzoites having minimal host mononuclear cells, mice should be more than 6–8 weeks of age and weigh approximately 22–25 g.)

2. Kill the mice 3 days later by CO₂ inhalation (avoid cervical dislocation as this may cause contamination of peritoneal fluid with red blood cells).

3. Pin the mouse out on its back on a clean cork mat. Reflect the abdominal skin with aseptic precautions, remove any peritoneal fluid by means of a 21-gauge needle attached to a 1 ml syringe and gently eject the harvested exudate into an equal volume of PBS.

4. The optimum time to collect tachyzoites is 72 hours after initial inoculation, when there will be sufficient organisms but before there is significant contamination by host cells. It is also important not to delay harvesting peritoneal fluid much past 3 days as the mice will die. (If tachyzoites for mouse inoculation are taken from a frozen stabalate, it may be necessary to harvest mice 4 or 5 days after the initial inoculation and passage the parasite once more through mice before using it as an antigen in the above tests.)

5. Centrifuge the fluid at 500 *g* for 5 minutes, aspirate supernatant and resuspend in Hanks' balanced salt solution (HBSS). Alternate between PBS and HBSS washes by centrifugation.

6. Calculate the concentration of tachyzoites and contaminating host cells with an improved Neubauer counting chamber (Count numbers of tachyzoites at 1/1000 dilution and cellular contamination at 1/10).

7. Carry out further washes (step iv above) as required to reduce cellular contamination to <0.5% host mononuclear cells and <0.25% for red blood cells.

8. Resuspend the tachyzoites in PBS to give a final concentration of $1 \times 10^7$/ml.

9. Tachyzoites may be maintained by continual passage in this manner without the addition of penicillin/streptomycin by observing strict aseptic procedures.

**Preparation of aliquots of a frozen stabalate of *T. gondii* tachyzoites**

1. Produce tachyzoites in mice or tissue culture as described.

2. Centrifuge at 500 *g* for 5 minutes and resuspend in Iscove’s modified Dulbecco’s medium (IMDM) (Gibco BRL, Paisley, UK) approximately three times.

3. Add the following solutions to give these concentrations: 10% dimethyl sulphoxide; 20% normal horse serum (free from antibody to *T. gondii*); 70% resuspended tachyzoites to give a final concentration of $1 \times 10^8$ tachyzoites/ml.

4. Allow the preparation to stand on the bench for 1 hour.

5. Dispense into 1-ml aliquots using screw-topped tubes appropriate for liquid nitrogen storage.

6. Put the tubes into a small container, wrap in thick insulating material and place in −70°C freezer to allow the tachyzoites to freeze gradually.

7. The next day transfer to liquid nitrogen, keeping well insulated while transferring.

8. This stabalate may then be used for mouse inoculation or tissue culture growth of the parasite. When removing from storage thaw the sample rapidly in warm water.

**Production of Toxoplasma tachyzoites in tissue culture**

1. *Toxoplasma gondii* can be grown and maintained in tissue culture by twice-weekly passage in African green monkey kidney (Vero) cells.

2. Cells and parasite are grown in IMDM supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin and 2% fetal bovine serum.

3. Tachyzoites are harvested from tissue culture flasks by scraping the cell monolayer using a sterile cell scraper.
iv) Using 25cm² vented tissue culture flasks that have each been seeded with $1 \times 10^5$ Vero cells, add tachyzoites at the rate of two tachyzoites per monolayer cell and incubate at 37°C in a 5% CO₂ humidified chamber. Harvest after 3–4 days.

**Preparation of whole tachyzoites for use in the IFA test**

i) Produce 4 $\times 10^7$/ml suspension of RH strain *T. gondii* tachyzoites in PBS.

ii) Add formaldehyde (40%) to give a final concentration of 0.2% (v/v).

iii) Incubate at 4°C overnight and divide into aliquots in suitable sealed tubes and store frozen until required.

**Production of soluble antigen for ELISA**

i) Produce a suspension of RH strain *T. gondii* tachyzoites in PBS.

ii) Centrifuge at 2000 $g$ for 15 minutes, retain the pellet and resuspend it in nine times its volume of distilled water.

iii) Rupture the tachyzoites by freezing and thawing three times.

iv) The antigen preparation is then sonicated for 20 seconds at 4°C at an amplitude of 20 microns.

v) Remove any cellular debris by centrifugation at 10,000 $g$ for 30 minutes at 4°C.

vi) Retain the supernatant and store at –20°C until required. (Protein estimation might be expected to give a value of between 2 and 4 µg/ml.)

**Protocol for the IFA test**

The following is a protocol for carrying out an IFA test for anti-*Toxoplasma* IgG antibodies in sheep serum. It only requires minor modifications for testing different species or for measuring IgM antibody.

i) Clean the required number of tissue culture 15-well multitest slides (Flow laboratories) and allow to dry.

ii) Layer 5 µl of a whole tachyzoite preparation on to each well and allow to dry.

iii) Fix in methanol for 10 minutes.

iv) Carry out two 10-minute washes in 0.3 M PBS, pH 7.4.

v) Add 5 µl of the given test sheep serum (diluted in PBS) to each well. (Prepare serial dilutions of the test sera, e.g. 1/16, 1/32, etc. up to 1/1024.) Ensure that positive and negative control sera are included in each test as well as a ‘PBS-only’ sample. Incubate for 30 minutes at room temperature.

vi) Carry out two 10-minute washes in PBS.

vii) Add 5 µl of an appropriate dilution of rabbit-anti-sheep IgG conjugated to fluorescein isothiocyanate, diluted in 0.2% filtered Evan’s blue dye in PBS, to each well and incubate for 30 minutes at room temperature.

viii) Carry out three 10-minute washes in PBS.

ix) Mount the slides under cover-slips with buffered glycerol (nine parts PBS one part glycerol) or Citifluor (Citifluor Ltd, London).

x) Examine using a fluorescence microscope, fitted with ×20 and ×40 objective lenses.

With a negative test serum result the whole parasites will appear red due to the autofluorescence of the Evan’s blue dye. They may also present with a green fluorescent cap at the parasite pole (nonspecific polar fluorescence). With a positive test serum the parasites will fluoresce red and at least 80% of them within a given well will be surrounded by an unbroken band of green fluorescence. In an adult sheep/goat a positive titre could be defined as 1/64 and a negative titre as 1/32. For lamb/kid and fetal sera, respective titres could be defined as 1/32 and 1/16.
An example slide set-up is shown below:

<table>
<thead>
<tr>
<th>Sample 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/16 → 1/32 → 1/64 → 1/128 → 1/256</td>
</tr>
<tr>
<td>1/512 → 1/1024 PBS only</td>
</tr>
<tr>
<td>1/1256 ← 1/128 ← 1/64 ← 1/32 ← 1/16</td>
</tr>
</tbody>
</table>

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

The only available vaccine is a commercially produced live preparation for sheep (Toxovax, Intervet BV, The Netherlands; Toxovax, AgVax, Ag Research, New Zealand), currently licensed for use in the UK, Ireland, France and Spain and New Zealand. It consists of tissue culture grown S48 T. gondii tachyzoites attenuated by over 3000 passages in mice. The vaccine stimulates effective protective immunity for at least 18 months following a single subcutaneous injection, but as it is unable to produce tissue cysts, sheep are not left with a persistent vaccinal infection. The vaccine has a short shelf life and is a potential risk to immunosuppressed and pregnant female operatives (2).

**REFERENCES**


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1126 OIE Terrestrial Manual 2004
CHAPTER 2.10.13.

VEROCYTOTOXIGENIC ESCHERICHIA COLI

SUMMARY

Escherichia coli are normal inhabitants of the gastrointestinal tract of animals and humans. Some strains have become highly adapted to cause diarrhoea and a range of extra-intestinal diseases. Since 1977, it has been recognised that some diarrhoegenic strains of E. coli produce toxins that have an irreversible cytopathic effect on cultured Vero cells. Such verocytotoxigenic E. coli (VTEC) belong to over 100 different serotypes. Escherichia coli O157:H7 is the predominant and most virulent serotype in a pathogenic subset of VTEC, designated enterohaemorrhagic E. coli (EHEC). This designation is based on their capacity to cause haemorrhagic colitis and haemolytic uremic syndrome in humans, their ability to produce verocytotoxins, their ability to cause attaching and effacing lesions on epithelial cells, and their possession of a characteristic large plasmid. In the past two decades, VTEC O157:H7 has risen in importance world-wide as a public health problem. Other non-O157 serogroups, including O26, O91, O103, O104, O111, O113, O117, O118, O121, O128 and O145, have been associated with occasional outbreaks of human disease, and others still with sporadic cases. VTEC have been isolated from the faeces of a variety of animals, mostly healthy carriers, including cattle, sheep, goats, pigs, cats, dogs, chickens and wild birds. Cattle are considered to be the main reservoir of E. coli O157:H7 infection for humans. Despite its pathogenicity for humans, infection in animals with E. coli O157:H7 is invariably asymptomatic. By contrast, the EHEC serogroups, O26, O111 and O103 may be pathogenic for both humans and animals. The presence of VTEC in animal faeces provides the potential for these organisms to enter the food chain by faecal contamination of milk products, contamination of meat with intestinal contents during the slaughter process or contamination of fruit and vegetables by contact with infected manure. VTEC are also transmitted through water and by direct contact with infected people or animals.

Identification of the agent: Diagnostic procedures for VTEC have been developed, primarily for E. coli O157:H7, and seek to overcome the problems of isolating low numbers of target organisms from complex matrices such as animal faeces, food and clinical specimens. Identification of E. coli O157:H7 in subclinical animal carriers depends on enrichment of faeces samples in liquid media, usually buffered peptone water with or without the addition of vancomycin, cefsulodin and cefixime, for 6 hours at 37°C followed by immunomagnetic separation using commercially available paramagnetic particles or beads coated with anti-O157 lipopolysaccharide antibody. Beads with bound bacteria are plated on to selective agar, commonly 1% sorbitol MacConkey agar containing cefixime and potassium tellurite, and incubated for 18 hours at 37°C. Non-sorbitol-fermenting colonies are confirmed biochemically as E. coli and by serum or latex agglutination as possessing the O157 somatic antigen and/or H7 flagellar antigen. Potential virulence for humans is confirmed by the demonstration of verocytotoxin production by Vero cell assay, enzyme-linked immunosorbent assay (ELISA) or agglutination tests or the demonstration of genes encoding verocytotoxin by polymerase chain reaction. Detection of non-O157 VTEC relies on direct analysis of colonies on nonselective plates by, for example, immunoblotting or DNA probing for verocytotoxin production. Numerous immunological and nucleic acid recognition tests have been described to provide a more rapid presumptive diagnosis of VTEC and many are available commercially. Phage typing and pulsed field gel electrophoresis are widely used by reference laboratories for subtyping VTEC O157 for epidemiological purposes.

Serological tests: Serological tests are not used routinely in animals to diagnose VTEC infection, but it has been shown that cattle infected with VTEC produce serum antibodies to the O157 lipopolysaccharide that can be detected by ELISA.
Requirements for vaccines and diagnostic biologicals: No vaccines are currently available for controlling VTEC infections in animals or humans, but a variety of experimental vaccines are being developed.

A. INTRODUCTION

*Escherichia coli* are normal inhabitants of the gastrointestinal tract of animals and humans of which only some strains have become highly adapted to cause diarrhoea and a range of extra-intestinal diseases. *Escherichia coli* are routinely characterised by serological identification of somatic O, flagellar H and capsular K antigens. However, while some serotypes correlate closely with certain clinical syndromes, differentiation of pathogenic strains from the normal flora depends on the identification of virulence characteristics. Since 1977, it has been recognised that some diarrhoeagenic strains of *E. coli* produce toxins that has an irreversible cytopathic effect on cultured Vero cells (16). Such verocytotoxigenic *E. coli* (VTEC) have been shown to belong to over 100 different serotypes (13, 26). They are also described as Shiga toxin-producing *E. coli* (STEC) due to the similarity demonstrated between verocytotoxins (VT) and Shiga toxins (Stx) of *Shigella dysenteriae* (22). In the past two decades, VTEC O157:H7 has risen in importance world-wide as a public health problem. *Escherichia coli* O157:H7 is the predominant and most virulent serotype in a pathogenic subset of VTEC, designated enterohaemorrhagic *E. coli* (EHEC). This designation is based on their capacity to cause haemorrhagic colitis and haemolytic uraemic syndrome in humans, their ability to produce VT, their ability to cause attaching and effacing lesions on epithelial cells, and their possession of a characteristic large plasmid (21). Other non-O157 serotypes, including O26:H11, O104:H21, O111:H– and O145:H–, have been associated with occasional outbreaks of human disease, and others still with sporadic cases (13).

VTEC have been isolated from the faeces of a variety of animals, mostly healthy, including cattle, sheep, goats, pigs, cats, dogs, chickens and wild birds (3, 13). Surveys have shown that O157 strains represent a minority of the VTECs that colonise the intestinal tract of animals. The presence of VTEC in animal faeces provides the potential for these organisms to enter the food chain by faecal contamination of milk, contamination of meat with intestinal contents during slaughter or contamination of fruit and vegetables by contact with infected manure. VTEC are also transmitted through water and by direct contact with infected people, animals or animal waste. Cattle are considered to be the main reservoir of *E. coli* O157:H7 infection for humans, although the organism has been isolated from a variety of farmed animals, horses, dogs, rabbits, birds and flies. Despite its ability to cause severe disease in humans (23), infection in animals with *E. coli* O157:H7 is invariably subclinical. Some non-O157 serotypes, however, are pathogenic for animals and humans and include O26:H11; O103:H2; O111:H– (2, 13).

VTEC are also associated with oedema disease in piglets with four serotypes responsible for the majority of outbreaks world-wide, namely O45:K+, O138:K81, O139:K82 and O141:K–. The main virulence factors are a fimbrial adhesin, F18, involved in colonisation and the VT2e toxin, which is responsible for clinical signs. A high degree of genetic relatedness between O101 strains harbouring stx2e genes of human and porcine origin has been demonstrated, the role of pigs as subclinical carriers of STEC in the epidemiology of human disease needs further research.

Because *E. coli* O157:H7 has become the predominant zoonotic VTEC, diagnostic methods have been developed to detect selectively this serotype in human clinical cases (26) and in food sources (27). In this chapter, however, emphasis will be given to the isolation and identification of O157 and other VTEC from carrier animals (8).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

   - Samples

   In most cases, samples taken from animals for VTEC isolation will be faeces collected for surveillance purposes or as part of an epidemiological trace-back exercise following an outbreak of disease in humans. Samples may be taken from the rectum or from freshly voided faeces on the farm or from intestinal contents after slaughter. A variety of VTEC are present in healthy animals and not all are thought to be pathogenic for humans. Some isolates of *E. coli* O157, particularly from pigs, are non-verocytotoxicigenic and nonpathogenic for humans. Diagnosis, therefore, must include the demonstration of known virulence factors in the isolates. These include the verocytotoxins VT1 (Stx1) and VT2 (Stx2) and their genes and an outer membrane adhesion protein associated with attaching and effacing lesions, intimin, which is encoded by the eae gene (18). *Escherichia coli* O157:H7, which is the most significant VTEC in human disease, is carried subclinically in animals. Cattle are
thought to be the most important reservoir of this serotype. In an infected herd, only a proportion of the animals will be detectably infected, the organism is usually present in carriers in low numbers and is shed intermittently in faeces. Shedding is influenced by the age of the animals, diet, stress, population density, geographical location and season (20). Isolation rates may be improved by taking faeces samples in preference to rectal swabs, by increasing the sample size, by increasing the number of individuals sampled and by repeat sampling. Precautions should be taken to avoid cross-contamination of samples in transit and at the laboratory. Samples should be kept cool and cultured as soon as possible after collection.

- **Safety**

Care should be exercised when handling VTEC-positive samples as the infective dose capable of causing severe human infection may be low (possibly 100 organisms for VTEC O157:H7) and laboratory-acquired infections have been reported (see Chapter 1.1.6. Human safety in the veterinary microbiology laboratory).

- **Isolation**

a) **Liquid enrichment media**

Clinical samples are routinely plated directly on to solid media for isolation of *E. coli*, but the number of target VTEC organisms in faeces from healthy carriers is usually low and enrichment in liquid media improves recovery. Commonly used enrichment media are buffered peptone water supplemented with 8 mg/litre vancomycin, 10 mg/litre ceftuxolin and 0.05 mg/litre cefixime (BPW-VCC) to suppress the growth of Gram-positive organisms, *Aeromonas* spp. and *Proteus* spp.; modified trypticase–soy broth (mTSB) supplemented with 20 mg/litre novobiocin or 10 mg/litre acriflavin to reduce the growth of Gram-positive organisms; or modified *E. coli* broth with 20 mg/litre novobiocin (mEC+n). *EHEC E. coli* grow poorly at 44°C. The optimal incubation for bovine faeces to minimise overgrowth by other organisms is 6 hours at 37°C. For meat samples, enrichment for 6 hours at 41–42°C is used and for water and dairy products, 24 hours at 41–42°C. Nonselective pre-enrichment is necessary for the effective recovery of low levels of stressed *E. coli* O157. Enrichment broths should be pre-warmed to prevent cold-shocking the organisms and slowing their initial growth; 24 hours' incubation may increase recovery if the organisms are stressed.

b) **Immunomagnetic separation**

Immunomagnetic separation (IMS) has been used as a selective concentration technique to improve isolation of *E. coli* O157:H7 where numbers of the organism are low (7). Commercially available paramagnetic particles or beads coated with anti-lipopolysaccharide (LPS) antibody are mixed with the test sample. Beads with bound bacteria are separated from the supernatant by a magnetic field and after washing are plated on to selective agar and incubated for 18 hours at 37°C to isolate suspect colonies. The technique is serogroup specific. Commercial systems are available for manual or automated separation (6). Recovery may be affected by the bead-to-organism ratio (optimum is 3:1), the enrichment broth used and the problem of nonspecific adsorption of *E. coli* to the magnetic beads (which can be reduced by the use of a low ionic strength solution in the IMS procedure and careful washing). These factors should be taken into account when trying to maximise the sensitivity of the technique for detecting target *E. coli*.

c) **Selective culture for *Escherichia coli* O157**

There are no biochemical characteristics that distinguish the majority of VTEC from other *E. coli*, however, the inability of most strains of *E. coli* O157:H7 to ferment D-sorbitol rapidly and their lack of beta-glucuronidase activity can be exploited in the isolation and identification of these organisms. However, the less common *E. coli* O157:H+ (nonmotile) isolates, which are sorbitol fermenters and beta-glucuronidase positive, will not be identified by isolation in such selective media chosen for these biochemical characteristics (15). MacConkey agar containing 1% D-sorbitol instead of lactose (SMAC) is a useful and inexpensive medium on which non-sorbitol-fermenting *E. coli* grow as small, round greyish-white colonies. Selectivity is improved by the addition of 0.5% rhamnose, and addition of 0.05 mg/litre cefixime (CR-SMAC) inhibits overgrowth by *Proteus* spp. While fewer presumptive colonies require testing on this medium, rhamnose is an expensive supplement. An alternative modification is the addition of 2.5 mg/litre potassium tellurite in addition to cefixime (CT-SMAC), which has a greater inhibitory effect against non-O157 *E. coli* and other non-sorbitol fermenters, such as *Aeromonas, Plesiomonas, Morganella* and *Providencia*, than against *E. coli* O157 (28). This is currently the most commonly used medium for isolating *E. coli* O157.

Media containing fluorogenic or chromogenic glucuronides are used to distinguish non-beta-glucuronidase-producing *E. coli* O157:H7 from beta-glucuronidase-producing *E. coli*. Hydrolysis of 4-methylumbelliferyl-beta-D-glucuronide (MUG) by beta-glucuronidase activity produces a fluorescent compound visible under UV light. The addition of 0.1 g/litre 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide (BCIG) to SMAC differentiates white colonies of *E. coli* O157:H7 from green-blue colonies of sorbitol negative, beta-glucuronidase positive organisms. Commercially available chromogenic and fluorogenic media may be found by reference to media catalogues. While advances have been made in improving the selectivity of media for *E. coli* O157:H7, isolation rates, particularly of stressed organisms, may be adversely affected by
the additives used. To mitigate against these effects, addition of recovery agents such as 1% sodium pyruvate to tryptone–soy agar or delaying exposure of stressed cells to selective agents can aid recovery of the organism (5).

d) Isolation of other VTEC
Non-O157 VTEC grow well on media that permit the growth of E. coli, such as blood agar or MacConkey agar, and the majority can only be differentiated from other E. coli by their ability to produce VT. The large number of different VTEC serotypes precludes the use of O-antisera for the routine screening and presumptive identification of colonies on these media. IMS can be used for selective concentration of serogroups O26, O103, O111 and O145 from a pre-enriched sample, as for the O157 strains. These serogroups are the non-O157 VTECs most commonly associated with human disease, but commercially produced beads are currently available for research purposes only.

The inability of O26 strains to ferment rhamnose has led to the recent development of media that may prove to be useful in differentiating O26 E. coli from other enteric organisms. The first is rhamnose-MacConkey agar (RMAC) in which the lactose in the MacConkey medium is replaced by 10 g/litre rhamnose. Addition of 2.5 mg/litre potassium tellurite and 0.05 mg/litre cefixime (CT-RMAC) is said to improve specificity. The second is a chromogenic rhamnose agar incorporating 10 g/litre rhamnose and 0.02 g/litre phenol red in ES coliform agar (an indicator medium for beta-galactosidase activity) to which is added 0.5 mg/litre potassium tellurite and 0.05 mg/litre cefixime. On this medium, O26 colonies are reported to be dark blue to black, other E. coli serotypes are green, and enterobacteria other than E. coli are green, yellow or colourless.

One potentially useful virulence marker for VTEC is enterohaemolysin production, which causes haemolysis of washed sheep erythrocytes after overnight incubation on blood agar supplemented with calcium. This characteristic is shared by 90% of VT-producing E. coli isolated from human infections. However, the finding that a proportion of disease-producing VTEC can be negative for enterohaemolysin production reduces the value of enterohaemolysin agar as a screen.

In most cases, therefore, isolation of VTEC relies on direct analysis of colonies on plates by immunoblotting or DNA probing for VT production to identify colonies for further characterisation. Colonies are first replicated so that positive colonies can be isolated after replicates have been tested. Colonies may be blotted on to suitable membranes (nitrocellulose or nylon) from which replicates are made or picked off into 96-well microtitre plates containing broth for replication before transferring aliquots to appropriate filters. Colonies are then analysed using nucleic acid probes or antibodies to identify any VTEC (26). Hull et al. (11) developed a mitomycin immunoblot assay for detecting VTEC in faeces that was simple enough to use in routine diagnostic laboratories. Serial dilutions of faeces in broth are inoculated on to MacConkey agar plates and incubated overnight at 37°C. Using standard replica plating techniques, growth from the plate yielding approximately 200 colonies is transferred to two 0.45-µm pore-size nitrocellulose filters laid on Syncase agar with 25 ng mitomycin/ml. This medium induces vegetative growth of bacteriophages carrying the genes for VT and enhances toxin expression. (Alternatively, bacterial or faecal suspensions may be plated directly on to the filters.) The plates are incubated overnight at 37°C. After overnight growth, filters are removed from the plates, immersed in a chloroform bath for 15 minutes, then blocked for 1 hour with 5% non-fat milk in 10 mM Tris, 150 mM NaCl, 0.05% Tween (pH 8) (TNT). The filters are incubated for 1 hour in antisera raised against VT1 or VT2, given three 5-minute washes in TNT, then incubated for 1 hour with alkaline phosphatase-conjugated anti-immunoglobulin G followed by three further 5-minute washes in TNT. Any reaction is visualised by colour development with nitroblue and 5-bromo-4-chloro-3-indolyl-phosphate. VT1, VT2 and VT-negative control E. coli are tested in parallel. The use of polyclonal antibodies results in some false positives that are eliminated by using monoclonal antibodies. When the use of DNA probes was compared with the use of the mitomycin immunoblot colony assay, it was shown that the results were comparable. The immunoblot assay has the advantage of being simpler to perform than DNA probing. Mitomycin plates have a long shelf life when stored in the dark at 4°C.

Colony immunoblotting or probing are labour intensive techniques and may be better applied to samples that have been screened and shown to be positive for the presence of VT or VT genes by, for example, enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR).

- **Identification and characterisation of suspect colonies**

Colonies growing on solid media that are suspected to be VTEC must be confirmed biochemically to be E. coli. Somatic ‘O’ and flagellar ‘H’ antigens are identified serologically, and to confirm virulence, the organisms have to be shown to be VT-producers. For VTEC O157 strains, subtyping methods are available in reference laboratories for epidemiological investigations.

- **Biochemical tests**

VTEC are biochemically similar to other E. coli. VTEC O157:H7 strains differ in failing to ferment sorbitol, failing to produce beta-glucuronidase and fermenting raffinose and dulcitol. *Escherichia coli* can be
distinguished from *E. hermanii* by lack of growth in the presence of potassium cyanide and failure to ferment cellulbiose. *Escherichia hermanii* is positive for both tests. Ninety-eight per cent of *E. hermanii* strains have a characteristic yellow pigment on nutrient agar that is not seen in VTEC. *Escherichia coli* may be confirmed by demonstration of the use of tryptophan and beta-galactosidase activity (see below) or by commercially available biochemical test strips.

b) **Serological tests**

Commercial latex kits are available for O157, O26, O91, O103, O111, O128, O145 and H7. Tests should be carried out according to the manufacturer’s instructions and should incorporate positive and negative control organisms and control latex. Presumptive diagnosis may also be made using slide or tube agglutination tests with anti-O LPS antiserum (antisera to 181 O– antigens are available). O157 antiserum has been shown to cross-react with other organisms including *E. hermanii* (frequently found in foods), *Salmonella* O group N, *Yersinia enterocolitica* serotype O9 and *Citrobacter freundii*, indicating the need to confirm putative VTEC colonies as *E. coli*. Isolates can be tested for the presence of flagellar antigen (antisera have been raised to 56 H-antigens), but this may require passage through motility medium. Some pathogens are nonmotile.

c) **Verocytotoxin production in Vero cell assay** (16)

The Vero cell assay remains a standard method for the confirmation of VT production (see below). Vero cells have a high concentration of globotriaosylceramide (Gb3) and globotetraosylceramide (Gb4) toxin-binding receptors in their plasma membranes and will detect all variants of VT. The test can be used on faecal suspensions, culture filtrates or live cultures. In mixed faecal cultures, sensitivity of the assay is increased by treating the suspension with polymyxin B or mitomycin to release cell-associated toxin. While the test is sensitive, it is not available in all routine diagnostic laboratories. It is labour intensive and results can take 3–4 days after the cell culture is inoculated. Where tissue culture facilities are not available, other methods may be used for detecting VT production, including ELISA or agglutination and PCR can detect the vt genes. All of these methods are now available as commercial kits.

d) **Subtyping of Escherichia coli O157 for epidemiological studies**

A variety of methods is available in reference laboratories to help discriminate between strains of *E. coli* O157:H7 to aid epidemiological investigations of outbreaks of human disease (10, 26). These methods vary in technical complexity and more than one technique is required to provide useful differentiation. Techniques include phage typing, biotyping and antimicrobial sensitivity testing, plasmid profiling, restriction fragment length polymorphism analysis, ribotyping, pulsed field gel electrophoresis (PFGE) and various PCR-based analyses (random amplification of polymorphic DNA; repetitive DNA element PCR; amplified fragment length polymorphism analysis). Of these, only phage typing and PFGE are widely used. Despite some difficulties with interpretation of profiles, PFGE has emerged as the standard method used by public health reference laboratories for subtyping VTEC O157 due to its high level of discrimination and accuracy and reproducibility. It is used in ‘Pulsenet’, a network of public health laboratories performing a standardised PFGE method that allows comparison of fingerprints held on an electronic database by the Centres for Disease Control and Prevention in the USA (www.cdc.gov/ncidod/dbmd/pulsenet/pulsenet.htm). The European Union’s ‘Enter-net’ system for the surveillance of *Salmonella* and VTEC relies largely on phage typing to subtype *E. coli* O157:H7 strains. Subtyping methods for non-O157 serotypes has been less well explored, however, similar approaches to those used for VTEC O157 can be taken.

- **Non-culture techniques for detecting VTEC**

Although definitive diagnosis of VTEC relies on the isolation and characterisation of pure cultures, cultural methods for VTEC are time-consuming and labour intensive. This has led to the development of a range of immunological and nucleic acid hybridisation tests for rapid identification of O and H antigens, VT or genes associated with VT production in the sample. As the tests have a detection level above the numbers at which the target organism is normally present in the food or faeces, an enrichment step (preferably nonselective for associated with VT production in the sample. As the tests have a detection level above the numbers at which the target organism is normally present in the food or faeces, an enrichment step (preferably nonselective for isolation of injured or stressed bacteria) is required to increase the numbers prior to testing.

a) **Immunological methods**

Immuonassays to identify O and H antigens and VT may be used to confirm the identity of the organisms once isolated from clinical, food or environmental samples, while others, including dipstick and membrane technologies, microplate assays, colony immunoblotting, immunofluorescence and ELISA, are used as rapid methods for detecting the presence of potential pathogens in samples prior to isolation thus shortening the time for a presumptive diagnosis. Most assays for somatic and flagellar antigens are designed to detect the O157 LPS and H7 flagellar antigen. Toxin assays have the advantage of detecting all VTEC. Enzyme immunoassays for O157 and VT, visual immunoassays for O157 and agglutination tests for O157, H7 and VT are available commercially as kits (4, 8, 21, 26). Not all have been validated for use with faeces. Specialised reagents in which anti-O157 LPS antibodies are conjugated to fluorescein, peroxidase
or phosphatase are also available. Of the enzyme immunoassays, the most commonly used format is a sandwich assay. Antibody is bound to a carrier surface to capture a specific VTEC antigen; following the addition of an appropriate substrate, a second antibody with an enzyme label binds to this antigen and produces a colour reaction. The kits have been validated with specific pre-enrichment protocols and reagents to ensure reproducible results. Some use heat-treated samples thus improving the safety of the test, and some incorporate an automated processing system to screen large numbers of samples. Others are blot ELISAs developed to screen colonies for O157 antigen. The commercial kits have the advantage of being easy to perform in routine laboratories, and tests should be carried out according to the manufacturers’ instructions. Kits validated for food and carcase samples or for human clinical samples may lack sensitivity for animal faeces samples. Immunological assays only give a presumptive result, which must be confirmed by isolation and characterisation of the organisms producing the O157 antigen or the toxin.

b) Nucleic acid recognition methods

i) Colony hybridisation assays

Colony hybridisation is a useful means of detecting VTEC in mixed culture for further characterisation. DNA probes and synthetic oligonucleotide probes are available labelled with digoxigenin or biotin and therefore suitable for use in routine diagnostic laboratories. Assays have been described to detect VT genes, the 60 MDa plasmid in *E. coli* O157 and the *eae* gene individually and in combination (21, 23, 26). Hybridisation assays are less sensitive for detecting VTEC in broth cultures or faecal extracts.

ii) PCR for VT genes and other virulence markers

Many PCRs are described in the literature for detection of VT1, VT2 and VT2 variant genes (21, 23, 26), and a number of these toxin-typing PCR methods has recently been compared (29). Demonstration of the genes associated with VT-production does not confirm gene expression and hence production of toxin. PCR can be used on pure or mixed plate or broth cultures, and extracts from food or faeces. It can also be used to detect genes in non-viable organisms. As well as its role in diagnosis, PCR has the potential to be used to screen samples for VTEC in epidemiological studies. Amplification of target genes in bacterial DNA extracts from faeces is less successful than from pure cultures, and careful preparation of the sample is required to improve sensitivity. Faeces contain non-specific PCR inhibitors and no single method of removing these is ideal. Sensitivity is improved by non-selective enrichment prior to testing, but remains lower than using IMS or the Vero cell cytotoxicity assay. Commercial assays are available.

DNA probes and PCR assays have also been developed to detect other genes in VTEC shown to be associated with virulence in humans, including *eae* (encoding for intimin), *ehx* (encoding for enterohaemolysin production), *fliC* (encoding the H7 antigen), O157 *rfb* (encoding O157 LPS), *uidA* (the mutant glucuronidase gene in beta-glucuronidase-negative *E. coli* O157:H7) and *katP* (a gene carried on the large plasmid of *E. coli* O157:H7 encoding a novel catalase peroxidase) (21, 23, 26). A variety of multiplex assays has been developed to detect simultaneously several diagnostic genes. These assays are of value in the characterisation of pure cultures. On mixed populations of bacteria in food or faeces samples, they may have a use in identifying samples to which isolation procedures should be targeted.

• Screening faeces for *Escherichia coli* O157:H7

*Escherichia coli* O157:H7 is the VTEC of greatest public health concern. Its carriage in the intestinal tract of healthy animals, particularly cattle, represents a source of direct and indirect infection to humans. Screening relies on cultural techniques designed to overcome the problems of isolating low numbers of organisms, possibly in a stressed state, from a competing background flora followed by identification of suspect colonies and demonstration of known virulence characteristics. These methods are still evolving and the following is a description of the methods routinely employed in one national veterinary laboratory. Suitable precautions should be taken to avoid human infection (see Chapter I.1.6.).

a) Pre-enrichment

i) Transport faeces in sterile, leak proof, closed containers at 4°C and culture as soon as possible, preferably within 2 hours of collection. Faeces intended for storage for longer than 24 hours should be frozen at −70°C.

ii) Mix faeces at a dilution of 1/10 in warmed buffered peptone water (BPW) in a labelled container.

iii) Incubate at 37°C±2°C for 6 hours.

iv) Include positive and negative control cultures.
b) Immunomagnetic separation

i) Use of Dynabeads® anti-\textit{E. coli} O157 product 710.04 (Dynal Biotech, ASA, Oslo, Norway) meets the requirements of AFNOR (DYN 16/02-0696 and DIN 10167); it is cited in the USA Food and Drug Administration's Bacteriological Analytical Manual (www.cfsan.fda.gov/~ebam/bam-4a.html) and the Health Canada Compendium of Analytical Methods (www.hc-sc.gc.ca/food-aliment/mh-dm/mhe-dme/compendium/volume_3/e_mflp9001.html) and is the official method of the Japanese Health Ministry.

ii) Following the instructions of the manufacturers, carry out immunomagnetic separation (IMS) on the pre-enriched samples using the manual (MIMS) or automated (AIMS) method. Care should be taken to mix the beads well before use and to avoid cross-contamination between prepared tubes. If using the manual method, adherence to the instructions for careful washing of the bead–bacteria complexes is essential.

iii) After the final wash, use a micropipette to transfer 50 µl of each bead–bacteria suspension to a labelled sorbitol MacConkey agar plate containing cefixime and potassium tellurite (CT-SMAC) (28) taking care to avoid cross-contamination.

iv) Using a sterile swab, spread the drop over one-third to one-half of the plate to break up the complexes. Using a sterile 1 µl loop, dilute the bead–bacteria complexes further over one quadrant by streaking out at right angles from the previously streaked area. Using a second sterile loop, streak out at right angles from this quadrant into the final unstreaked area of the plate to obtain single colonies. Incubate at 37°C±2°C for 16–18 hours (sorbitol-fermenting colonies lose colour after this time and may be confused with non-sorbitol fermenting \textit{E. coli} O157). An alternative method for isolating sorbitol-negative colonies is to spread the entire inoculum over the surface of a dry CT-SMAC plate with a sterile bent rod.

c) Colony identification

i) Pick off up to 10 white, sorbitol-negative colonies per plate and test by O157 latex agglutination following the manufacturer’s instructions (include appropriate positive and negative control organisms and latex control).

ii) Subculture agglutination-positive colonies on to solid medium without antibiotics (e.g. 5% sheep blood agar). streak to obtain single colonies. Incubate at 37°C±2°C overnight.

d) Confirmation of \textit{Escherichia coli}

i) Inoculate o-nitrophenyl beta-D-galactopyranoside (ONPG) broth. Set up positive and negative controls. Incubate overnight, aerobically at 37°C. \textit{Escherichia coli} produce a positive result indicated by a change to yellow colouration confirming beta-galactosidase activity.

ii) Place a circle of 0.45 µm cellulose nitrate membrane filter paper on to a plate of tryptone bile agar (TBA) using sterile forceps. Use a 1 µl loop to remove a loopful of growth to be tested and inoculate a pea-sized area on the surface of the Millipore filter. Set up positive and negative controls. Incubate at 44°C for at least 17 hours. Transfer the membrane to filter paper soaked with indole reagent for the detection of the use of tryptophan. \textit{Escherichia coli} show a positive reaction indicated by a purple/pink colouration.

iii) A commercial reagent for detection of indole is available. The reagent is placed on to filter paper and a portion of the colony rubbed into the reagent spot. This requires less than 5 minutes and can be backed up by the described test if suspicious colonies appear negative.

iv) Alternatively, use commercially available biochemical test strips to confirm \textit{E. coli}.

e) Somatic determination (24)

i) Using a sterile loop, pick off a single colony obtained by the colony-identification method described in point c above and subculture into 4 ml of Schlecht broth. Incubate at 37°C±2°C overnight.

ii) Boil the Schlecht broth for a minimum of 1 hour at 100°C.

iii) Dispense 25 µl of 0.85% saline into wells 2 to 12 of a U-well microtitre plate. Dispense 50 µl of O157 antiserum into well 1. Make a doubling-dilution series of the antiserum to 1/1024, discarding 25 µl after mixing well. Add 50 µl of boiled broth suspension to wells 1 to 12. Cover the plate to prevent evaporation and incubate at 37°C for 6 hours. Use a black background to identify agglutination in the wells.

f) Vero cell assay

i) Using a sterile loop, pick off a single colony obtained by the colony-identification method described in point c above and subculture into 4 ml of Mundell broth. Incubate at 37°C±2°C overnight.
ii) Set up broths with control strains of organisms producing no toxin, thermolabile enterotoxin (LT), cytotoxic necrotising factor (CNF) and verocytotoxin (VT). Incubate at 37°C±2°C overnight.

iii) Dispense Vero cells (African green monkey kidney cells, reference ATCC CCL81, seeding rate 2 × 10^5/ml) into flat-well microtest plates, 200 µl to each well, 24 hours before inoculation. Incubate at 37°C±2°C in 5% CO₂ for 24 hours.

iv) Add 100 µl of a 400,000 units/ml solution of polymyxin B sulphate in sterile distilled water to each overnight broth culture. Incubate at 37°C±2°C for 5 hours.

vi) Centrifuge the broths at 3000 rpm for 30 minutes.

vii) Remove supernatants into labelled sterile containers (approximately 1.5 ml required).

viii) Place the Vero cell plate on a numbered worksheet to identify each well. Incubate 10 µl of prepared supernatant into the relevant well of Vero cells. Return Vero cells to the CO₂ incubator and incubate for 3 days.

ix) Examine cells after 24 hours, 48 hours and 72 hours to observe any cytopathic effect. Compare with positive and negative test controls. With VT-positive samples, the cell sheet becomes disintegrated with blackened, shrivelled cells observed between 24 and 72 hours.

g) **Multiplex PCR for VT1, VT2 and eae** (1, 12, 25)

Multiplex PCR is used to confirm the presence of virulence determinants using primers as shown below:

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Accession no.</th>
<th>Primer sequence</th>
<th>Nucleotide position</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VT1</td>
<td>M19437</td>
<td>F (5'-CGC-TCT-GCA-ATA-GGT-ACT-CC-3')</td>
<td>287–306</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R (5'-CGC-TGT-TGT-ACC-TGG-AAA-GG-3')</td>
<td>522–541</td>
<td></td>
</tr>
<tr>
<td>VT2</td>
<td>X07865</td>
<td>F (5'-TCC-ATG-ACA-ACG-GAC-AGC-AG-3')</td>
<td>623–642</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R (5'-GC-TTC-TGC-TGT-GAC-AGT-GAC-3')</td>
<td>788–807</td>
<td></td>
</tr>
<tr>
<td>eaeA</td>
<td>X60439</td>
<td>F (5'-GC-TTA-GTG-CTG-GTT-TAG-GAT-TG-3')</td>
<td>271–293</td>
<td>618</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R (5'-CCA-GTG-AA-AC-GAC-TAC-GCG-AAG-3')</td>
<td>871–890</td>
<td></td>
</tr>
</tbody>
</table>

i) Using a sterile loop, pick off a single colony obtained by the colony-identification method described in point c above and subculture into 1 ml of Luria-Bertani broth. Set up three appropriate control broths. Incubate at 37°C±2°C overnight.

ii) Boil the broths for 15 minutes at 100°C. Remove from waterbath and allow to cool.

iii) Prepare master mix for 48 µl per sample containing:

1 × Saiki buffer (50 mM KCl; 10 mM Tris, pH 8.5; 100 µg/ml gelatin); 3 mM MgCl₂; 0.5 U Taq polymerase; 25 pmol of each primer (forward and reverse primers for VT1, VT2 and eaeA); 0.2 mM each of dATP, dCTP, dGTP and dTTP.

iv) Mix by inverting tubes and dispense 48 µl into each PCR reaction tube.

v) Add 2 µl of boiled culture (crude DNA extract) to the bottom of each reaction tube (include three control extracts and a media blank).

vi) Run the PCR using cycling parameters of initial denaturation at 94°C for 2 minutes; 25 cycles of 94°C for 1 minute, 62°C for 1.5 minutes and 72°C for 2 minutes; with a final extension of 72°C for 5 minutes. The reaction is held at 4°C until required for electrophoresis.

vii) Electrophorese 15 µl of each PCR sample on a 1.5% agarose gel in E buffer (10× strength solution made by adding to distilled water in the following order: 109 g/litre Tris, 55.6 g/litre ortho-boric acid, 9.3 g EDTA, made up to 1 litre with distilled water and adjusted to pH 8.0 with 10 ml concentrated hydrochloric acid diluted in distilled water before use). Run 100 bp step ladder molecular weight marker for comparison.

ix) Stain in ethidium bromide and view by transillumination.

x) Inspect control lanes to identify positions of VT1, VT2 and eae amplicons. Compare with bands present in test sample lanes. Record the results.
2. Serological tests

In humans, serodiagnosis of VTEC can be valuable, particularly later in the course of the disease when the causative organism becomes increasingly difficult to isolate from faeces. LPS has emerged as the antigen of choice, and production of serum antibodies to the LPS of a wide range of prevalent serotypes of VTEC has been demonstrated. Serological tests are not used for diagnosis of animal infection with VTEC. However, it has been shown that exposure of cattle to E. coli O157:H7 infection results in the production of antibodies against the O157 LPS, which persist for months, demonstrable by the indirect ELISA (14). Cross-reactions have been demonstrated between O157-LPS and the LPS antigens of other bacteria including E. coli O55, Salmonella spp., Yersinia enterocolitica, Brucella abortus and V. cholerae non-O1 strains. To reduce cross-reactivity, a blocking ELISA using a monoclonal antibody specific for E. coli O157 as the competing antibody has been developed for detection of serum antibodies to O157 antigen in cattle (17). Serum antibodies to VT1, but not to VT2, have been demonstrated in cattle by toxin neutralisation tests in Vero cell assays (14). Other studies have shown greater prevalence of VT1 neutralising antibodies in cattle sera than VT2 which may be explained by the greater prevalence of VT1-producing VTEC in cattle and/or the lesser immunogenicity of VT2.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are currently no vaccines available to control zoonotic VTEC. Various approaches to the immunological control of EHEC infections in humans are being explored (19). These are aimed at preventing colonisation, intestinal disease or the serious sequelae of haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura. They include the use of conjugate vaccines (e.g. O157 polysaccharide linked to the B-subunit of VT1 and VT2 as carrier proteins), live-vector vaccines, toxoid vaccine or passive immunisation with hyperimmune globulin or monoclonal antibodies against VT. However, were an effective vaccine to become available, there is debate about the social, political and economic consequences of widespread vaccination of people against pathogens in their food. As animals, mainly cattle, are thought to be the reservoirs of infection for the human population, a novel strategy being explored is to vaccinate cattle in order to reduce colonisation with pathogenic VTEC and thereby reduce contamination of food and the environment (i.e. to make food safer as opposed to protecting people against their food). One approach is to use a live, toxin-negative colonising strain as an oral vaccine to induce antibodies against surface components, and another is to deliver colonisation factors, such as intimin, as an edible vaccine in transgenic plants (9).

REFERENCES

Chapter 2.10.13. — Verocytotoxigenic Escherichia coli


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OIE Terrestrial Manual 2004 1137
CHAPTER 2.1.0.14.

LISTERIA MONOCYTogenes

SUMMARY

Listeriosis is one of the most important food-borne diseases. The disease manifestations in humans include septicaemia, meningitis (or meningoencephalitis) and encephalitis, usually preceded by influenza-like symptoms including fever. In pregnant women, intrauterine or cervical infections may result in spontaneous abortion or stillbirths. Listeria monocytogenes has also been associated with gastroenteric manifestations with fever. Although the morbidity of listeriosis is relatively low, the mortality of the systemic/encephalitic disease can be very high, with values in the vicinity of 30%. The elderly, pregnant women, newborns and the immunocompromised are considered to be at high risk of contracting the disease.

A wide variety of animal species can be infected with L. monocytogenes, but clinical listeriosis is mainly a ruminant disease, with occasional sporadic cases in other species. The main clinical manifestations of animal listeriosis are encephalitis, septicaemia and abortion, and the disease is primarily feed-borne. Post-mortem findings and histopathology depend on the clinical presentation.

A number of molecular and cellular determinants of virulence have been identified for this intracellular pathogen, and although there is evidence of polymorphism among different strains of L. monocytogenes for some of these virulence determinants, this heterogeneity cannot be correlated with the ability or inability of the organism to produce disease. Therefore, all L. monocytogenes strains are considered to be potentially pathogenic.

Identification of the agent: A variety of conventional and rapid methods are available for the detection and identification of L. monocytogenes in food samples and specimens from animal listeriosis. Conventional methods remain the ‘gold standard’ with which other methods are compared. They are usually very sensitive. These methods use selective agents and enrichment procedures to reduce the number of contaminating microorganisms and allow multiplication of L. monocytogenes.

Although not required for regulatory purposes, different levels of subtyping L. monocytogenes strains are available, including serotyping, phage typing, multilocus enzyme electrophoresis, DNA restriction enzyme digestion patterns (conventional and pulse-field gel electrophoresis [PFGE]), nucleic acid sequence-based typing and random amplification of polymorphic DNA (RAPD).

Serological tests: Serological tests have not been traditionally used for the diagnosis of listeriosis. A number of formats have been tried and they have all been found to be largely unreliable, lacking sensitivity and specificity. Experimental serological assays based on the detection of anti-listeriolysin O have been used in some epidemiological investigations and as support for the diagnosis of culture-negative central nervous system infections.

Requirements for vaccines and diagnostic biologicals: It has proven very difficult to develop effective vaccines against L. monocytogenes which, as an intracellular organism, requires effector T cells for an effective immune response. Experimental vaccines in laboratory animals are being explored to confer protection to L. monocytogenes infection by a number of different approaches, including immunisation with plasmid DNA, CD40 signalling along with heat-killed L. monocytogenes, listeriolysin O-deficient mutants inoculated along with liposome-encapsulated listeriolysin O, and immunisation with listerial antigens and IL-12.
A. INTRODUCTION

Although Listeria monocytogenes has been recognised as an animal pathogen for many years, its significant role as a food-borne human pathogen became evident only in the 1980s, when documented reports of listeriosis outbreaks, traced to contaminated food, started to appear in the literature (45). Today, L. monocytogenes is considered to be one of the most important agents of food-borne disease. Possible explanations for the emergence of human food-borne listeriosis as a major public health concern include major changes in food production, processing and distribution, increased use of refrigeration as a primary preservation means for foods, changes in the eating habits of people, particularly towards convenience and ready-to-eat foods, and an increase in the number of people considered to be at high risk for the disease (elderly, pregnant women, newborns, immunocompromised) (42, 49).

A wide variety of animal species can be infected by L. monocytogenes, including mammals, birds, fish and crustaceans, although most of the clinical listeriosis occurs in ruminants; pigs rarely develop disease and birds are generally subclinical carriers of the organism. Most infections in animals are subclinical, but listeriosis can occur either sporadically or in epidemic form. In addition to the economic impact of listeriosis in animals, there is a link between animals and their role as a source of infection for humans, either as a result of direct contact with infected animals, especially during calving or lambing, or after consumption of contaminated animal products (54). However, the relative importance of the zoonotic transmission of the disease to humans is not clear, and contamination from the food processing environment is apparently more relevant to public health (41).

The primary manifestation of listeriosis in humans may include septicaemia, meningitis (or meningoencephalitis) and encephalitis, usually preceded by influenza-like symptoms including fever. Gastroenteric manifestations with fever also occur. Although the morbidity of listeriosis is relatively low, the mortality can reach values around 30%. In pregnant women, infection may result in abortion, stillbirth or premature birth (42, 47).

In animals, the clinical manifestations of listeriosis include encephalitis, septicaemia and abortion, especially in sheep, goats and cattle. The septicaemic form is relatively uncommon and generally, but not invariably, occurs in the neonate. It is marked by depression, inappetence, fever and death. The encephalitic form is sometimes referred to as ‘circling disease’ because of a tendency to circle in one direction, and it is the most common manifestation of the disease in ruminants. The signs include depression, anorexia, head pressing or turning of the head to one side, unilateral facial paralysis, and bilateral keratoconjunctivitis. Abortion is usually late term (after 7 months in cattle and 12 weeks in sheep) (26, 53). Only one clinical form of listeriosis usually occurs in a particular group of animals. Ovine ophthalmitis has also been described (52). Mastitis of ruminants has also been associated with L. monocytogenes infection. When listeriosis occurs in pigs, the primary manifestation is septicaemia, with encephalitis reported less frequently and abortions rarely. Although birds are usually subclinical carriers, sporadic cases of listeriosis have been reported, most frequently septicaemia and far less commonly meningoencephalitis. Avian listeriosis may be the result of a secondary infection in viral disease conditions and salmonellosis (54).

The post-mortem findings and histopathology, in animal listeriosis, depend on the clinical presentation. In the encephalitic form, the cerebrospinal fluid may be cloudy and the meningeal vessels congested. Gross pathological lesions of the brain are rare. On occasion, the medulla shows areas of softening. However, the histopathology is characteristic of the disease, consisting of foci of inflammatory cells with adjacent perivascular cuffing, predominantly of lymphocytes and histiocytes, plasma cells and occasional neutrophils. The microabscesses in the brain stem are usually unilateral and may show liquefaction of the neuropil. The medulla and pons are most commonly involved. In the septicaemic form, multiple foci of necrosis in the liver and, less frequently the spleen, may be noted. Aborted fetuses of ruminants show very little gross lesions, but autolysis may be present if the fetus was retained before being expelled (38, 53).

The evidence indicates that animal listeriosis is predominantly a feed-borne disease, with the environment as the main source of contamination of feed. Silage is the most frequent source of feed-borne listeriosis (22, 55). The intestinal mucosa is the main route of entry, after oral ingestion, in the case of septicaemic abortive listeriosis. The incubation period can be as short as 1 day. Although the pathogenesis of encephalitic listeriosis is controversial, it appears that the microorganism can enter nerve endings through abrasions in the buccal mucosa, lips, nostril, conjunctiva or teeth and then migrate centripetally to cause a central nervous system infection. An alternative route of infection, for this form of listeriosis, might be haematogenous. The incubation period for the encephalitic form is usually 2–3 weeks, and the course of the disease is short; 1–4 days (41).

Listeria monocytogenes is a Gram-positive rod and is responsible for almost all infections in humans, although rare cases of infection due to L. ivanovii and L. seeligeri have been reported. In animals, L. monocytogenes is responsible for the majority of infections, but 10–15% of listeric septicaemia in sheep is due to L. ivanovii.

Although L. monocytogenes has definite zoonotic potential, it is also an important environmental contaminant of public health significance.
Chapter 2.10.14. – Listeria monocytogenes

Subtyping of *L. monocytogenes* strains by a variety of methods is available for epidemiological investigations, but the fundamental question of whether all strains of *L. monocytogenes* are capable of causing disease remains unanswered (23, 33, 35, 36).

Several molecular virulence determinants have been identified that play a role in the cellular infection by *L. monocytogenes* and the unravelling of their mechanism of action has made of *L. monocytogenes* one of the most exciting models of host-pathogen interaction at the cellular and molecular levels. These virulence determinants include, among others, the internalins, listeriolysin O (LLO), ActA protein, two phospholipases, a metalloprotease and a bile salt hydrolase (16, 19). Although there is polymorphism among different strains of *L. monocytogenes* for some of these virulence determinants, it cannot be correlated with the ability or inability of the organism to produce disease (33).

### B. DIAGNOSTIC TECHNIQUES

1. **Identification of the agent**

There is a variety of conventional and rapid methods currently available for the detection and identification of *L. monocytogenes* in food samples and specimens from animal listeriosis. Conventional bacteriological methods are important for various reasons: Their use results in a pure culture of the organism, which is useful for regulatory purposes. They remain the ‘gold standards’ against which other methods are compared and validated. These methods are usually very sensitive and they do not require sophisticated and expensive equipment. Some of the disadvantages of this group of methods include the relatively long period of time that the protocols require for completion, several ‘hands-on’ manipulations, the requirement for many different chemicals, reagents and media, the possibility of contaminating microorganisms masking the presence of the target ones, including overgrowth, the potential overlook of atypical variants of the target organism and the relative subjectivity involved when interpreting bacterial growth on selective and differential agar plates (1).

The isolation and identification of *L. monocytogenes* from food, environmental samples and animal specimens require the use of selective agents and enrichment procedures that keep the levels of contaminating microorganisms to reasonable numbers and allow multiplication of *L. monocytogenes* to levels that are enough for detection of the organism. In the early days of listerial clinical bacteriology, cold enrichment (24) was regularly used to this end, exploiting the ability of the organism to multiply at refrigeration temperatures, whereas contaminating bacteria would not multiply under these conditions. However, this procedure requires very long incubation times, often months, making it unacceptable for current investigations of food-borne outbreaks and sporadic cases, as well as for the implementation of effective hazard analysis critical control points (HACCP) programmes in food production and processing establishments. A number of selective compounds that allow growth of *L. monocytogenes* at normal incubation temperatures have been incorporated into culture media, thus shortening the time required for selective growth of the organism. Examples of these selective compounds include cycloheximide, colistin, cefotetan, fosfomycin, lithium chloride, nalidixic acid, acriflavine, phenylethanol, ceftazidime, polymixin B and moxalactam (2, 3, 7, 27, 31, 51).

Bacteriological diagnosis of animal listeriosis has traditionally involved direct plating of specimens on blood agar or other enriched media and concomitant use of the ‘cold enrichment’ technique, with weekly subculturing for up to 12 weeks (24, 40, 53). The introduction of alternative enrichment procedures and selective agents for the isolation of *L. monocytogenes* from food and environmental samples has opened up the possibility of using some of these techniques for the bacteriological analysis of samples from animal listeriosis.

In spite of advances made in the selective isolation of *L. monocytogenes* from food, there is still room for improvement in a number of areas. No single procedure can be credited with being sensitive enough to detect *L. monocytogenes* from all types of food (17). In addition, sublethally injured *L. monocytogenes* cells can be found in processed food due to freezing, heating, acidification and other types of chemical or physical treatment. These sublethally injured bacteria require special culture conditions for damage repair, before being able to be detected in culture.

a) **Culture methods**

Conventional methods for the isolation of *L. monocytogenes* from food that have gained acceptance for international regulatory purposes include the United States Food and Drug Administration (FDA) method (27), the Association of Official Analytical Chemists (AOAC) official method (7), the ISO 11290 Standards (31, 32), the United States Department of Agriculture (USDA)-Food Safety and Inspection Service (FSIS) method (51) and the French Standards (2, 3).

Depending on the nature of the sample, a particular method might be more suitable than others. The International Organization for Standardization Technical Committee ISO/TC 34, Agricultural Food Products, Subcommittee SC 9, Microbiology, claims that the ISO Standard 11290, parts 1 and 2 (31, 32), can be used for the detection of *L. monocytogenes* in a large variety of food and feed products. Although they recognise
that this standard might not be appropriate in every detail in certain instances, they recommend that every effort should be made to apply this horizontal method as far as possible.

The FDA and AOAC methods can be used for milk and dairy products. The USDA-FSIS method is recommended for red meat and poultry (raw or cooked ready-to-eat), eggs, egg products and environmental samples.

The traditional procedure for the isolation of L. monocytogenes from animal tissues has been direct plating of specimens on sheep blood agar or other rich culture media and concomitant use of the 'cold enrichment' technique, with weekly subculturing for up to 12 weeks (24, 40, 53). Isolation of the organism by direct plating is relatively easy when numbers are large in a normally sterile site, like in the case of the septicaemic form of the disease, but isolation is difficult when the organism is present in low numbers, as in the case of the encephalitic form or when samples are heavily contaminated. A comparison of the efficiency of direct plating, cold enrichment and what became the AOAC method, has clearly established the superiority of the latter over the other two, for the isolation of L. monocytogenes from a variety of animal necropsy material, both in terms of the time required for the isolation and identification of the organism, and the isolation rates (20).

For the enumeration of L. monocytogenes, the ISO Standard 11290, part 2 (32) applies, as well as optional protocols mentioned in the FDA and USDA-FSIS methods (27, 51).

i) Isolation

Samples intended for analysis must be representative of the food, including the outer surface and the interior. In the case of animal listeriosis, the samples should be chosen according to the clinical presentation of the disease: material from lesions in the liver, kidneys and/or spleen, in the case of the septicaemic form; spinal fluid, pons and medulla in the case of the encephalitic form; and placenta (cotyledons), fetal abomasal contents and/or uterine discharges in the case of abortion. Refrigeration temperatures (4°C) must be used for handling, storing and shipping specimens. If the sample is already frozen, it should be kept frozen until analysis.

All culture media prepared should be subjected to quality control and must support growth of the suspect organism from a small inoculum. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are working correctly.

These conventional culture methods include an enrichment procedure based on the use of liquid culture media containing selective agents. The nature of the media and the selective agents vary with the method. Both the FDA (27) and the ISO methods include a pre-enrichment step that is intended for the recovery of sublethally injured L. monocytogenes cells, whereas in the USDA-FSIS (51) and the AOAC methods (7) the samples are processed directly into enrichment broth. In the case of the FDA method, the pre-enrichment is carried out at 30°C for 4 hours in trypticase–soy broth containing yeast extract (TSB YE) without selective agents. The ISO protocol uses a 'primary enrichment' for 24 hours at 30°C in the presence of selective agents, but at half the concentration ('half Fraser broth'). When dealing with samples from animal listeriosis, the amount of animal tissue available for analysis might not be enough to use the same amount of inoculum recommended for enrichment of food samples (25 g or ml). If that is the case, as much sample material as possible (aiming at 10–25 g or ml) is used (20).

Samples are enriched for 24–72 hours at 30°C, 35°C or 37°C, depending on the method. The FDA method uses TSB YE containing acriflavine, nalidixic acid and cycloheximide. The USDA-FSIS method uses two enrichment steps: The 'primary' enrichment is done in University of Vermont medium (UVM), containing nalidixic acid and acriflavine; the 'secondary' enrichment is carried out in Fraser broth, containing nalidixic acid, lithium chloride and acriflavine. The ISO standard indicates Fraser broth for the 'secondary' enrichment, containing the selective agents at full concentration, whereas the 'primary' enrichment is carried out in 'half Fraser broth', as indicated above. The AOAC method calls for selective enrichment in tryptone soy broth containing acriflavine, nalidixic acid and cycloheximide ('selective enrichment medium').

After selective enrichment, cultures are then plated on to selective/differential agar plates for isolation of presumptive colonies of L. monocytogenes. All methods use Oxford agar, except the USDA-FSIS method, which uses a modified Oxford (MOX) agar formulation. Oxford agar contains lithium chloride, cycloheximide, colistin, acriflavine, cefotetan and fosfomycin as selective agents, and typical colonies of Listeria spp are small, black and surrounded by a black halo. In addition to Oxford agar, the FDA includes lithium chloride/phenylethanol/moxalactam (LPM) or PALCAM agar and the ISO standard includes the latter, which contains lithium chloride, polymixin B, acriflavine and ceffazidime. The MOX agar, used in the USDA-FSIS method, contains lithium chloride, colistin and moxalactam.

ii) Identification

Typical Listeria spp. colonies, on the above selective/differential agar plates, are then selected for further identification to the species level, using a battery of tests. The tests include the Gram-staining
reaction, catalase, motility (both in a wet mount observed under phase-contrast microscopy and by inoculation into motility test media), haemolysis and carbohydrate use. Some of the protocols accommodate conventional and nonconventional commercially available tests e.g. Vitek, API, MICRO-ID, enzyme-linked immunosorbent assay (ELISA) kits and nucleic acid assay kits to help in the identification of \textit{L. monocytogenes}.

The Christie–Atkins–Munch–Peterson (CAMP) test is a very useful tool to help identify the species of a \textit{Listeria} spp. isolate. It is used in the ISO and AOAC protocols and it is considered to be optional in the FDA and USDA-FSIS methods. The test is simple to perform and easy to read. It consists of streaking a \(\beta\)-haemolytic \textit{Staphylococcus aureus} (ATCC strain 49444 or 25923, NCTC strain 7428 or 1803) and \textit{Rhodococcus equi} (ATCC strain 6939, NCTC strain 1621) in single straight lines in parallel, on a sheep blood agar plate or a double-layered agar plate with a very thin blood agar overlay. The streaks should have enough separation to allow test and control \textit{Listeria} strains to be streaked perpendicularly, in between the two indicator organisms, without quite touching them (separated by 1–2 mm). After incubation for 24–48 hours at 35–37°C (12–18 hours if using the thin blood agar overlay), a positive reaction consists of an enhanced zone of \(\beta\)-haemolysis, at the intersection of the test/control and indicator strains.

### Table 1. Differentiation of \textit{Listeria} species

<table>
<thead>
<tr>
<th>Species</th>
<th>Haemolysis</th>
<th>Production of acid</th>
<th>CAMP test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rhamnose</td>
<td>Xylose</td>
</tr>
<tr>
<td>\textit{L. monocytogenes}</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>\textit{L. innocua}</td>
<td>–</td>
<td>V</td>
<td>–</td>
</tr>
<tr>
<td>\textit{L. ivanovii}</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>\textit{L. seeligeri}</td>
<td>(+)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>\textit{L. welshimeri}</td>
<td>–</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>\textit{L. grayi} subsp. \textit{Grayi}</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>\textit{L. grayi} subsp. \textit{Murrayi}</td>
<td>–</td>
<td>V</td>
<td>–</td>
</tr>
</tbody>
</table>

V: variable; (+): weak reaction; +: >90% positive reactions; –: no reaction.

Serology, lysogenic typing and the immunocompromised mouse pathogenicity assay are considered to be optional in some of these methods.

### b) Rapid identification methods

i) \textit{MICRO-ID} \textit{Listeria}

\textit{MICRO-ID} \textit{Listeria} is a commercially available system (Organon Teknika Corp., 100 Akzo Ave., Durham, NC 27712, USA) that has been validated by the AOAC (method 992.18) (4) for the presumptive identification of \textit{Listeria} species isolated from food and environmental samples. It provides an alternative to conventional biochemical testing of \textit{Listeria} spp. isolates by the FDA and USDA-FSIS methods. It is based on the principle that the test inoculum contains preformed enzymes that can be detected after 24 hours at 37°C. Differentiation of \textit{Listeria} species is based on an octal code derived after adding the numerical values for each group of three tests and on the reactions obtained from the CAMP test and haemolysis characteristics, which are assayed separately.

ii) \textit{Vitek Automicrobic System}

The \textit{Vitek Automicrobic System} (bioMérieux Vitek, Inc., 595 Anglum Dr., Hazelwood, MO, USA) is an automated microbial identification system that can be used for the presumptive identification of food-borne \textit{Listeria} species and for screening of non-\textit{Listeria} isolates. It has been validated by the AOAC as method 992.19 (5). The system uses an incubator chamber with an optical reader, a filler/sealer unit for test kit inoculation and a computer. Gram-positive (GPI) and Gram-negative (GNI+) identification cards each contain 30 biochemical tests. Changes are analysed by the computer, which then assigns the test organism a genus and/or species. The identification of the \textit{Listeria} species requires the use of the GPI card and two reactions on the GNI+ card. However, for identification of some \textit{Listeriae}, the analyst must perform the CAMP, haemolysis and/or nitrate reduction tests as described under the FDA method.

Organisms placed in the ‘LM’ category are identified as \textit{L. monocytogenes} or \textit{L. innocua}; in the ‘LI’ category, as \textit{L. ivanovii} or \textit{L. seeligeri}; in the ‘LW’ category, as \textit{L. welshimeri}; and in the ‘LG’ category, as \textit{L. grayi} or \textit{L. murrayi} (a subspecies of \textit{L. grayi}). Organisms in the ‘O’ category are classified as non-\textit{Listeria}
species. Further tests should be conducted to identify the species within each category according to the FDA method.

Other commercially available methods for the identification of Listeria species include the API LISTERIA (bioMérieux), the MICROBACT 12L (Microgen), the MicroLog System (Biolog), the Sherlock Microbial Identification System (MIS) (Microbial ID; based on fatty acid patterns) and the Walk/Away System (MicroScan).

iii) **Rapid immunological detection methods**

A number of immunological methods have been developed to identify *L. monocytogenes* in foods and the following commercially available methods have been validated by one or more recognised formal validation systems (18, 46).

- **Colorimetric monoclonal enzyme-linked immunosorbent assay (Listeria-Tek)**

The Listeria-Tek is AOAC official method 994.03 (8) and it is intended for the detection of *Listeria* spp. in dairy products, seafood and meats. Because the monoclonal antibodies (MAbs) used in the test may cross react with other *Listeria* spp., the test is not confirmatory for *L. monocytogenes*.

The kit is commercially available from Organon Teknika Corp., 100 Akzo Ave, Durham, NC 27704, USA.

Enrichment cultures found positive by this method are streaked on selective media and suspect colonies are biochemically identified as *L. monocytogenes* according to the FDA method. A positive result is only valid when the positive and negative controls give acceptable absorbance readings.

- **Colorimetric polyclonal enzyme immunoassay screening method (TECRA® Listeria Visual Immunoassay [TLVIA])**

The TLVIA is AOAC official method 995.22 (9) and it is intended for the detection of *Listeria* spp. in dairy foods, seafood, poultry, meats (except raw ground meat), and leafy vegetables.

The commercial kit is available from TECRA International Pty Ltd, P.O. Box 788, Willoughby, NSW, Australia.

Enrichment cultures that are positive must be inoculated on to selective media and suspect colonies identified according to the criteria specified under the FDA and USDA methods.

- **Assurance® polyclonal enzyme immunoassay method**

The Assurance® *Listeria* Enzyme Immunoassay is AOAC official method 996.14 (10) and it can be used for the detection of *Listeria* spp., including *L. monocytogenes*, in dairy foods, red meats, pork, poultry products, fruits, nutmeats, seafood, pasta, vegetables, cheese, animal meal, chocolate, and eggs.

Readings above the cut-off value are considered presumptive positive and the enriched cultures are then confirmed by culture and identification procedures as described under the FDA method.

The commercial kit is available from BioControl Systems, Inc., 12822 SE 32nd St., Bellevue, WA 98005, USA.

- **Visual Immunoprecipitate assay (VIP™)**

The VIP™ assay is AOAC official method 997.03 (11). It can be used for the detection of *L. monocytogenes* and other *Listeria* spp. in dairy foods, red meats, pork, poultry and poultry products, seafood, fruits, vegetables, nutmeats, pasta, chocolate, eggs, and bone meal.

The test is performed with an enriched culture of the test samples. Presumptive positive tests must be confirmed by culture and identification procedures as described under the FDA method.

The VIP units are commercially available from BioControl Systems, Inc., 12822 SE 32nd St., Bellevue, WA 98005, USA.

- **VIDAS LIS assay screening method**

This enzyme-linked immunofluorescent assay (ELFA) is AOAC official method 999.06 (12). It has also been validated by the Association Français de Normalisation (AFNOR) and by the European Microbiological Methods Assessment Scheme (EMMAS) (13). It is used for screening dairy products, vegetables, seafood, raw meats and poultry, as well as processed meats and poultry, for *Listeria* spp. antigens.
This immunoassay is performed in the automated VIDAS® instrument. The computer compares this value with a standard and a positive or negative report is generated. Positive results must be confirmed by standard culture methods as described under the FDA method.

The VIDAS system is available from bioMérieux, Inc., 595 Anglum Rd., Hazelwood, MO 63042, USA.

Other commercially available immunological methods that have been validated by formal systems include the VIDAS *Listeria monocytogenes* (LMO) ELISA (bioMérieux), validated by AFNOR; the Transia Plate *Listeria* ELISA (Transia, Diffchamb Ltd), validated by AFNOR; the ElAFOSS *Listeria* automated ELISA (Foss Electric), validated by the AOAC Research Institute; the immunochromatographic method REVEAL for *Listeria* (Neogen Corporation), validated by the AOAC Research Institute; the immunochromatographic method Clearview *Listeria* Rapid Test (Oxoid), validated by AFNOR, EMMAS and the AOAC Research Institute and the immunomagnetic separation-based Listertest (Vicam), validated by the AOAC Research Institute (13).

Other commercially available immunologically based methods include the Transia Plate *Listeria monocytogenes* ELISA (Transia, Diffchamb Ltd), the immunomagnetic separation-based Dynabeads anti-*Listeria* (Dynal Ltd), the *Listeria* UniQue™ ELISA (TECRA), the Microscreen *Listeria* latex agglutination test (Microgen BioProducts Ltd), and the *Listeria* Rapid Test EIA (Oxoid).

**iv) Nucleic acid recognition methods**

A number of methods based on nucleic acid recognition have been developed to identify *L. monocytogenes* in foods. A few of them have been validated by one or more recognised formal validation systems and are commercially available (39).

- **GENE-TRAK *Listeria* assay**
  
  The GENE-TRAK *Listeria* assay is a colorimetric DNA hybridisation method for the detection of *Listeria* sequences, which has been validated by the AOAC as method 993.09 (6) for use with dairy products, meats and seafood. This assay has also been validated by AFNOR. Due to the possibility of encountering false-positive reactions, positive samples must be confirmed by standard cultural methods.

  Test portions found positive by this DNA hybridisation assay must be confirmed by streaking a phosphate buffered saline growth suspension on a *Listeria* selective plate and continuing with biochemical identification of presumptive *Listeria* isolates as described under the FDA method.

  The GENE-TRAK *Listeria* assay is commercially available from GENE-TRAK™ Systems, 94 South Street, Hopkinton, MA 01748, USA.

- **BAX® System**

  The USDA-FSIS has adopted the PCR-based BAX® System (Qualicon) (50) as their new screening method for *L. monocytogenes* in enriched meat and poultry samples. It reduces the report out time for true negative samples by 24 hours and reduces false-positive results, with a detection limit better that 1 cfu/g in a 25 g sample. All samples that are identified as presumptive positive for *L. monocytogenes* are then subject to cultural confirmation by the conventional method.

- **GENE-TRAK test for *Listeria monocytogenes***

  The GENE-TRAK test for *L. monocytogenes* (GENE-TRAK™ Systems) is a hybridisation probe-based method validated by AFNOR (13).

- **Gen-Probe (AccuProbe®) *Listeria monocytogenes* confirmatory test**

  The Gen-Probe (AccuProbe®) *Listeria monocytogenes* Confirmatory Test (Gen-Probe) is another hybridisation probe-based method validated by AFNOR (13).

- **AD713 method**

  The FDA uses a *L. monocytogenes*: a combination of invasion-associated protein and haemolysin (hly) gene probes – AD713 method (25). This method combines the detection of the *L. monocytogenes* haemolysin (also called listeriolysin O) gene by use of the oligonucleotide probe AD13, and the detection of the invasion-associated protein gene by a synthetic probe, AD07. Both probes are used in combination (designated AD713) to avoid false-negative results because of ‘silent’ mutations in the gene (nucleotide changes that affect DNA probe binding but do not change the gene function). Positive samples must be confirmed with conventional procedures as described under the FDA method.
Other commercially available methods based on nucleic acid recognition, include the Foodproof® *Listeria monocytogenes* polymerase chain reaction (PCR) assay (Bioteccon Diagnostics) and the PROBELIA™ (*L. monocytogenes*) PCR assay (Sanofi Diagnostics). The application of the real-time PCR as a quantitative detection method, specific for *L. monocytogenes*, has also been developed (28) and it shows good potential for routine analytical use. Table 2 summarises some of the available commercial systems for rapid *Listeria* screening and confirmation.

<table>
<thead>
<tr>
<th>Test</th>
<th>ID level</th>
<th>Principle</th>
<th>Approx. test time</th>
<th>Company</th>
<th>Main use</th>
</tr>
</thead>
<tbody>
<tr>
<td>MICRO-ID Listeria</td>
<td><em>L. monocytogenes/innocua complex</em></td>
<td>Enzyme reaction</td>
<td>24 hours</td>
<td>Organon Teknika</td>
<td>Confirmation</td>
</tr>
<tr>
<td>Vitek System</td>
<td><em>L. monocytogenes/innocua complex</em></td>
<td>Biochemical tests</td>
<td>24 hours</td>
<td>bioMérieux</td>
<td>Confirmation</td>
</tr>
<tr>
<td>API Listeria</td>
<td><em>L. monocytogenes</em></td>
<td>Biochemical tests</td>
<td>24 hours</td>
<td>bioMérieux</td>
<td>Confirmation</td>
</tr>
<tr>
<td>MicroLog System</td>
<td><em>L. monocytogenes</em></td>
<td>Carbon source substrates</td>
<td>4 or 24 hours</td>
<td>Biolog</td>
<td>Confirmation</td>
</tr>
<tr>
<td>MICROBACT 12L</td>
<td><em>L. monocytogenes</em></td>
<td>Carbohydrate use and micro haemolysis test</td>
<td>4–6 or 24 hours</td>
<td>Microgen</td>
<td>Confirmation</td>
</tr>
<tr>
<td>Sherlock Microbial Identification System (MIS)</td>
<td><em>L. monocytogenes/innocua complex</em></td>
<td>Fatty acid patterns</td>
<td>90 minutes</td>
<td>Microbial ID</td>
<td>Confirmation</td>
</tr>
<tr>
<td>Gen-Probe (AccuProbe)</td>
<td><em>L. monocytogenes</em></td>
<td>Nucleic Acid Hybridization Probe</td>
<td>30 minutes</td>
<td>Gen Probe</td>
<td>Confirmation</td>
</tr>
<tr>
<td>Microscreen</td>
<td>Listeria spp.</td>
<td>Latex agglutination</td>
<td>1 minute</td>
<td>Microgen BioProducts</td>
<td>Confirmation</td>
</tr>
<tr>
<td>Listeria Tek</td>
<td>Listeria spp.</td>
<td>ELISA</td>
<td>50 hours</td>
<td>Organon Teknika</td>
<td>Screening</td>
</tr>
<tr>
<td>TECRA Listeria Visual Immunonassay (TLVIA)</td>
<td>Listeria spp.</td>
<td>ELISA</td>
<td>50 hours</td>
<td>TECRA</td>
<td>Screening</td>
</tr>
<tr>
<td>Assurance Listeria EIA</td>
<td>Listeria spp.</td>
<td>ELISA</td>
<td>50 hours</td>
<td>BioControl Systems</td>
<td>Screening</td>
</tr>
<tr>
<td>VIP Listeria</td>
<td>Listeria spp.</td>
<td>Immuno-chromatography</td>
<td>2 minutes (post-enrichment)</td>
<td>BioControl Systems</td>
<td>Screening</td>
</tr>
<tr>
<td>VIDAS Listeria (LIS)</td>
<td>Listeria spp.</td>
<td>ELISA</td>
<td>50 hours</td>
<td>bioMérieux</td>
<td>Screening</td>
</tr>
<tr>
<td>VIDAS Listeria monocytogenes (LMO)</td>
<td><em>L. monocytogenes</em></td>
<td>ELISA</td>
<td>50 hours</td>
<td>bioMérieux</td>
<td>Screening</td>
</tr>
<tr>
<td>Foodproof Listeria monocytogenes</td>
<td><em>L. monocytogenes</em></td>
<td>PCR</td>
<td>48 hours</td>
<td>Bietecon Diagnostics</td>
<td>Screening</td>
</tr>
<tr>
<td>Transia Plate Listeria</td>
<td>Listeria spp.</td>
<td>ELISA</td>
<td>50 hours</td>
<td>Diffchamb</td>
<td>Screening</td>
</tr>
<tr>
<td>Transia Plate Listeria monocytogenes</td>
<td><em>L. monocytogenes</em></td>
<td>ELISA</td>
<td>50 hours</td>
<td>Diffchamb</td>
<td>Screening</td>
</tr>
<tr>
<td>Dynabeads anti-Listeria</td>
<td>Listeria spp.</td>
<td>Immunomagnetic separation</td>
<td>48–72 hours</td>
<td>Dynal</td>
<td>Screening</td>
</tr>
<tr>
<td>EIAFOSS Listeria</td>
<td>Listeria spp.</td>
<td>Automated ELISA</td>
<td>48 hours</td>
<td>Foss Electric</td>
<td>Screening</td>
</tr>
</tbody>
</table>

1: Adapted and expanded from ref. 13
2: When used for confirmation, the test time indicated is after enrichment and agar isolation
### Table 2 cont. Some commercial systems for rapid Listeria screening and confirmation

<table>
<thead>
<tr>
<th>Test</th>
<th>ID level</th>
<th>Principle</th>
<th>Approx. test time</th>
<th>Company</th>
<th>Main use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene Trak Listeria Assay</td>
<td>Listeria spp.</td>
<td>Nucleic acid hybridisation probe</td>
<td>50 hours</td>
<td>Gene Trak</td>
<td>Screening</td>
</tr>
<tr>
<td>Gene Trak test for L. monocytogenes</td>
<td>L. monocytogenes</td>
<td>Nucleic acid hybridisation probe</td>
<td>50 hours</td>
<td>Gene Trak</td>
<td>Screening</td>
</tr>
<tr>
<td>REVEAL for Listeria</td>
<td>Listeria spp.</td>
<td>Immuno-chromatography</td>
<td>43 hours</td>
<td>Neogen</td>
<td>Screening</td>
</tr>
<tr>
<td>REVEAL for Listeria (Oxoid Listeria Rapid Test)</td>
<td>Listeria spp.</td>
<td>Immuno-chromatography</td>
<td>43 hours</td>
<td>Oxoid</td>
<td>Screening</td>
</tr>
<tr>
<td>BAX for screening L. monocytogenes</td>
<td>L. monocytogenes</td>
<td>PCR</td>
<td>48 hours</td>
<td>Qualicon</td>
<td>Screening</td>
</tr>
<tr>
<td>BAX for screening Listeria Genus</td>
<td>Listeria spp.</td>
<td>PCR</td>
<td>48 hours</td>
<td>Qualicon</td>
<td>Screening</td>
</tr>
<tr>
<td>PROBELIA</td>
<td>L. monocytogenes</td>
<td>PCR</td>
<td>48 hours</td>
<td>Sanofi Diagnostics Pasteur</td>
<td>Screening</td>
</tr>
<tr>
<td>Listertest</td>
<td>Listeria spp.</td>
<td>ELISA</td>
<td>32 hours</td>
<td>TECRA</td>
<td>Screening</td>
</tr>
<tr>
<td>Listertest</td>
<td>Listeria spp.</td>
<td>Immunomagnetic separation</td>
<td>24–48 hours</td>
<td>Vicam</td>
<td>Screening</td>
</tr>
<tr>
<td>SwabCheck System</td>
<td>Listeria spp.</td>
<td>ELISA</td>
<td>24 hours</td>
<td>Biopath</td>
<td>Screening</td>
</tr>
</tbody>
</table>

1: Adapted and expanded from ref. 13
2: When used for confirmation, the test time indicated is after enrichment and agar isolation

v) **Subtyping**

Regulatory identification of *L. monocytogenes* does not require any specific subtyping of the isolates. However, subtyping schemes can be useful in outbreak investigations, environmental tracking and public health surveillance.

*Listeria monocytogenes* can be subtyped by a number of different approaches including serotyping, phage typing, multilocus enzyme electrophoresis (MEE), DNA restriction enzyme analysis (either using high-frequency cutting enzymes and conventional gel electrophoresis to separate fragments, or using rare-cutting enzymes and pulse-field gel electrophoresis [PFGE] to separate fragments), nucleic acid sequencing-based typing and random amplification of polymorphic DNA (RAPD).

Because of the requirement for specific reagents, stringent quality assurance procedures and some sophisticated equipment, it is recommended that subtyping of *L. monocytogenes* isolates be referred to the appropriate reference centre. A list of international centres and subtyping methods can be found in ref. 14 (pp. 258–259)

- **Serotyping**

Strains of *Listeria* can be assigned to 13 different serotypes, based on their combination of somatic (O) and flagellar (H) antigens. Although all of them are considered to be potentially pathogenic, most (>95%) human clinical isolates belong to three serotypes 1/2a, 1/2b, and 4b. Compared with other subtyping methods, serotyping has poor discriminatory power, but can provide valuable information to facilitate the ruling out of isolates that are not part of an outbreak. Isolates from foods and from environmental sources are frequently nontypable with standard typing antisera.

- **Phage typing**

Bacteriophage typing is a technique with very good discriminatory power and that can be used to subtype a large number of isolates. However, the current available phage sets are unable to type a high proportion of strains (20–51% in the case of the international phage typing set). Because of stringent standardisation requirements and the biological nature of the reagents, this technique is performed only at specialised national and international reference laboratories and is subject to considerable experimental and biological variability. In spite of these problems, phage typing remains the most practical and suitable method for application in large acute outbreaks (23).
• **Multilocus enzyme electrophoresis**

This technique takes advantage of nucleotide sequence differences, which result in distinct electrophoretic mobilities of selected metabolic enzymes in starch gels, and can be applied to subtype-related bacterial strains. However, MEE is only moderately discriminatory when used in epidemiological investigations involving *L. monocytogenes*. Some strains may lack certain enzyme activities and therefore the technique can get complicated. Because of its very nature, the interlaboratory results are highly variable (23).

• **Chromosomal DNA restriction endonuclease analysis**

Restriction endonuclease analysis (REA) of chromosomal DNA is a useful subtyping method for *L. monocytogenes*. As these enzymes are highly specific in recognising nucleotide sequences, the resulting DNA digestion fragments, of different size and electrophoretic mobility, reflect genomic differences, resulting in specific ‘fingerprints’ among otherwise related strains. Because of the restriction endonuclease specificity, the method is highly reproducible. Of the restriction endonuclease tests used on *L. monocytogenes* in a World Health Organization (WHO) Multicentre study, *HaeIII, Hhal* and *CfoI* were the most useful (23). However, because of a potentially large number of enzyme recognition sites in the bacterial genome, sometimes complex fingerprints evolve, with overlapping or poorly resolved bands that are difficult to interpret. The technique is therefore not adequate for comparing a large number of strain patterns or for building dynamic databases (23).

By combining REA with Southern hybridisation, using chromosomal-labelled probes, only the particular restriction fragments associated with the corresponding chromosomal loci are detected, thereby significantly reducing the number of DNA fragments to be analysed. This technique is known as restriction fragment length polymorphism (RFLP) analysis. When ribosomal RNA/DNA probes are used, only the particular restriction fragments associated with the chromosomal loci for rRNA are detected. This technique is known as ribotyping and it has been widely used for subtyping *L. monocytogenes*, mainly through the use of the restriction endonuclease *EcoRI*. However, the technique was found to be less discriminating than phage typing, REA or MEE. Qualicon has designed an automated ribotyping system, the RiboPrinter, which generates, analyses and stores riboprint patterns of bacteria, including *Listeria*.

When restriction endonuclease enzymes that cut infrequently are used to digest unsheared chromosomal DNA, such as *ApaI, SmaI, NotI* and *Ascl*, very large fragments are obtained. Because of their size, these large fragments do not separate when run under conventional agarose gel electrophoresis. However, by periodically changing the orientation of the electric field across the gel, through pulses, the large fragments can ‘crawl’ through the agarose matrix and are separated according to size differences. This technique is known as pulsed-field gel electrophoresis (PFGE) and has revolutionised the precise separation of DNA fragments larger than 40 kilobases. PFGE has been applied to the subtyping of *L. monocytogenes* and has been found to be a highly discriminating and reproducible method. PFGE is particularly useful for subtyping serotype 4b isolates, which are not satisfactorily subtyped by most other subtyping methods. The main disadvantages of PFGE are the time required to complete the procedure (2–3 days), the large quantities of expensive restriction enzymes required, and the need for specialised, expensive equipment (23). The Centers for Disease Control and Prevention (CDC) in the United States of America has established PulseNet, a network of public health and food regulatory laboratories that routinely subtype food-borne pathogenic bacteria by PFGE. PulseNet laboratories use highly standardised protocols and can quickly compare PFGE patterns from different locations via the Internet. *Listeria monocytogenes* was added to PulseNet in 1999 (49).

• **Nucleic acid sequence-based typing**

Although there have been some reports on the sequence analysis of single genes as a means to type *L. monocytogenes* strains, determination of allelic variation of multiple genes, has been recently introduced as a very promising subtyping methodology for this microorganism. This approach has been reported for a handful of other microorganisms and it is known as multi locus sequence typing (MLST) (48). Direct amplification and nucleotide sequencing (15, 44), as well as an alternative approach that targets the variable genetic changes directly in a DNA array format (43) have both been used with good discrimination between the strains analysed. Because MLST is based on nucleotide sequence, it is highly discriminatory and provides unambiguous results.

Short repetitive sequence elements are widely distributed among bacteria and palindromic units, known as repetitive extragenic palindromes (REP), are the best-characterised family of repetitive bacterial sequences. REP elements are present in *L. monocytogenes* and a PCR based on the sequence of these elements (rep-PCR) has been used successfully to subtype strains of the organism. The four major strain clusters identified by this method matched the origin of their isolation (34).

• **Random amplification of polymorphic DNA**

When arbitrarily selected primers are used in the PCR under low stringency conditions, with chromosomal *L. monocytogenes* DNA as a template, amplicon patterns are generated that are useful for subtyping
strains. RAPD is a viable alternative to phage typing and is highly discriminating. However, despite its relative simplicity and discriminating ability, its main drawback is the inconsistent reproducibility of patterns. The low stringency conditions for primer annealing results in polymerisation with various efficiencies, and therefore the quantities of DNA produced may be widely variable among the different amplicons from a given isolate, which makes it difficult to compare and interpret the RAPD patterns. The technique requires a great deal of standardisation and consistency to obtain reliable results (23).

Based on the results of the WHO multicentre L. monocytogenes subtyping study (21), which compared several different subtyping methods using a well defined set of isolates, serotyping, phage typing, REA, PFGE and RAPD were selected for standardisation in Phase II. This effort should eventually result in a selected set of standardised L. monocytogenes subtyping methods (23).

2. Serological tests

Serological tests have not been traditionally used for the diagnosis of listeriosis. They have been largely unreliable, lacking sensitivity and specificity. A number of formats, including ELISA, complement fixation and microagglutination have been largely unsuccessful in the diagnosis of culture-proven human listeriosis, even in the absence of immunosuppression. Considerable cross-reactivity with antigenic determinants of other Gram-positive organisms has been observed. On the other hand, L. monocytogenes is a ubiquitous organism, and regular exposure of animals and humans to this microorganism is very common. Many healthy individuals are intestinal carriers (2–6%) and anti-L. monocytogenes serum antibody prevalence as high as 53% have been reported in humans. Carriage rate for animals is similar to that of humans, with some differences depending on the species and a little higher rate during indoor season, as compared to animals on pasture (29, 30).

The discovery that the L. monocytogenes haemolysin, listeriolysin O (LLO), is a major virulence factor and that it can stimulate an antibody response, has recently renewed interest in the possibility of using serological tests for the diagnosis of listeriosis, particularly in central nervous system patients, with sterile blood and cerebrospinal fluid, and in perinatal listeriosis. An indirect ELISA based on the detection of anti-LLO was used for the diagnosis of experimental listeriosis in sheep (37). However, LLO is antigenically related to a number of cytolysins, including streptolysin O (SLO) from Streptococcus pyogenes, pneumolysin from S. pneumoniae and perfringolysin from Clostridium perfringens. Problems of cross-reactivity of anti-LLO antibodies with these cytolysins, particularly SLO and pneumolysin, have hampered the development of specific reliable serological tests based on the detection of anti-LLO antibodies. In addition, anti-LLO antibodies have been found in a proportion of healthy individuals and patients with other bacterial, fungal or viral infections (27%, all combined), although at lower titres than in patients with listeriosis. Absorption of diagnostic antisera with SLO is only partially effective in eliminating all cross-reactivity. These experimental assays have been used in some epidemiological investigations and as support for the diagnosis of culture-negative central nervous system infections. Recombinant forms of LLO have been explored as alternatives to wild LLO as a diagnostic antigen in Western blot assays. This is currently an evolving field and we will have to wait for the development of reliable, validated serological tests for the diagnosis of listeriosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

It has proven very difficult to develop effective vaccines against L. monocytogenes which, as an intracellular organism, requires effector T cells for an effective immune response. Experimental vaccines in laboratory animals are being explored to confer protection to L. monocytogenes infection by a number of different approaches, but these are still far from becoming available for human and farm animal use. These experimental approaches include immunisation with plasmid DNA, CD40 signalling along with heat-killed L. monocytogenes, LLO-deficient mutants inoculated along with liposome-encapsulated LLO, and immunisation with listerial antigens and IL-12.

Genetically modified L. monocytogenes is also being considered as an effective vaccine vector for the expression, secretion and intracellular delivery of foreign antigens for the induction of potent immune responses against viral antigens and tumour cells.

However, the most feasible and practical means to reduce the risk of listeriosis in humans is through dietary and food preparation measures that not only decrease the risk of acquiring listeriosis, but also contribute to the prevention of other common food-borne infections such as those caused by Escherichia coli O157:H7, Salmonella and Campylobacter. These preventive measures include thorough cooking of raw food of animal origin, keeping uncooked meats separate from vegetables, cooked foods, and ready-to-eat foods, thoroughly washing raw vegetables before eating, washing hands, knives, and cutting boards after handling uncooked foods, and avoiding unpasteurised milk or products made from it. Immunocompromised persons, pregnant women and other groups at increased risk of listeriosis should avoid foods that have been epidemiologically linked to this disease, e.g. soft cheeses and pâté. These individuals should also avoid other ready-to-eat foods, unless they are heated until steaming hot before being consumed.
The food industry and public health agencies play a pivotal role in the prevention of food-borne listeriosis by developing and implementing effective HACCP programmes to reduce the presence of L. monocytogenes at all critical points in the food production and distribution chain (from the farm to the market).

Likewise, the lack of well designed and tested vaccines for animal use, means that control of listeriosis in animals is most feasible by preventing the environmental conditions that favour its presentation. There is a well-established linkage between silage feeding and listeriosis and, as L. monocytogenes is widely distributed in nature, with animals and birds acting as carriers, contamination of silage and feed is inevitable. Emphasis should therefore be placed on reducing the likelihood of the multiplication of the organism, which occurs more frequently at pH values greater than 5, particularly where ineffective fermentation has occurred and where there is concomitant growth of moulds. Every effort should be made to produce silage of good quality, with early cutting of grass and minimal contamination with soil or faeces. The best silage for feeding should be selected, especially in the case of sheep, avoiding obviously moulded material and that from the superficial few inches of a clamp. Leftover silage should be removed (38).

REFERENCES


Chapter 2.10.14. — Listeria monocytogenes


* *
PART 3

OIE REFERENCE EXPERTS

AND DISEASE INDEX
**LIST OF OIE REFERENCE LABORATORIES IN 2004**

## List A diseases

### 2.1.1. Foot and mouth disease – Fièvre aphteuse – Fiebre aftosa

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For certain diseases, two institutions have been jointly designated as an OIE Reference Laboratory or Collaborating Centre. Where this occurs, the superscript (1) or (2) will be placed after the name of the designated Reference Expert.

### 2.1.2. Vesicular stomatitis – Stomatite vésiculeuse – Estomatitis vesicular

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Fax: (55.21) 36.61.90.01  
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### 2.1.3. Swine vesicular disease – Maladie vésiculeuse du porc – Enfermedad vesicular porcina

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2.1.4. Rinderpest – Peste bovine – Peste bovina

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2.1.5. Peste des petits ruminants – Peste des petits ruminants – Peste de pequeños ruminantes

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Fax: (254.154) 32.450

2.1.6. Contagious bovine pleuropneumonia – Péripneumonie contagieuse bovine – Perineumonía contagiosa bovina

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2.1.7. Lumpy skin disease – Dermatose nodulaire contagieuse – Dermatosis nodular contagiosa

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2.1.8. Rift Valley fever – Fièvre de la Vallée du Rift – Fiebre del Valle del Rift

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### 2.1.9. Bluetongue – Fievre catarrhale du mouton – Lengua azul

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<tr>
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### 2.1.10. Sheep pox and goat pox – Clavelée et variole caprine – Viruela ovina y viruela caprina

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### 2.1.11. African horse sickness – Peste équine – Peste equina

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### 2.1.12. African swine fever – Peste porcine africaine – Peste porcina africana

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**OIE Terrestrial Manual 2004**
2.1.13. Classical swine fever – Peste porcine classique – Peste porcina clásica

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†  Reference Laboratory for Highly pathogenic avian influenza only
### List B diseases

**Multiple species diseases/Maladies communes à plusieurs espèces/Enfermedades comunes a varias especies**

#### 2.2.1. Anthrax – Fièvre charbonneuse – Carbunco bacteridiano

<table>
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<tbody>
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</tr>
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</table>

#### 2.2.2. Aujeszky's disease – Maladie d'Aujeszky – Enfermedad de Aujeszky

<table>
<thead>
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<th>Laboratory 1</th>
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<th>Phone 1</th>
<th>Fax 1</th>
<th>Email 1</th>
</tr>
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<tr>
<th>Laboratory 2</th>
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<th>Phone 2</th>
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</thead>
<tbody>
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<td></td>
</tr>
</tbody>
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#### 2.2.3. Echinococcosis/hydatidosis – Echinococcose/hydatidose – Equinococosis/hidatidosis

<table>
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<th>Address</th>
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<tbody>
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<thead>
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‡ There are currently four diseases on OIE List B that are included under the umbrella term ‘Brucellosis’. These are: 2.3.1. Bovine brucellosis – Brucellose bovine – Brucellosis bovina; 2.4.1. Ovine: epididymitis – Epididymite ovine – Epididimitis ovina (Brucella ovis); 2.4.2. Caprine and ovine brucellosis – Brucellose caprine et ovine – Brucelosis ovina y caprina (excluding B. ovis); and 2.6.2. Porcine brucellosis – Brucellose porcine – Brucelosis porcina.
2.3.13. Bovine spongiform encephalopathy – Encéphalopathie spongiforme bovine – Encefalopatía espongiforme bovina

2.4.8. Scrapie – Tremblante – Prurigo lumbar

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2.3.5. Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis – Rhinotrachéite infectieuse bovine/vulvovaginite pustuleuse infectieuse – Rinotraqueítis infecciosa bovina/vulvovaginitis pustular infecciosa

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2.3.15. Trypanosomosis (tsetse-transmitted) – Trypanosomose (transmise par tsé tsé) – Tripanosomosis (transmitida por tsetse)

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2.4.4. Caprine arthritis/encephalitis – Arthrite/encéphalite caprine – Artritis/encefalitis caprina
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2.5.3. Equine encephalomyelitis (Eastern and Western) – Encéphalomyélite équine de l’Est ou de l’Ouest –  
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2.5.12. Venezuelan equine encephalomyelitis – Encéphalomyélite équine vénézuélienne – Encefalomielitis equina venezolana

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Dr T.W. Drew  
VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB  
UNITED KINGDOM  
Tel: (44.1932) 34.11.11, Fax: (44.1932) 34.70.46  
E-mail: t.w.drew@vla.defra.gsi.gov.uk

Dr Y. Fukunaga  
Epizootic Research Station, Equine Research Institute, The Japan Racing Association, Kokubunji-machi, Shimosuga-gun, Tochigi 329-0412  
JAPAN  
Tel: (81.285) 44.00.90, Fax: (81.285) 44.56.76  
E-mail: yoshiof@epizoo.equihl.go.jp
2.6.4. Transmissible gastroenteritis – Gastro-entérite transmissible – Gastroenteritis transmisible

Dr L.J. Saif
Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University
1680 Madison Avenue, Wooster, OH 44691-4096
UNITED STATES OF AMERICA
Tel: (1.330) 263.37.44, Fax: (1.330) 263.36.77
E-mail: saif.2@osu.edu

2.6.5. Porcine reproductive and respiratory syndrome – Syndrome dysgénésique et respiratoire du porc – Síndrome disgenésico y respiratorio porcino

Dr R. Magar
Laboratoire d’hygiène vétérinaire et alimentaire
Agence canadienne d’inspection des aliments
3400 Casavant ouest, Saint-Hyacinthe, Québec J2S 8E3
CANADA
Tel: (1.450) 773.77.30, Fax: (1.450) 773.81.52
E-mail: magarr@inspection.gc.ca

2.7.1. Infectious bursal disease (Gumboro disease) – Bursite infectieuse (Maladie de Gumboro) – Bursitis infecciosa (Enfermedad de Gumboro)

Dr Y.M. Saif
Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University
1680 Madison Avenue, Wooster, OH 44691-4096
UNITED STATES OF AMERICA
Tel: (1.330) 263.37.43, Fax: (1.330) 263.36.77
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Dr N. Eterradossi
AFSSA Ploufragan, Unité de virologie, immunologie et parasitologie aviaires et cunicoles (VIPAC), BP 53, 22440 Ploufragan
FRANCE
Tel: 33 (0)2 96.01.62.88, Fax: 33 (0)2 96.01.62.63
E-mail: n.eterradossi@ploufragan.afssa.fr

2.7.2. Marek’s disease – Maladie de Marek – Enfermedad de Marek

Dr K. Venugopal
Institute for Animal Health, Compton Laboratory, Compton Newbury, Berkshire RG20 7NN
UNITED KINGDOM
Tel: (44.1635) 57.84.11, Fax: (44.1635) 57.72.37
E-mail:venu.gopal@bbsrc.ac.uk

Dr J.L. Spencer
Animal Diseases Research Institute, 3851 Fallowfield Road P.O. Box 11300, Station H, Nepean, Ontario K2H 8P9
CANADA
Tel: (1.613) 228.66.98, Fax: (1.613) 228.66.69
E-mail: spencerl@inspection.gc.ca
2.7.3. Avian mycoplasmosis – Mycoplasmose aviaire – Micoplasmosis aviar (Mycoplasma gallisepticum)

Dr S.H. Kleven  
The University of Georgia, College of Veterinary Medicine  
Department of Avian Medicine, 953 College Station Road  
Athens, Georgia 30602-4875  
UNITED STATES OF AMERICA  
Tel: (1.706) 542.56.44, Fax: (1.706) 542.56.30  
E-mail: skleven@uga.edu

Dr I. Kempf  
AFSSA Ploufragan, UR Mycoplasmologie-Bactériologie  
Unité de pathologie aviaire, Zoopôle Beaucemaine-Les Croix  
BP 53, 22440 Ploufragan  
FRANCE  
Tel: 33 (0)2 96.01.62.81, Fax: 33 (0)2 96.01.62.73  
E-mail: i.kempf@ploufragan.afssa.fr

Lagomorph diseases/Maladies des lagomorphes/Enfermedades de los lagomorfos

2.8.2. Tularemia – Tularémie – Tularemia

Dr T. Mörner  
National Veterinary Institute, Department of Wildlife  
751 89 Uppsala  
SWEDEN  
Tel: (46.18) 67.42.14 Fax: (46.18) 30.91.62  
Email: torsten.morner@sva.se

2.8.3. Rabbit haemorrhagic disease – Maladie hémorragique du lapin – Enfermedad hemorrágica del conejo

Dr L. Capucci  
Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia  
Romagna ‘B. Ubertini’, Via A. Bianchi n° 7/9, 25124 Brescia  
ITALY  
Tel: (39.30) 229.03.66, Fax: (39.30) 229.03.11  
E-mail: capucci@bs.izs.it

Bee diseases/Maladies des abeilles/Enfermedades de las abejas

2.9.1.—2.9.5. Bee diseases – Maladies des abeilles – Enfermedades de las abejas

Mr J.-P. Faucon  
AFSSA Sophia Antipolis, Laboratoire de pathologie des petits ruminants et des abeilles, Unité Pathologie de l’abeille ‘Les Templiers’, 105 Route des Chappes, BP 111  
06902 Sophia Antipolis  
FRANCE  
Tel: 33 (0)4 92.94.37.13, Fax: 33 (0)4 92.94.37.01  
E-mail: jp.faucon@sophia.afssa.fr

Dr W. Ritter  
Chemisches und Veterinäruntersuchungsamt Freiburg  
P.O.B. 100462, D-79123 Freiburg  
GERMANY  
Tel: (49.761) 150.20.75, Fax: (49.761) 150.22.99  
E-mail: wolfgang.ritter@cvuafr.bwl.de
2.10.3. Salmonellosis – Salmonellose – Salmonelosis

**Dr R. Davies**  
VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB  
UNITED KINGDOM  
Tel: (44.1932) 35.73.61, Fax: (44.1932) 35.75.95  
E-mail: r.h.davies@vla.defra.gsi.gov.uk

**Dr C. Poppe**  
Health Canada, Population and Public Health Branch, Laboratory for Foodborne Zoonoses, 110 Stone Road West  
Guelph, Ontario N1G 3W4  
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Tel: (1.519) 822.33.00, Fax: (1.519) 822.22.80  
E-mail: cornelius_poppe@hc-sc.gc.ca

**Dr M. Hartung**  
BfR- Bundesinstitut für Risikobewertung (Federal Institute for Risk Assessment), P.O. Box 330013, 14191 Berlin  
GERMANY  
Tel: (49.1888) 412.22.12 Fax: (49.1888) 412.47.41  
E-mail: m.hartung@bfr.bund.de

2.10.6. Bovine viral diarrhoea – Diarrhée virale des bovins – Diarrea viral bovina

**Dr T.W. Drew**  
VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB  
UNITED KINGDOM  
Tel: (44.1932) 34.11.11, Fax: (44.1932) 34.70.46  
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**Dr D. Deregt**  
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CANADA  
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E-mail: deregtd@inspection.gc.ca

2.10.7. West Nile encephalitis – Encéphalite du Nil occidental – Encefalitis del Oeste del Nile

**Dr E.N. Ostlund**  
Diagnostic Virology Laboratory, National Veterinary Services Laboratories, P.O. Box 844, Ames, IA 50010  
UNITED STATES OF AMERICA  
Tel: (1.515) 663.75.51, Fax: (1.515) 663.73.48  
E-mail: eileen.n.ostlund@aphis.usda.gov

2.10.10. Hendra and Nipah virus diseases – Maladies dues aux virus Hendra et Nipah – Enfermedades causadas por los virus Hendra y Nipah

**Dr Peter Daniels**  
CSIRO Australia Animal Health Laboratory (AAHL), Geelong  
Victoria 322  
AUSTRALIA  
Tel: (61.3) 52.27.50.00, Fax: (61.3) 52.27.55.55  
E-mail: peter.daniels@csiro.au
Control of Veterinary Medicinal Products in Sub-Saharan Africa – Contrôle des médicaments vétérinaires en Afrique subsaharienne – Control de los Medicamentos Veterinarios en África subsahariana

Dr François Abiola
Ecole Inter-Etats de Science et Médecine Vétérinaire (EISMV)
BP 5077, Dakar
SENEGAL
Tel: (221) 865.10.08, Fax: (221) 825.42.83
E-mail: faabiola@refer.sn

Application of Polymerase Chain Reaction Methods for Diagnosis of Viral Diseases in Veterinary Medicine – Application des méthodes d’amplification en chaîne par polymérase pour le diagnostic des maladies virales en médecine vétérinaire – Aplicación de los Métodos PCR para el Diagnóstico de las Enfermedades Virales en Medicina Veterinaria

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E-mail: sandor.belak@sva.se
List of OIE Reference Laboratories in 2004

List of OIE Collaborating Centres

Veterinary Medicinal Products/Médicaments vétérinaires/Medicamentos veterinarios

AFSSA Fougères, Agence nationale du médicament vétérinaire
B.P. 203, 35302 Fougères Cedex
FRANCE
Tel: 33 (0)2 99.94.78.78
Fax: 33 (0)2 99.94.78.99
E-mail: p.dehaumont@anmv.afssa.fr

ELISA and Molecular Techniques in Animal Disease Diagnosis
Diagnostic des maladies animales par l’ELISA et les techniques moléculaires
Diagnóstico de enfermedades animales por el método ELISA y las técnicas moleculares

FAO/IAEA Centre for ELISA and Molecular Techniques in Animal Disease Diagnosis
International Atomic Energy Agency, Wagramerstrasse 5, P.O. Box 100
A-1400 Vienna
AUSTRIA
Tel: (43.1) 2600.283.55
Fax: (43.1) 2600.282.22
E-mail: a.diallo@iaea.org

Diagnosis and Control of Animal Diseases in Tropical Regions
Diagnostic et contrôle des maladies animales en régions tropicales
Diagnóstico y control de las enfermedades animales en las regiones tropicales

CIRAD/EMVT, Campus international de Baillarguet, TA 30/B, Montferriez-sur-Lez
B.P. 5035, 34032 Montpellier Cedex 01
FRANCE
Tel: 33 (0)4 67.59.37.11
Fax: 33 (0)4 67.59.37.95
E-mail: emmanuel.camus@cirad.fr

Surveillance and Control of Animal Diseases in Africa
Surveillance et contrôle des maladies animales en Afrique
Vigilancia y control de enfermedades animales en África

Onderstepoort Veterinary Institute, Agricultural Research Council
Private Bag X5, Onderstepoort 0110
SOUTH AFRICA
Tel: (27.12) 529.91.06
Fax: (27.12) 565.46.67
E-mail: fred@moon.ovi.ac.za

Animal Disease Surveillance Systems and Risk Analysis
Systèmes de surveillance des maladies animales et analyse des risques
Sistemas de vigilancia de enfermedades animales y análisis de riesgos

Centers for Epidemiology and Animal Health
USDA/APHIS-VS, 2150 Centre Av. Building B
Fort Collins, Colorado 80526
UNITED STATES OF AMERICA
Tel: (1.970) 490.70.01
Fax: (1.970) 472.26.68
E-mail: cristobal.zepeda@aphis.usda.gov
List of OIE Reference Laboratories in 2004

**Diagnosis and Control of Animal Diseases in Eastern Europe, Central Asia and Transcaucasia**

*Diagnostic et contrôle des maladies animales en Europe de l’Est, en Asie centrale et en Transcaucassie*

*Diagnóstico y control de las enfermedades animales en Europa oriental, Asia central y Transcaucasia*

Federal Government Institute(1) Federal Centre for Animal Health (FGI ARRIA) 600900 Yur’evets, Vladimir RUSSIA

All-Russia State Research Institute for Control(2) Standardisation and Certification of Veterinary Preparations Ministry of Agriculture and Food, 5 Zvenigorodskoye shosse, 123022 Moscow, RUSSIA

Tel: (7.0922) 26.06.14/26.19.14 
Fax: (7.0922) 26.15.25/26.15.51 
E-mail: mail@arriah.elcom.ru 
Telex: 218124 URAL RUS

**Veterinary Training, Epidemiology, Food Safety and Animal Welfare**

*Formation vétérinaire, l’épidémiologie, la sécurité sanitaire des aliments et le bien-être animal *

*Entrenamiento Veterinario, Epidemiología, Seguridad Sanitaria de los Alimentos y Bienestar Animal*

Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise ‘G. Caporale’ 
Via Campo Boario, 64100 Teramo ITALY 
Tel: (39.0861) 33.22.79 
Fax: (39.0861) 33.22.51 
E-mail: caporale@izs.it

**Diagnosis of Animal Diseases and Vaccine Evaluation in the Americas**

*Diagnostic des maladies animales et évaluation des vaccins dans les Amériques*

*Diagnóstico de las enfermedades animales y evaluación de las vacunas en las Américas*

National Veterinary Services Laboratory(1) Institute for International Cooperation in Animal Biologics(2) VS/APHIS/USDA College of Veterinary Medicine P.O. Box 844 Iowa State University Ames, Iowa 50010 Ames, Iowa 50010 UNITED STATES OF AMERICA UNITED STATES OF AMERICA 
Tel: (1.515) 663.72.66 Tel: (1.515) 294.18.50 
Fax: (1.515) 663.73.97 Fax: (1.515) 294.82.59 
E-mail: nvsl.concerns@aphis.usda.gov E-mail: iicab@iastate.edu

Center for Veterinary Biologics(3) 
VS/APHIS/USDA P.O. Box 844 Ames, Iowa 50010 UNITED STATES OF AMERICA 
Tel: (1.515) 663.73.31 
Fax: (1.515) 663.76.73 
E-mail: cvb@aphis.usda.gov
### Food-Borne Zoonotic Parasites

**Les zoonoses parasitaires d'origine alimentaire**  
**Las zoonosis parasitarias de origen alimentario**

Canadian Food Inspection Agency  
Centre for Animal Parasitology  
116 Veterinary Road  
Saskatoon, Saskatchewan S7N 2R3  
CANADA  
Tel: (1.306) 975.40.71  
Fax: (1.306) 975.57.11  
E-mail: agajadhar@inspection.gc.ca

### Training of Official Veterinarians

**Formation des vétérinaires officiels**  
**Formación de Veterinarios Oficiales**

Ecole nationale des Services vétérinaires  
1, avenue Bourgelat  
BP 83  
69280 Marcy-l’Etoile  
FRANCE  
Tel: 33 (0)4 78.87.25.45  
Fax: 33 (0)4 78.87.25.48  
E-mail: veronique.bellemain@agriculture.gouv.fr

### New and Emerging Diseases

**Maladies nouvelles et émergentes**  
**Nuevas Enfermedades y Enfermedades Emergentes**

CSIRO Australia Animal Health Laboratory (AAHL)  
Geelong Victoria 322  
AUSTRALIA  
Tel: (61.3) 52.27.50.00, Fax: (61.3) 52.27.55.55  
E-mail: martyn.jeggo@csiro.au

### OIE COLLABORATING CENTRE FOR AQUATIC ANIMAL DISEASES

**Information on Aquatic Animal Diseases**  
**Información sobre las maladies des animaux aquatiques**  
**Información sobre las enfermedades de los animales acuáticos**

The Centre for Environment, Fisheries and Aquaculture Sciences (CEFAS)  
Weymouth Laboratory, Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB  
UNITED KINGDOM  
Tel: (44.1305) 20.66.26  
Fax: (44.1305) 20.66.27  
E-mail: b.j.hill@cefas.co.uk
2.1.1. Epizootic haematopoietic necrosis – Nécrose hématopoïétique épizootique – Necrosis hematopoyética epizoótica

Dr A. Hyatt
Australian Animal Health Laboratory, CSIRO, Private Bag 24
Geelong, Victoria 3220
AUSTRALIA
Tel: (61.3) 52.27.50.00, Fax (61.3) 52.27.55.55
E-mail: alex.hyatt@li.csiro.au

Dr R. Whittington
Faculty of Veterinary Science, University of Sydney, 425 Werombi Road, Private Bag 3, Camden NSW 2570, AUSTRALIA
Tel.: (61.2) 93.51.16.19., Fax: (61.2) 93.51.16.18
E-mail: richardw@camden.usyd.edu.au

2.1.2 Infectious haematopoietic necrosis – Nécrose hématopoïétique infectieuse – Necrosis hematopoyética infecciosa (Rhabdoviruses)

Dr J. Winton
Western Fisheries Research Center, 6505 N.E. 65th Street
Seattle, Washington 98115
UNITED STATES OF AMERICA
Tel: (1.206) 526.65.87, Fax: (1.206) 526.66.54
E-mail: jim_winton@usgs.gov

2.1.3. Oncorhynchus masou virus disease – Herpèsvirose du saumon masou – Herpesvirosis del salmón masou

Dr M. Yoshimizu
Laboratory of Microbiology, Graduate School of Fisheries
Sciences, Hokkaido University, 3-1-1 Hakodate
Hokkaido 041-0821
JAPAN
Tel/Fax: (81.138) 40.88.10
E-mail: yosimizu@fish.hokudai.ac.jp

2.1.4. Spring viraemia of carp – Virémie printanière de la carpe – Viremia primaveral de la carpa

Prof. B.J. Hill
The Centre for Environment, Fisheries and Aquaculture Sciences (CEFAS), Barrack Road, The Nothe Weymouth
Dorset, DT4 8UB
UNITED KINGDOM
Tel: (44.1305) 20.66.26, Fax: (44.1305) 20.66.27
E-mail: b.j.hill@cefas.co.uk

* The numbers correspond to the chapters in the Aquatic Animal Health Code
Les références correspondent aux chapitres dans le Code sanitaire pour les animaux aquatiques
Las referencias corresponden a los capítulos en el Código Sanitario para los Animales Acuáticos

OIE Terrestrial Manual 2004 1173
2.1.5. Virus hemorrhagic septicaemia – Septicémie hémorragique virale – Septicemia hemorrágica viral

Dr N.J. Olesen
Danish Veterinary Laboratory, Hangøvej 2, DK-8200 Aarhus N
DENMARK
Tel: (45) 89.37.24.31, Fax: (45) 89.37.24.70
E-mail: njo@svs.dk

2.1.6. Channel catfish virus disease – Herpèsvirose du poisson-chat – Virosis del bagre de canal

Dr L.A. Hanson
Fish Diagnostic Laboratory, College of Veterinary Medicine
Mississippi State University, Box 6100, Mississippi 39762
UNITED STATES OF AMERICA
Tel: (1.662) 325.12.02, Fax: (1.662) 325.10.31
E-mail: hanson@cvm.msstate.edu

2.1.7. Viral encephalopathy and retinopathy – Encéphalopathie et rétinopathie virales – Encefalopatía y retinopatía virales

Dr G. Bovo
Istituto Zooprofylattico Sperimentale delle Venezie, Dipartimento di Ittiovirologia, Viale dell’Università 10, 35020 Legnaro PD
ITALY
Tel: (39.049) 88.30.380, Fax: (39.049) 88.30.046
E-mail: bovo.izs@interbusiness.it

Dr T. Nakai
Laboratory of Fish Pathology, Graduate School of Biosphere Science, Hiroshima University, Higashihiroshima 739-8528
JAPAN
Tel: (81.824) 24.79.47, Fax: (81.824) 22.70.59
E-mail: nakait@hiroshima-u.ac.jp

2.1.8. Infectious pancreatic necrosis – Nécrose pancréatique infectieuse – Necrosis pancreática infecciosa

Prof. B.J. Hill
The Centre for Environment, Fisheries and Aquaculture Sciences (CEFAS), Barrack Road, The Nothe Weymouth
Dorset, DT4 8UB
UNITED KINGDOM
Tel: (44.1305) 20.66.26, Fax: (44.1305) 20.66.27
E-mail: b.j.hill@cefas.co.uk

2.1.9. Infectious salmon anaemia – Anémie infectieuse du saumon – Anemia infecciosa del salmón

Dr B. Dannevig
National Veterinary Institute, Ullevålsvien 68
P.O. Box 8156 Dep., 0033 Oslo
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Tel: (47.23) 21.64.04, Fax: (47.23) 21.63.01
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Dr Frederick Kibenge
Atlantic Veterinary College, Department of Pathology and Microbiology, Faculty of Veterinary Medicine
University of Prince Edward Island, 550 University Avenue
Charlottetown, Prince Edward Island, C1A 4P3
CANADA
Tel: (1.902) 566-0967, Fax: (1.902) 566-0851
E-mail: kibenge@upei.ca
2.1.10. Epizootic ulcerative syndrome – Syndrome ulcératif épizootique – Síndrome ulcerante epizootico

Dr S. Kanchanakhan  
Aquatic Animal Health Research Institute (AAHRI)  
Department of Fisheries, Kasetsart University Campus  
Phaholyothin Road, Jatuchak, Bangkok 10900  
THAILAND  
Tel: (66.2) 579.41.22, Fax: (66.2) 561.39.93  
E-mail: somkiatkic@fisheries.go.th

2.1.11. Bacterial kidney disease (Renibacterium salmoninarum) – Rénibactérioise – Renibacteriosis

Dr R.J. Pascho  
Western Fisheries Research Center, 6505 N.E. 65th Street  
Seattle, Washington 98115  
UNITED STATES OF AMERICA  
Tel: (1.206) 526.65.88, Fax: (1.206) 526.66.54  
E-mail: ron_pascho@usgs.gov


Dr L.A. Hanson  
Fish Diagnostic Laboratory, College of Veterinary Medicine  
Mississippi State University, Box 6100, Mississippi 39762  
UNITED STATES OF AMERICA  
Tel: (1.662) 325.12.02, Fax: (1.662) 325.10.31  
E-mail: hanson@cvm.msstate.edu

2.1.13. Piscirickettsiosis (Piscirickettsia salmonis) – Piscirickettsiose – Piscirickettsiosis

Dr M. Kent  
Center for Fish Disease Research, Department of Microbiology,  
220 Nash Hall, Oregon State University, Corvallis  
Oregon 97331-3804  
UNITED STATES OF AMERICA  
Tel: (1.541) 737.44.41, Fax: (1.541) 737.04.96  
E-mail: michael.kent@orst.edu

2.1.14. Gyrodactylosis (Gyrodactylus salaris) – Gyrodactylose – Girodactilosis

Dr T.A. Mo  
National Veterinary Institute, Fish Health Section  
Ullevålsvien 68, P.O. Box 8156 Dep., 0033 Oslo  
NORWAY  
Tel: (47.23) 21.61.10, Fax: (47.23) 21.61.01  
E-mail: tor-atle.mo@vetinst.no
2.1.15. Red sea bream iridoviral disease – Iridovirose de la daurade japonaise – Iridovirosis de la dorada japonesa

Dr K. Nakajima
Fisheries Research Agency, Research Promotion and Development Division, 2-12-4 Fukuura, Kanagawa-ku Yokohama-shi, Kanagawa 236-8648 JAPAN
Tel: (81.45) 788.75.12, Fax: (81.45) 788.50.90
E-mail: kazuhiro@fra.affrc.go.jp

3.1.1–3.1.3, 3.1.5, 3.1.6. Bonamiosis and Marteiliosis – Bonamiose et Marteiliose – Bonamiosis y Marteiliosis

Dr F. Berthe
Laboratoire de Génétique Aquaculture et Pathologie, IFREMER
BP 133, 17390 La Tremblade FRANCE
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E-mail: fberthe@ifremer.fr


Dr E.M. Burreson
Director for Research and Advisory Services, Virginia Institute of Marine Science, P.O. Box 1346, College of William and Mary Gloucester Point, VA 23062 UNITED STATES OF AMERICA
Tel: (1.804) 684.71.08, Fax: (1.804) 684.70.97
E-mail: gene@vims.edu

3.1.7. Mikrocytosis – Mikrocytose – Mikrocytosis

Dr S. Bower
Department of Fisheries and Oceans, Pacific Biological Station 3190 Hammond Bay Road, Nanaimo, British Columbia V9R 5K6 CANADA
Tel: (1.250) 756.70.77, Fax: (1.250) 756.70.53
E-mail: bowers@dfo-mpo.gc.ca

4.1.1. 4.1.2, 4.1.4.–4.1.6 Crustacean pathogens – Agents pathogènes des crustacés – Agentes patógenos de los crustáceos

Prof. D.V. Lightner
Aquaculture Pathology Laboratory, Department of Veterinary Science and Microbiology, University of Arizona, Building 90 Room 202 Pharmacy/Microbiology, Tucson, AZ 85721 UNITED STATES OF AMERICA
Tel: (1.520) 621.84.14, Fax: (1.520) 621.48.99
E-mail: dvl@u.arizona.edu

** Reference Laboratory for the following diseases: Taura syndrome, White spot disease, Tetrahedral baculovirosis (Baculovirus penaei), Spherical baculovirosis (Penaeus monodon-type baculovirus) & Infectious hypodermal and haematopoietic necrosis – Syndrome de Taura, Enfermedad de las manchas blancas, Baculovirosis tetraédrique (Baculovirus penaei), Baculovirosis sphérique (Baculovirus spécifique de Penaeus monodon) & Nécrose hypodermique et hématoipoïétique infectieuse – Síndrome de Taura, Enfermedad de las manchas blancas, Baculovirosis tetraédrica (Baculovirus penaei), Baculovirosis esférica (Baculovirus de tipo Penaeus monodon) & Necrosis hipodérmica y hematopoyética infecciosa
4.1.2. White spot disease – Maladie des points blancs – Enfermedad de las manchas blancas

Dr Grace Lo
Department & Institute of Zoology, National Taiwan University
1, Sec. 4, Roosevelt Rd
TAIPEI CHINA
Tel: (886-2) 23.63.02.31/22.62, Fax: (886-2) 23.63.68.37
E-mail: gracelow@ccms.ntu.edu.tw

4.1.3. Yellowhead disease – Maladie de la tête jaune – Enfermedad de la cabeza amarilla

Dr Peter Walker
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AUSTRALIA
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4.1.5. Spherical baculovirosis (*Penaeus monodon*-type baculovirus) – Baculovirose sphérique (Baculovirus spécifique de *Penaeus monodon*) – Baculovirosis esférica (Baculovirus de tipo *Penaeus monodon*)

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4.1.7. Crayfish plague – Peste de l'écervisse – Plaga del cangrejo de río (*Aphanomyces astaci*)

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## ALPHABETICAL LIST OF DISEASES COVERED IN THIS TERRESTRIAL MANUAL

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### Alphabetical list of diseases covered in this Terrestrial Manual

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