Mistakes

Room temp: 16-18

Contents: Rabies p. 205 (not 204)

767

57 (sent by Yajima)

615 (incomplete ref) remains

ix Next Manual: add a note re fish diseases?

297

Ent. enc. makes ref to tests for sterility in chapter on general into & change to chapter on Tests for sterility when ready

* Marken vaccines = deletion

Sep. 15
MANUAL OF STANDARDS
FOR DIAGNOSTIC TESTS AND VACCINES
for Lists A and B diseases
of mammals, birds and bees
1992
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List of OIE Reference Laboratories (as of May 1992) | - | 767 |
Alphabetical list of diseases | - | 781 |
The purpose of this Manual is to provide a uniform approach to the diagnosis of important animal diseases. Its aim is to describe standard methods for laboratory disease diagnosis and the production and control of biological products (mainly vaccines), for veterinary use in laboratories all over the world. This should increase efficiency and promote improvements in animal health worldwide.

This is an ambitious objective requiring the co-operation of veterinary experts in many countries. The Office International des Epizooties (OIE) was clearly one of the organisations best equipped to undertake this task at a worldwide level. The OIE was established in 1924 in order to promote world animal health, and its main activities are as follows:

1. To collect and disseminate to its Member Countries, information (including emergency information) on the occurrence, course and treatment of animal diseases

2. To provide guidelines and standards for health regulations applicable in the international trade of animals

3. To promote and co-ordinate research on the pathology, treatment and prevention of animal diseases when international collaboration in such research is desirable.

In pursuing these activities (especially the second one), the OIE had already produced the International Animal Health Code (previously known as the Zoo-Sanitary Code). The Manual is a companion volume to the Code and provides standards for the diagnostic tests and vaccines prescribed in the Code. However, the Manual is not limited to those diseases for which the Code prescribes tests, but covers all of the OIE Lists A and B diseases except fish diseases. A few additional related diseases of importance for trade are also covered.

The task of compiling the Manual was assigned to the OIE Standards Commission (previously known as the Norms Commission) by the International Committee in May 1985. Under the editorship of the Standards Commission and initially of Dr J.H. Darbyshire, and later of Dr Y. Ozawa, manuscripts were requested from specialists in each of the diseases covered. Each chapter was circulated to all OIE Member Countries for review and the Standards Commission took the resultant comments into account before finalising the chapters. The World Health Organisation and the Food and Agriculture Organisation of the United Nations, with whom the OIE maintains close co-ordination in matters of mutual interest, were also consulted. The chapters were then published in three separate volumes between 1989 and 1991.

In this book, the three volumes have been combined, some supplementary information has been added, and some chapters have been updated. However, continual revision is needed in such a changing field, and the OIE intends to issue an updated version of the Manual approximately once every four years.
We extend our most sincere gratitude to Drs Ozawa and Darbyshire for their role in coordinating this project; to the authors of the chapters; and to the many other people listed in the Acknowledgements whose work made it possible to produce this book.

Dr J. Blancou
Director General, OIE

Prof. M. Truszczynski
President, OIE Standards Commission

July 1992
ACKNOWLEDGEMENTS

I am most grateful to the many people whose combined efforts have gone into the preparation of this Manual. In particular, I would like to express my thanks to:

- Dr L. Blajan, Director General of the OIE from 1980 to December 1990, who gave his encouragement and support to the project of producing this Manual

- Dr J.H. Darbyshire, who effectively set the whole process in motion by serving as editor from 1986 to September 1988

- The authors listed on pp xvii to xxv, who contributed their precious time to write the chapters, which of course constitute the essence of the Manual

- Dr G.A. Cullen of the Central Veterinary Laboratory, Weybridge, UK, who acted as assistant editor and did a great deal in harmonising the contents and providing missing information where required

- Dr J. Pearson of the National Veterinary Services Laboratories, Ames, Iowa, USA, who also provided supplementary details, having carried out a comparative review of information in the Manual and the International Animal Health Code

- Those OIE Member Countries who submitted comments on the draft chapters that were circulated to them. These were most helpful in making the Manual internationally acceptable

- The members of the OIE Standards Commission (listed below*), who proposed the idea and the format of the Manual and were responsible for finalising the chapters, taking into account the comments received

- Staff members of the OIE Technical and Publications Departments, for their assistance

- Miss Grace Townshend, who made a major contribution as editorial assistant.

Dr Y. Ozawa
Principal Scientific Adviser

*Members of the OIE Standards Commission

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About this volume

In this book, Volumes I, II and III of the OIE Manual (published in 1989, 1990 and 1991 respectively) have been combined. Some of the chapters have been revised in order to ensure that all the 'prescribed tests' listed in the table on p. xi are covered. Information has been added or revised where there was a pressing need for this to be done. However, we are aware that some chapters need much more extensive revision in the light of the rapid changes taking place in the fields of disease diagnosis and vaccine production. The OIE therefore intends to revise the Manual completely and to produce a new version approximately once every four years.

Diseases covered in the Manual and numbering system

The Manual covers diseases in the approximate order in which they appear in OIE Lists A and B. List A includes those diseases which spread rapidly, the scope of which extends beyond national borders. These diseases 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 includes the diseases which are considered of socio-economic and/or public health importance within countries, and which are also, of course, significant in the international trade of animals and animal products.

A few additional related diseases of importance for trade (e.g. S. typhimurium and S. enteritidis) are also included.

The numbering system (A1, B2, etc.) used in previous volumes of the Manual has been retained for ease of reference. These numbers indicate whether a disease is on List A or B and the sequence of appearance, and are used in the OIE International Animal Health Code when cross-references are made to the Manual. (A different numbering system is used in the FAO/OIE/WHO Animal Health Yearbook due to the requirements of computerisation.)

Teschen disease, previously on List A, has been moved to List B and is now called enterovirus encephalomyelitis.

There is an alphabetical index of the diseases at the back of the book.

Format of chapters

Each 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 chapter, Part A deals with laboratory diagnosis of the disease, and Part B (where appropriate) with the requirements for vaccines or
other biological products. Bibliographic references that provide further information are listed at the end of each chapter.

Most diseases are treated independently in a separate chapter. In some cases, a chapter may include more than one disease; for example *Brucella ovis* infection, caprine/ovine brucellosis and porcine brucellosis are treated together. Trypanosomiasis applies to both cattle and horses.

**Explanation of the tests described and of the table on p. xi**

The table on page xi lists diagnostic tests in three categories: 'prescribed', 'alternative', and 'other'. Prescribed tests are those which are required by the *International Animal Health Code* for the screening of animals before they are moved internationally. In the *Manual* these tests are indicated by a line in the margin next to them. At present it is not possible to have prescribed tests for every disease of Lists A and B. 'Alternative tests' are those which are suitable for the diagnosis of disease within a local setting and can also be used in the import/export of animals after bilateral negotiation. The third group listed in the table are 'other tests', which may also be of some practical value in local situations or which may still be in a state of development.

A booklet containing French and Spanish translations of the prescribed tests only, will be available shortly after publication of this *Manual*. It will be sent together with the *Manual* to all purchasers in French- and Spanish-speaking countries. Purchasers in other countries may request a copy if desired.

**List of OIE Reference Laboratories**

A list of OIE Reference Laboratories appears on p. 767 of the *Manual*. These laboratories have been designated by the OIE as centres of expertise in their particular field, able to provide advice on methodology. In some cases standard strains or reagents (antisera, antigens, etc.) 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, and the revised list will be published in the May issue of the *OIE Bulletin*.

*(or, for disease is not listed in the Code, the best tests available)*
**LIST OF PRESCRIBED AND ALTERNATIVE TESTS (a)**

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(a) An explanation of this table is given on p. x. In the text prescribed tests are indicated by a line in the margin next to them.

Amendments to this list will be published in the May issue of the Bulletin of the Office International des Epizooties.
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Disease Changes approved May 95

- Vesicular stomatitis virus: ELISA, VN, CF
- African horse sickness: ELISA, CF
- Paratuberculosis: -
- Bovine babesiosis: -
- Bovine brucellosis: BBAT, CF, ELISA
- Infectious bovine rhinotracheitis: VN, ELISA
- Dourine: CF
- Equine viral arteritis: VN
- Agent identification (semen only)
- Brucellosis in pigs: BBAT
- Trichinellosis: Agent identification
- Enferovirus encephalomyelitis: -
## Abbreviations used in the Manual

**Note:** Abbreviations are usually defined the first time they appear in each chapter. For convenience, this list covers some of the most frequently used abbreviations.

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<thead>
<tr>
<th>Abbreviation</th>
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<tr>
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<td>Acidified antigen plate tests</td>
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<td>AGID</td>
<td>Agar gel immunodiffusion</td>
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<td>ASF</td>
<td>African swine fever</td>
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<td>BHK</td>
<td>Baby hamster kidney (cell line)</td>
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<td>BPAT</td>
<td>Buffered plate antigen test</td>
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<td>BVD(V)</td>
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<td>ECE</td>
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<td>MEM</td>
<td>Minimum essential medium</td>
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**Abbreviations:**
- **BPL** beta-prolactrone
- **OPD** o-phenylenediamine (both in VHD)
- **CAM** chorioallantoic membrane (DVE)
- **ID<sub>50</sub>** |
- **PD<sub>50</sub>** |
- **LA** latex agglutination
- **LD<sub>50</sub>**
MV(V) Maedi-visna (virus)
ND (V) Newcastle disease virus
NPLA Neutralisation peroxidase-linked assay
OD Optical density
PBS Phosphate buffered saline
PFU Plaque-forming unit
PPD Purified protein derivative
PPLO Pleuropneumonia-like organisms
PPR Peste des petits ruminants
PRN Plaque reduction neutralisation
RBC Red blood cell
RFFIT Rapid fluorescent focus inhibition test
RK Rabbit kidney
RREID Rapid rabies enzyme immunodiagnosis
SPF Specific pathogen free
TCID Tissue culture infective dose
TGE Transmissible gastroenteritis
USDA US Department of Agriculture
VHD Viral haemorrhagic disease
VIA Virus infection associated
VN Virus neutralisation
VS(V) Vesicular stomatitis (virus)

Note: A line in the margin indicates a test that is prescribed for the purposes of international trade by the International Animal Health Code.

\[ \text{ICFTU} \quad (812) \]
\[ \text{SRK} \quad \text{Sheep red blood cell} \]
\[ \text{W/V} \]
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<td>G.A. Cullen</td>
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<td>A4 Rinderpest</td>
<td>W.P. Taylor</td>
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<td>A5 Peste des petits ruminants</td>
<td>O.A. Durojaiye</td>
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<td>A6 Contagious bovine pleuropneumonia</td>
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<td>A7 Lumpy skin disease</td>
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<td>Veterinary Research Laboratory, P.O. Kabete, Kenya</td>
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<td>A8 Rift Valley fever</td>
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<td>Director of Veterinary Services and Animal Health, Ministry of Agriculture, P.O. Box 12, Beit Dagan, Israel</td>
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<td>Animal Disease</td>
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<td>A10 Sheep and goat pox</td>
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<td>A11 African horse sickness</td>
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<td>A12 African swine fever</td>
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<td>A15 Highly pathogenic avian influenza</td>
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<td>B1 Anthrax</td>
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<td>B6 Leptospirosis</td>
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| 830  | 43     | Nairobi sheep disease | F.G. Davies  
Veternary Research Laboratory, P.O. Kabete, Kenya |
| 831  | 44     | Salmonellosis (with particular reference to S. abortus ovis/ S. abortus equi) | P. Pardon  
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| 836  | 49     | Dourine | J. Donnelly  
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<td>66</td>
<td>Avian infectious laryngotracheitis</td>
<td>R.C. Jones University of Liverpool Department of Veterinary Pathology, Veterinary Field Station, Jordan Building, Leahurst, Neston Wirral, L64 7TE, UK</td>
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<td>67</td>
<td>Avian tuberculosis</td>
<td>E. Boughton Central Veterinary Laboratory, New Haw, Weybridge, Surrey KT15 3NB, UK</td>
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<td>Duck hepatitis</td>
<td>P. R. Woolcock Cornell University Duck Research Laboratory, P.O. Box 217, Old Country Road, Eastport, Long Island, NY 11941, USA</td>
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<td>K.R. Rhodes USDA-ARS, Midwest Area, National Animal Disease Center, P.O. Box 70, Ames, Iowa 50010, USA</td>
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<td>71</td>
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<td>S.B. Houghton Hoechst Animal Health, A Division of Hoechst UK Ltd, Walton Manor, Walton, Milton Keynes, Bucks MK7 7AJ, UK</td>
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<td>D.N. Tripathy College of Veterinary Medicine, University of Illinois, Urbana, IL 61801, USA</td>
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<td>Compton New, Near Newbury, Berkshire RG16 0NN, UK</td>
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**Extracted Text**

- A56 66 Avian infectious laryngotracheitis
- B57 67 Avian tuberculosis
- B58 68 Duck hepatitis
- B59 69 Duck virus enteritis
- B60 70 Fowl cholera
- B61 71 Fowl pox
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- B63 73 Infectious bursal disease
- B64 74 Marek's disease
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- B66 76 Avian chlamydiosis

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<td>Introductory note on bee diseases and co-ordination of bee diseases section</td>
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GENERAL INFORMATION

I. Sampling methods

Introduction

Samples may be taken from animals, or their environment, for the purpose of establishing a disease diagnosis, for health surveillance or for the monitoring of response to vaccines. A great variety of different combinations of sample and species of animal may occur. In all cases the samples need to be appropriate for the purpose required, and adequate in number to provide a statistically valid result. Samples must be taken with care, to avoid undue stress or damage to the animal or danger to the operator. It is usually important to adopt aseptic techniques and care must be taken to avoid cross-contamination between samples. Having obtained suitable material, it must be carefully packaged and labelled and transmitted to the laboratory by the fastest practicable method. Relevant postal regulations must be obeyed. If material is sent to a laboratory in another country, this must be consulted in advance, to ensure that the laboratory is willing to receive the material. All specimens must be accompanied by a written note indicating the origin of the material, the relevant history and the tests required.

Collection of specimens

Blood

Blood samples may be taken for haematology or for culture for bacteria, viruses, or protozoa, in which case the blood is added to anti-coagulants such as heparin. They may also be taken for serology, in which case a clotted sample is required. A blood sample is taken, as cleanly as possible, by venepuncture. In most large mammals the jugular vein or a caudal vein are selected, but brachial veins and mammary veins are also used. In birds a wing vein (brachial vein) is usually selected. Blood may be taken by syringe and needle, by needle and vacuum tube (not easy in delicate veins but convenient in strong veins) or by the use of a stab with a triangular needle and dripping into an open tube. The latter method is economical but does not give such a clean specimen. Ideally the skin at the site of venepuncture should first be shaved (plucked) and swabbed with 70% ethyl alcohol and allowed to dry.

Whole blood samples can have antibiotics added to reduce bacterial growth, taking
care that the antibiotics are chosen so as to avoid interference with the growth of the pathogens concerned. For samples with anticoagulant and/or antibiotics, thorough mixing is necessary as soon as the sample has been taken. For serum samples, the blood should be left to stand at ambient temperature (but protected from excessive heat) until the clot begins to contract. The clot can then be ringed round with a rod and the bottles then placed in a refrigerator at +4°C. Later, the serum can be decanted or removed after slow centrifugation. If serum cannot be transported to the laboratory within 48 hours, antibiotics such as penicillin and dihydrostreptomycin should be added. Chemical preservatives such as boric acid or merthiolate should be avoided in sera to be used in virus neutralisation tests. A new development is to transport a drop of dried blood on a filter paper disk, which contains enough material for sensitive antibody assay systems.

Tissues

Tissues may be collected for culture or for histopathology and occasionally for use as antigen in serological tests. The person removing the tissues should be experienced in post-mortem technique and have a knowledge of pathology sufficient to select the right organs and the most promising lesions for sampling. The skin of the dead animal may be removed with ordinary instruments but the body cavities should be opened with sterile instruments and a fresh set of sterile instruments used to collect the pieces of the various organs required. Each piece of tissue should be placed in a separate sterile screw-capped jar, fully labelled with the date, tissue and animal identification. Care must be taken not to contaminate one tissue with another. Instruments can be sterilised in the field in a simple boiler which can be heated on a burner with portable packs of liquid gas.

The specimens should be forwarded to the laboratory by the fastest direct route. If they can reach the laboratory within 24 hours they should be forwarded in a wide mouthed vacuum flask with wet ice. An alternative is to use polystyrene containers and chemical refrigeration bricks. Only if the specimens are likely to take more than 24 hours to reach the laboratory is it necessary to freeze the specimens and send them in this state. The tissues may be sent to the laboratory dry or in virus transport medium (VTM) depending on the examinations required. For histopathology, blocks of tissue not more than 0.5 cm thick and 1-2 cm² are cut and placed in neutral buffered formalin which should be at least ten times the volume of the tissue sample. For some procedures, e.g. rabies, larger portions of brain are required, some fresh and some in fixative.

Faeces

Freshly voided faeces should be selected, which may be sent either dry or in VTM, depending on the laboratory tests required. An alternative and sometimes preferable method is to take swabs from the rectum (or cloaca) taking care to swab the mucosal surface. Swabs may also be transported either dry or in VTM.
Skin

In diseases producing vesicular rashes or where lesions are exclusively in the skin, specimens are taken from the lesions themselves. Scrapings of the lesion may be taken, and additionally the vesical fluid should be sampled where unruptured vesicles are present.

Genital tract

Samples may be taken by vaginal or preputial washing, or by the use of suitable swabs. Sometimes the same urethra is also sampled by swabbing.

Eye

A gentle swab of the surface of the conjunctiva is taken, which is broken off into VTM. Scrapings may also be taken, onto a microscope slide.

Environment

Samples may be taken to monitor hygiene or as part of a disease enquiry, for example, from litter, ventilation ducts, drains, hatcheries and slaughter houses.

Selection of samples

Considerable skill and care are required to decide on the correct samples to be sent to the laboratory in each case. Frequently a combination of blood samples for serology and tissues from dead or culled animals for microbiological culture will be required. These matters are considered in detail in the chapters dealing with each disease and will not be discussed here. There are, however, some general statistical rules which should be borne in mind, particularly when sampling herds or flocks for a health surveillance scheme. It is possible to calculate how many animals have to be sampled from a herd/flock of a certain size, to achieve a 95% probability of detecting infection assumed to be present in a certain percentage of the animals. For example, if disease were present in 5% of a herd of 500 animals, it would be necessary to sample 56 animals to be 95% confident of finding one positive (See "Livestock Disease Surveys 1982").

Information to be sent with specimens

Information or case history should always accompany the specimens to the laboratory with a duplicate to follow in a letter. The information should include the following points:

1. Name and address of owner
2. Different species on the farm and number of each
3. Species affected
4. Date of first cases and of subsequent cases or losses
5. Description of the spread of infection in the herd or flock
6. Number of animals dead, the number showing clinical signs and their age
7. The clinical signs and their duration including the condition of mouth, eyes and feet, and milk or egg production data
8. Type and standard of husbandry, including the type of feed available
9. A list and description of the specimens submitted for examination
10. Any medication already applied to the animals
11. The tests required
12. Name and address of sender

Transport of specimens

Samples must be carefully packed, to avoid any possibility of leakage. Screw-capped bottles should be used and should be additionally sealed with adhesive tape or paraffin wax. Samples in individually identified containers should be placed in larger strong outer containers and packed with enough absorbent material to protect from damage. Official regulations should be consulted. For air transport, parcels should be placed in the pressurized hold of the aircraft. It is advisable to contact the laboratory in advance by telephone or telex, especially in the case of unusual requests. It is essential to do so where material is sent to a laboratory overseas. Many countries require a special import licence to be obtained in advance for any biological material, especially for material which could contain animal pathogens.

References

II. Tests for sterility and freedom from contamination of biological materials

Definitions

**Sterility:** the absence of living organisms.

Sterility is achieved by heating, by filtration, by treatment with ethylene oxide, or by ionising irradiation, and by conducting any subsequent processing aseptically.

**Freedom from contamination:** the absence of specific living organisms or groups of organisms.

This may be achieved by selecting materials from sources shown to be free from the organisms specified, and by conducting subsequent processing aseptically.

Principles

Adequate assurance of sterility and freedom from contamination can be achieved only by proper control of the primary materials used in manufacture and of the manufacturing process.

Tests on the final product are necessary as a check that this control has been achieved, but the assurance of quality rests primarily on the in-process control system.

A. General procedures

1. Primary materials shall be collected and handled in such a way as to minimise contamination and the opportunities for any contaminants to multiply.

2. Materials that can be sterilised without their biological activities being affected unduly shall be sterilised by a method effective for the materials concerned. The method should reduce the level of contamination to no more than one micro-organism per million "units", that is, containers or doses, of product, as determined by an appropriate sterility test (See paragraph B3 below).

3. If a sterilisation process is used, it shall be adequately controlled to show that it has functioned properly on each occasion.
4. Materials that are not sterilised and those which are to be processed further after sterilisation shall be handled aseptically.

5. The environment in which any aseptic handling is carried out shall be maintained in a clean state and protected from external sources of contamination, and shall be controlled so as to reduce internal contamination to a minimum. The control should be sufficient to reduce the level of contamination to no more than three micro-organisms per thousand "units" as determined by an appropriate sterility test (See paragraph B3 below).

B. Living viral vaccines for administration by injection

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

2. Seed lots of virus and of any continuous cell line used for virus growth shall be shown to be free from bacteria, fungi, mycoplasmas, and pathogenic viruses and other pathogens which can be transmitted from the species of origin to the species to be vaccinated.

3. Each batch of vaccine shall pass a test for sterility that conforms to the General Requirements for Sterility of Biological Substances (Requirements for Biological substances No. 6) of the World Health Organisation. Suitable test methods are published in the European Pharmacopoeia and the United States Pharmacopoeia.

4. Each batch of vaccine shall pass tests appropriate to prove that the vaccine is free from extraneous viruses. (Such tests include tests in cell cultures susceptible to viruses of the species to be vaccinated, tests in embryonated eggs, and, where necessary, tests in animals.)

5. Some countries require that each batch of vaccine pass a test for freedom from mycoplasmas. Suitable test methods are published in the General Requirements for the Sterility of Biological Substances of the World Health Organisation, in the British Veterinary Pharmacopoeia, and, for avian mycoplasmas, in the European Pharmacopoeia.

6. Tests for freedom from certain specific bacteria may be required, e.g. for *Salmonella pullorum* on viral vaccines for poultry.
C. Living viral vaccines for administration through drinking water, spray, or skin scarification

1. Paragraphs B1, 2, 4, 5, and 6 apply.

2. A limited number of contaminating, non-pathogenic bacteria and fungi may be permitted.

D. Inactivated viral vaccines

1. Paragraphs B2 and 3 apply.

2. Each batch of vaccine shall pass a test for inactivation of the vaccinal virus.

3. Demonstration that the method of inactivation also inactivates representative pathogens may be required unless the vaccine satisfies the conditions of Paragraphs B1 and B4.

E. Living bacterial vaccines

1. Paragraph B1 applies.

2. Seed lots of bacteria shall be shown to be free of other bacteria as well as fungi and mycoplasmas.

3. Each batch of vaccine shall pass a test for sterility carried out using solid media and ignoring the growth of the vaccinal bacterium.

4. Some countries require that each batch of vaccine pass a test for freedom from mycoplasmas. Suitable test methods are published in the General Requirements for the Sterility of Biological Substances of the World Health Organisation, in the British Veterinary Pharmacopoeia and, for avian mycoplasmas, in the European Pharmacopoeia.

F. Inactivated bacterial vaccines

1. Paragraphs B1, B3, and E2 apply.

2. Each batch of vaccine shall pass a test for inactivation of the vaccinal bacterium. If appropriate, the test for sterility may be used for this purpose.
G. Sera for administration to animals

1. Paragraph B1 applies.

2. Paragraph B2 or E2 applies, as appropriate, if a virus or a bacterium is used in serum production.

3. Each batch of serum shall pass a test for sterility that conforms to the General Requirements for Sterility of Biological Substances (Requirements for Biological substances No. 6) of the World Health Organisation. Suitable test methods are published in the European Pharmacopoeia and the United States Pharmacopoeia.

4. Each batch of serum shall pass tests appropriate to provide that the serum is free from extraneous viruses. (Such tests include tests in cell cultures susceptible to viruses of the species to be treated, tests in embryonated eggs and, where necessary, tests in animals.)

5. Some countries require that each batch of serum pass a test for freedom from mycoplasmas. Suitable test methods are published in the General Requirements for the Sterility of Biological Substances of the World Health Organisation, in the British Veterinary Pharmacopoeia and, for avian mycoplasmas, in the European Pharmacopoeia.

H. Diagnostic agents for administration to animals

1. Paragraphs B1 and 3 apply.

2. Paragraphs B2 and D2 apply if a virus is used in the production of the diagnostic agent; E2 and F2 apply if a bacterium is used.

I. Embryos, ova, and semen

See "Diseases transmissible by semen and embryo transfer techniques", OIE Technical Series No. 4, 1985.
Laboratory work of the type described in this Manual should be carried out without risk to the health of the staff. This requires careful consideration of the risks involved in a particular procedure followed by appropriate measures to avoid human infection. This is a complex subject which can only be considered in outline in this chapter. References for further reading are given below.

Existing national and international reference laboratories have considerable experience in the operation of safe working practices and provision of appropriate facilities. When new laboratories are being established it would be prudent to seek advice from the competent authorities at established institutes.

Assessment of risk from pathogens

To assess the risk from a particular pathogen it is necessary to know whether infection with that organism can cause death, disease or inconvenience to the people working with it and whether it could then spread to cause disease in the general population or in animals. To assess this risk it is necessary to know the epidemiological background to the organism and also such attributes of the organism as infectivity for man, stability in the environment, ability to infect by different routes and susceptibility to specific treatments or prophylaxis. It is relatively easy to do this when working with a known pathogen but the problem is more complex in a diagnostic laboratory, receiving clinical material which may be infected with a variety of pathogens, some of which could be extremely hazardous to human health. Some of the considerations to take into account are:

1. Known occurrence of human infection with the organism or related organisms with similar characteristics. Production of toxins or allergens.

2. The volume of culture to be handled and the concentration of the organism likely to be present. (Procedures such as antigen or vaccine production which require large quantities of organisms carry a higher risk than attempted isolation procedures.)

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

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

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

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

**Grouping of micro-organisms**

Micro-organisms can be conveniently divided into four groups representing increasing hazards to human health. These are:

- **Group 1** Organisms which are unlikely to cause human disease.
- **Group 2** Organisms which may cause human disease but are unlikely to be spread in the community and for which effective prophylaxis and treatment are available.
- **Group 3** Organisms which can cause severe human disease and may spread in the community but for which there is usually effective prophylaxis and treatment.
- **Group 4** Organisms which cause severe human disease, may represent a high risk of spread in the community and for which there is usually no effective prophylaxis or treatment.

An additional group may be required for animal disease agents which are controlled by veterinary authorities.

Such categorisation of pathogens makes no allowance for people who are particularly susceptible, for example due to pre-existing disease, a compromised immune system or pregnancy. Infectious organisms which might be encountered in laboratory work in Great Britain have been assigned to hazard groups 1-4 by the Advisory Committee on Dangerous Pathogens (Report 1984), and a similar classification is given in the WHO Laboratory Biosafety Manual (5). Some of the more important pathogens which may be found in a veterinary laboratory are listed in Table I.
TABLE I. Some of the micro-organisms in Containment Groups 2 and 3 which may be present in a veterinary laboratory

<table>
<thead>
<tr>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Influenza viruses types A, B, C</em></td>
<td><em>Rabies virus</em></td>
</tr>
<tr>
<td><em>Newcastle disease virus</em></td>
<td><em>Eastern, Western and Venezuelan equine encephalomyelitis</em></td>
</tr>
<tr>
<td><em>Respiratory syncytial virus</em></td>
<td><em>Japanese B encephalitis</em></td>
</tr>
<tr>
<td><em>Orf (parapox virus)</em></td>
<td><em>Louping ill</em></td>
</tr>
<tr>
<td><em>Alcaligenes spp.</em></td>
<td><em>Bacillus anthracis</em></td>
</tr>
<tr>
<td><em>Arizona spp.</em></td>
<td><em>Brucella spp.</em></td>
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<tr>
<td><em>Campylobacter spp.</em></td>
<td><em>Chlamydia psittaci (avian strains only)</em></td>
</tr>
<tr>
<td><em>Chlamydia</em></td>
<td><em>Coxiella burnetii</em></td>
</tr>
<tr>
<td><em>Clostridium tetani</em></td>
<td><em>Mycobacterium avium</em></td>
</tr>
<tr>
<td><em>Clostridium botulinum</em></td>
<td><em>Mycobacterium bovis</em></td>
</tr>
<tr>
<td><em>Corynebacterium spp.</em></td>
<td><em>Pseudomonas mallei</em></td>
</tr>
<tr>
<td><em>Erysipelothrix rhusiopathiae</em></td>
<td><em>Aspergillus fumigatus</em></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td><em>Echinococcus spp.</em></td>
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<tr>
<td><em>Haemophilus spp.</em></td>
<td><em>Microsporum spp.</em></td>
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<tr>
<td><em>Leptospira spp.</em></td>
<td><em>Trichophyton spp.</em></td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td><em>Cryptosporidium spp.</em></td>
</tr>
<tr>
<td><em>Moraxella spp.</em></td>
<td><em>Fasciola hepatica</em></td>
</tr>
<tr>
<td><em>Mycobacterium bovis</em></td>
<td><em>Toxocara canis</em></td>
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<tr>
<td><em>Mycoplasma pneumoniae</em></td>
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<tr>
<td><em>Pasteurella spp.</em></td>
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<tr>
<td><em>Proteus spp.</em></td>
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<tr>
<td><em>Pseudomonas spp.</em></td>
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<tr>
<td><em>Salmonella spp.</em></td>
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<tr>
<td><em>Staphylococcus spp.</em></td>
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<tr>
<td><em>Yersinia enterocolitica</em></td>
<td></td>
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<tr>
<td><em>Yersinia pseudotuberculosis</em></td>
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</tr>
</tbody>
</table>
Requirements for work with infectious agents

Having decided the risk level of certain work it is then possible to decide the appropriate containment level to avoid risk of human disease. Laboratories normally appoint a Safety Officer, who should be of sufficient seniority, to oversee and advise on all safety matters. The working methods for a particular procedure or work station should be written out and readily available. Staff must be fully trained and fully aware of any health risks associated with their work. Staff should also be given a medical card indicating pathogens to which they might be exposed. In some cases staff can be specially vaccinated to give additional protection, e.g. when working with rabies virus. This should also be recorded on the medical card. This information is useful for a medical practitioner in the event of illness occurring. Regular medical examinations of employees are recommended and, as appropriate, testing of employees working with the organisms that cause certain serious human diseases, such as brucellosis and tuberculosis.

Much information is available on containment of pathogens and sophisticated apparatus and buildings are produced for containment of the more hazardous organisms. A basic list of requirements for any work with infectious agents, however innocuous they may seem, is as follows:

- The laboratory should be easy to clean, with surfaces which are impervious to water and resistant to chemicals. There must be a wash-hand basin in each laboratory.

- Personnel access to the laboratory should be restricted.

- Protective clothing, including gloves, masks and eyeshields as appropriate, must be worn in the laboratory and removed when leaving the laboratory.

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

- Food and drink must not be stored or consumed in laboratories.

- Smoking and application of cosmetics must not take place in the laboratory.

- Pipetting must not be done with the mouth.

- Care must be taken to minimise the production of aerosols.

- In the event of some spillage or contamination, effective disinfectants must be available and hands must be immediately disinfected. Bench tops should be cleaned after use.
- Used laboratory glassware and other materials must be stored safely before disinfection. Materials for disposal must be transported without spillage in strong containers. Waste material should be incinerated or otherwise made safe before disposal. Reusable material must be decontaminated by appropriate means.

- Material contaminated with communicable disease agents (particularly Groups 3 and 4) should not be allowed to enter any drain leading directly to the municipal sewage system.

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

- With Group 2 pathogens a microbiological safety cabinet should be used but can be operated in the open front mode (Class I cabinet).

- At the Group 3 level of containment it is advisable to site the laboratory in some isolation and to restrict entry to named individuals. Appropriate signage is required at all entry doors to indicate the hazard present and the name and telephone number of the person(s) responsible. Emergency protocols should be posted within the laboratory to advise personnel of procedures to follow in case of a pathogen spill or the need to evacuate the laboratory in the event of a fire.

A ventilation system is required which removes air from the laboratory through a High Efficiency Particulate Air (HEPA) filter. This is conveniently done by drawing air through a microbiological safety cabinet, which may be either Class I or Class III. The laboratory should be under negative pressure to surrounding areas where less hazardous work is done. Staff must shower and change their clothes before leaving the building.

- For Group 4 containment the most stringent precautions are required, including access to the building through a system of air locks, the building being maintained under negative air pressure. Inlet air to the laboratory must be filtered through a single HEPA filter and extract air through double HEPA filters. All work with infective materials must be conducted in a Class III microbiological safety cabinet. Other precautions as described for Group 3 would also apply.

**Microbiological safety cabinets**

These are of three types:

Class I  
An open-fronted cabinet designed specifically to provide operator protection and not to give protection to the work being handled.
Class II  An open-fronted cabinet, sometimes referred to as a laminar flow recirculating cabinet. They are designed primarily to give product protection but also give some operator protection.

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

Storage of live pathogens requires appropriate containment and security to avoid risks due to breakage or unauthorised use of material. Storage facilities should be appropriately labelled to indicate the nature of the pathogens (e.g. their group) and the person(s) responsible for them. A complete inventory of the pathogens in storage should be kept up to date and available.

Many of the considerations given above relate not only to human safety but also to prevention of spread of infection to animals. In a veterinary laboratory an important responsibility is to minimise any risk of escape of pathogens to animals, either wild or domestic, in the outside community.

Laboratory animal facilities

Work with pathogens in laboratory animals poses special hazards. Animal rooms have to be constructed to appropriate standards and containment levels in much the same way as laboratories. Similar considerations also apply regarding the training of staff, protective clothing and the recording of working procedures. Special care must be taken to avoid injury to staff, e.g. through animals biting and kicking. Any such incidents should be recorded and wounds appropriately treated. There must be provision for incineration of carcasses and for the thorough cleansing and disinfection of animal rooms. The animal rooms should not only provide a suitable environment for the animals themselves but should be constructed and ventilated in such a way as to ensure comfort for the attendants.

Bibliography


FOOT AND MOUTH DISEASE
(A1)

SUMMARY

Foot and mouth disease (FMD) is the most contagious disease of animals and has a great potential for causing heavy loss in susceptible cloven-hoofed animals. There are seven serotypes of FMD virus, namely, O, A, C, SAT 1, SAT 2, SAT 3 and Asia I. Infection with any particular serotype does not confer immunity against another. FMD cannot be reliably differentiated clinically from other vesicular diseases, including swine vesicular disease (SVD), vesicular exanthema of swine (VES), and vesicular stomatitis (VS). Laboratory diagnosis of any suspected FMD case is therefore a matter of urgency.

In typical cases there is a vesicular condition of the feet, buccal mucosa and, in females, the mammary glands. The clinical signs can vary from mild to severe, and acute fatalities may occur, especially in young animals. The preferred tissue for diagnosis is epithelium from unruptured or freshly ruptured vesicles. Where this is not possible, saliva and blood and/or oesophageal-pharyngeal fluid samples taken by probang cup in ruminants or throat swabs from swine provide an alternative source of virus. Myocardial tissue or blood can be submitted from fatal cases, but vesicles are again preferable if present.

It is vital that samples from suspected cases be transported under secure special conditions and according to international regulations. They should only be dispatched to authorised laboratories.

Diagnosis of FMD can be based on the demonstration of FMD viral antigen in samples of tissue or fluid. Detection of a specific humoral antibody response is another means of diagnosis, but this generally requires an absence of any history of vaccination, since the differentiation of a serological response to natural infection from that of vaccination can be problematical. Diagnosis based on serological response may also be problematical in endemic areas due to the possibility of previous infection.

Identification of the agent: The demonstration of FMD viral antigen is sufficient for a positive diagnosis.

Complement fixation (CF) has been the traditional test for diagnosis, but is being progressively replaced by the enzyme-linked immunosorbent assay (ELISA), which is more accurate, sensitive and objective and is not affected by pro- or anti-complementary factors. If the sample is inadequate or the test result inconclusive, it will be necessary to grow the virus in cell cultures or in unweaned mice. The cultures should preferably be of primary bovine thyroid (BTY), or else pig, lamb or calf kidney cells, or cell lines of comparable sensitivity. The mice should be 2 to 7 days old. When a cytopathic effect (CPE) appears in the cultures, the fluids can be used in CF or ELISA tests.
Similar tests can be performed on homogenate suspensions of the dissected musculo-skeletal tissues of any mice that die. In the absence of CPE or any dead mice, two further passages should be made at 48-hour intervals, with freeze-thawing of the cells, before the sample is declared to be negative.

**Serological tests:** The demonstration of specific antibody titres in non-vaccinated animals, where a vesicular condition is present, is sufficient for a positive diagnosis. This is useful in mild cases or where epithelial tissue cannot be collected.

Virus neutralisation (VN) tests and ELISA are used. VN tests depend on tissue cultures and are more variable than ELISA; they are also slower, subject to contamination and yield a higher proportion of false positive results. ELISA tests for antibody to virus-infection-associated (VIA) antigen may be employed to differentiate between humoral responses to vaccination and natural infection, but this method is not yet suitable for routine use, since repeatedly vaccinated animals may also respond.

**Requirements for biological products:** Inactivated virus vaccines of varying composition are commercially available. Typically, virus is used to infect a suspension cell culture and the resulting preparation clarified, inactivated with ethyleneimine and blended with adjuvant. Many FMD vaccines are multivalent to provide cover against the different serotypes likely to be encountered in a given field situation.

The finished vaccine must be shown to be free of residual live virus. This is usually done by a combination of in vitro tests on the inactivated virus preparation and in vivo tests with the finished vaccine. Challenge tests are also conducted in vaccinated cattle to establish a PD$_{50}$ value, although a serum neutralisation test is considered satisfactory where the vaccine producer has established a statistically significant correlation between protection and specific serum neutralising antibody.

**A. DIAGNOSTIC TECHNIQUES**

There are seven serotypes of foot and mouth disease (FMD) virus, namely O, A, C, SAT 1, SAT 2, SAT 3, and Asia 1. Infection with any particular serotype does not confer immunity against another. Within serotypes many subtypes and strains can be identified.

Infection of susceptible animals with FMD virus leads to the appearance of vesicles on the feet, in and around the oral cavity and on the mammary glands of females. Vesicles can also occur at other sites, such as inside the nostrils and at pressure points on the limbs - especially in pigs. The severity of clinical signs varies with the strain of virus, the exposure dose, the age and breed of animal, the host species and its degree of immunity. The signs can range from a mild or inapparent infection to one that is severe. Death may result in some cases. Mortality from a focal myocarditis is most commonly seen in young animals; myositis may also occur in
other sites. Adult animals may occasionally succumb (7, 11).

On premises with a history of sudden death in young cloven-hoofed livestock, close examination of adult animals may often reveal the presence of vesicular lesions if FMD is involved. The presence of vesicles in fatal cases is variable.

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

FMD virus can replicate and be excreted from the respiratory tract of animals. Airborne excretion of virus occurs during the acute phase of infection. Ruminants, but not pigs, will carry virus during convalescence, or if immune, following exposure to infection. Virus can persist in the pharynx for up to two years in cattle, for five years in African buffalo, and for several months in sheep and goats. The domesticated (Asian) buffalo can carry virus for several months.

On account of the highly contagious nature and economic importance of FMD for many countries, the laboratory diagnosis and serotype identification of the virus should be done in a disease-secure laboratory. Countries lacking access to such a specialised national or regional laboratory should send specimens to the World Reference Laboratory for FMD, Institute for Animal Health, Pirbright Laboratory, Pirbright, Woking, Surrey GU24 ONF, England. Laboratories designated as Regional Laboratories include: Botswana Vaccine Institute, Gaborone, Botswana; Pan American FMD Centre, Rio de Janeiro; Pakchong Institute, Nong-Serai, Thailand; and Foreign Animal Disease Diagnostic Laboratory, Greenport, NY, USA.

The tissue of choice for laboratory diagnosis is epithelium. Ideally at least 1 g of tissue from an unruptured or recently ruptured vesicle should be collected. To avoid injury to personnel collecting the samples, as well as for humane reasons, it is recommended that animals be sedated before any samples are obtained.

Epithelial samples should be placed in a transport medium comprised of equal amounts of glycerol and 0.04 M phosphate buffer pH 7.2-7.6, preferably with added antibiotics. If 0.04 M phosphate buffer is not available, tissue culture medium or phosphate buffered saline (PBS) can be used instead, but it is important that the final pH of the glycerol-buffer mixture is in the range pH 7.2-7.6.

Where epithelial tissue is not available from ruminant animals, for example in advanced or convalescent cases, or where infection is suspected in the absence of clinical signs, samples of oesophageal-pharyngeal (OP) fluid can be collected by means of a probang (sputum) cup (or in swine by swabbing the throat) for submission to a laboratory for virus isolation.

Before the collection of OP samples from cattle or large ruminants (e.g. buffaloes), 2 ml transport fluid (comprised of 0.08 M phosphate buffer containing 0.01%
bovine serum albumin, phenol red (0.002%) and antibiotics, adjusted to pH 7.2) should be added to a container of around 5 ml capacity capable of withstanding freezing above solid carbon dioxide or liquid nitrogen.

After collection of OP fluid by probang, the contents of the cup should be poured into a wide-necked bottle of around 20 ml capacity. The fluid is examined for quality, and should contain some visible cellular material. Of this, 2 ml is then added to the 2 ml of transport fluid; the mixture is shaken gently and should have a final pH around pH 7.6. Samples contaminated with ruminal contents will be acidic and must be discarded. Samples seen to contain blood should also be discarded. Repeat sampling can be done after the mouth of the animal has been rinsed with water or PBS.

OP samples from small ruminants are collected by putting 2 ml of transport fluid into a wide-necked bottle of about 20 ml capacity and after collection, rinsing the probang cup to discharge the OP sample in this fluid. This is then transferred to a container of about 5 ml capacity for transport. The small container should be capable of withstanding freezing above solid carbon dioxide or liquid nitrogen.

Samples should be refrigerated or frozen immediately after collection. If they are to remain in transit for more than a few hours they should be frozen by being placed either above solid carbon dioxide or liquid nitrogen. Before freezing, the containers should be carefully sealed using airtight screw caps or silicone. They should reach the laboratory in a frozen state.

Special precautions are required when sending perishable suspect FMD material both within and between countries. These regulations are mainly designed to prevent leakage and consequent contamination but are also important in ensuring that the specimens arrive in a satisfactory state. If wet ice is put inside a package, escape of water must be prevented.

For samples destined for the World Reference Laboratory for FMD (10), the preferred method of shipment is by air freight. The package should be addressed to: World Reference Laboratory for Foot and Mouth Disease, Institute for Animal Health, Pirbright Laboratory, Pirbright, Woking, Surrey GU24 ONF, UK, and marked "To be collected by the addressee at London Airport; perishable; keep at 4°C". Before shipment, the World Reference Laboratory should be notified by telex (no. 859137 AVRI G) or telefax (no. 44-483-232621) of the details of airline flight, date and expected time of arrival. It is important that the air weighbill number for the consignment is provided as this facilitates location of the parcel on arrival at London Airport, and clearance and collection by personnel from the World Reference Laboratory. Authorised collection at the airport is made under a special import licence issued for this purpose to the World Reference Laboratory.

1. **Identification of the agent**

   a) **ELISA**

At the laboratory, the preferred procedure for the detection of FMD viral
antigen and identification of viral serotype is the ELISA (6, 12). This is an indirect sandwich test in which different rows in multiwell plates are coated with rabbit antisera to each of the 7 serotypes of FMD virus. These are the "trapping" sera. Test sample suspensions are added to each of the rows, and appropriate controls are also included. Guinea pig antisera to each of the serotypes of FMD virus are added next, followed by rabbit anti-guinea pig serum conjugated to an enzyme. Extensive washing is carried out between each stage to remove unbound reagents. On the addition of enzyme substrate, a colour reaction indicates a positive reaction. With strong positive reactions this will be evident to the naked eye, but results can also be read spectrophotometrically at 492 nm. In this case an optical density reading greater than 0.1 above background indicates a positive reaction; the serotype of FMD virus can also be identified. Values close to 0.1 should be confirmed by retesting or by amplification of antigen by tissue culture passage and testing the supernatant once a CPE has developed.

Depending on the geographical origin of samples, it may be appropriate to simultaneously test for swine vesicular disease (SVD) virus or vesicular stomatitis (VS) virus.

b) Complement fixation test

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

Antisera to each of the seven types of FMD virus are diluted in veronal buffer diluent (VBD) in 1.5-fold dilution steps from an initial 1:16 dilution to leave 25 μl of successive antiserum dilutions in U-shaped wells across a microtitre plate. Fifty microlitres of 3 units of complement are then added, followed by 25 μl of test sample suspension(s). The test system is incubated at 37°C for one hour prior to the addition of 25 μl of 1.4% standardised sheep red blood cells (SRBC) in VBD sensitised with 5 units of rabbit anti-SRBC. The reagents are incubated at 37°C for a further 30 minutes and the plates subsequently centrifuged and read. Appropriate controls for the test suspension(s), antisera, cells and complement are included. CF titres are expressed as the reciprocal of the serum dilution producing 50% haemolysis. A CF titre of equal to or greater than 36 is taken as a positive reaction and the serotype so read. Titre values of 24 should be confirmed by retesting an antigen that has been amplified through tissue culture passage.

c) Virus isolation

Suspensions of field samples suspected to contain FMD virus should be clarified and inoculated into cell cultures or unweaned mice. Sensitive cell culture systems include primary bovine thyroid cells and primary pig, calf or lamb kidney cells. Established cell lines such as BHK-21 and IB-RS-2 cells may be used but are less sensitive than primary cells for detecting low amounts of
infectivity. Mice are an alternative to cell cultures and should be 2-7 days of age and of selected inbred strains. Some field viruses may require several passages before they become adapted to mice (13).

2. Serological tests

FMD virus infection can be diagnosed by the detection of a specific antibody response. The tests generally used are virus neutralisation (VN) and ELISA (8, 9). The VN is specific, sensitive and quantitative and takes two to three days to provide a result. Low titre false-positive reactions can be expected in a small proportion of sera. The ELISA is also specific, sensitive and quantitative, but it has the advantage that it is more rapid to perform, is less variable, gives fewer false-positive results and is not dependent on tissue culture systems.

Tests for antibodies to virus-infection-associated antigen (VIA) using ELISA have shown promise under research conditions for distinguishing between infected and vaccinated animals but these tests require further evaluation before they can be employed routinely.

a) Virus neutralisation

VN tests are generally carried out in tissue culture grade microtitre plates with flat-bottomed wells, using susceptible cells such as IB-RS-2, BHK-21, or lamb or pig kidney monolayers.

Sera for VN tests are diluted 1 in 4, inactivated at 56°C for 30 minutes and diluted in a duplicate, 2-fold dilution series across the plates. Virus at a pre-titrated concentration of 100 TCID$_{50}$ is added to each well and the mixtures incubated at 37°C for 60 minutes to allow neutralisation to take place. Controls in each test include homologous antisera of known titres, a cell control, a medium control and a virus titration. A cell suspension is then added to each well to test for the presence of unneutralised virus. The plates are incubated at 37°C to permit cell growth. End-point titres are determined when wells containing the virus control titration indicate a concentration around 100 TCID$_{50}$ (between 30 and 300 TCID$_{50}$ is the acceptable range).

Wells with any evidence of CPE are considered to be infected and neutralisation titres are expressed as the reciprocal of the final dilution of serum present in the serum-virus mixtures at the 50% end-point.

Tests are valid when the amount of virus per well in the test is between 30 and 300 TCID$_{50}$ and when the titre of the reference serum is within 2-fold of its mean titre as estimated from previous titrations. The tests should be repeated if the controls are outside these limits.

In the tests described here, an end-point titre of 1:11 or less is taken as negative. In screening tests, sera are usually tested up to a final dilution of 1:32 initially. Titres of 1:16 or higher in the screening test are doubtful. Animals giving doubtful results may be re-tested, a second serum sample being
Foot and mouth disease (A1)

requested. A titre equal to or more than 1:16 in the second sample (in the absence of a history of vaccination) is positive. Humoral neutralising antibodies can generally be detected within 4 to 5 days after infection and reach high titres (1:250) within three weeks.

b) ELISA

The ELISA for the detection and quantitation of antibody to FMD virus is a blocking type test: duplicate, 2-fold dilution series of each test serum, starting at 1:4, are prepared in U-bottomed multiwell plates (carrier plates). An equal volume of a constant dose of homologous viral antigen is added to each well and the mixtures are left overnight at 4°C. These serum-antigen mixtures are then transferred to multiwell ELISA plates previously coated with rabbit anti-viral serum and incubated at 37°C for 60 minutes on a rotary shaker. After washing, guinea pig antiserum homologous to the viral antigen is added to each well. Plates are again incubated as before and washed. Finally, rabbit anti-guinea pig immunoglobulin, conjugated to enzyme, followed by enzyme substrate, is added to each well. The plates have to be washed between each stage. The reaction is stopped after 15 minutes by the addition of 1.25 M sulphuric acid. The optical density (OD) of the colour reaction is read spectrophotometrically at 492 nm in an automatic reader.

Controls are included. For each antigen used, there are 48 wells containing antigen in diluent but no serum; a duplicate, 2-fold dilution series of homologous bovine reference antiserum; and a duplicate 2-fold dilution series of negative bovine serum.

Antibody titres are expressed as the final dilution of test serum giving 50% of the mean OD values recorded in the virus control wells where test serum is absent. Titres in excess of 1:40 are considered positive.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Typically, FMD vaccines are chemically inactivated impure preparations of the virus which have been blended with a suitable adjuvant. A strain(s) of the virus appropriate to the field situation is used to infect a suspension of a transformed cell line, such as BHK (baby hamster kidney), or the surviving epithelial cells obtained from healthy bovine tongues. When the virus has reached its maximum titre, which is variously determined by infectivity, complement fixation or other serological tests, the culture is filtered, often with centrifugation, and virus subsequently inactivated by addition of ethyleneimine (often called binary ethyleneimine). This is carried out over a 24- to 48-hour period at a temperature of 20 to 37°C and is a crucial step with respect to satisfying regulatory authorities of the lack of infectivity of the final vaccine. A number of manufacturers still employ formalin as an inactivant although its use has often been implicated in outbreaks in Europe attributed to inadequately inactivated FMD vaccine. Neither chemical inactivant causes disruption of the virus.
The inactivated virus is then blended with aluminium hydroxide gel \((\text{Al(OH)}_3)\). The adsorption which occurs facilitates concentration of the virus by removal of virus-free supernatant fluids. A second adjuvant, saponin, is added at this stage as well as a small volume of chloroform to act as a preservative. In the case of vaccines destined for use in swine, oil adjuvants are preferred.

Because of the presence of multiple serotypes of the virus, many FMD vaccines are multivalent and it is common practice to prepare vaccines from three different virus strains.

1. **Cells, viruses and reagents**

   a) **Cells**

   The cell stocks and cultures made from them should be susceptible to FMD virus and free from contaminating microorganisms. In the case of BHK cells, it is common practice to keep stocks of cells over liquid nitrogen and revive as necessary. On revival, they are expanded in nutrient medium to a volume and cell density appropriate to seeding the main culture. As an approximation, the main culture is seeded to give initially 0.2 to 0.5 \(10^6\) cells/ml which are allowed to multiply to 2 to 3 \(10^6\) cells/ml before infection with virus.

   Viability of the cells is usually assessed by trypan blue exclusion. The susceptibility of the cells to the particular strain of FMD virus should also be assessed. Yields may vary considerably between different cultures of BHK (1). The cells must be proven free of contaminating microorganisms – i.e. bacteria, fungi, mycoplasma and viruses – by culturing on a comprehensive range of nutrient media or cells.

   b) **Viruses**

   Seed virus must also be tested and proven free of contaminating microorganisms. Seed viruses may be stored at -20°C if glycerinated or lower temperatures if not glycerinated. Working seed viruses may be expanded in one or several passages from the mother seed stock and used to infect the final cell culture at an approximate rate of 1 PFU (plaque forming unit) per 100 cells.

   Many manufacturers of FMD vaccines base their vaccine strains on local field isolates and, in the case of cell line systems, adapt them to growth in suspension cells by serial passage. The number of passages should be kept to a minimum as there is evidence of antigenic 'drift' during passage of FMD virus.

   c) **Reagents**

   The saponin chosen for the vaccine should be selected carefully. Only saponin derived from the South American tree, *Quillaja saponaria mollina*, should be used and its suitability for vaccine preparations assessed by vaccine potency and toxicity trials in guinea pigs. Cattle serum is used in the cell maintenance medium and must be free of extraneous microorganisms, including a number
of viruses of cattle, notably bovine virus diarrhoea. The quality of the serum is also important with respect to cell viability and production and should be screened accordingly.

Other components of the maintenance medium/vaccine must be sterilised by filtration or autoclaving before use. These include antifoam, phenol red dye (if permitted by the country requiring vaccine), aluminium hydroxide, lactalbumin hydrolysate, tryptose phosphate broth, antibiotic solutions, amino acids and vitamins, and buffer salts.

2. Manufacture and process control

The broad outlines of the manufacturer's process are given above in the foreword to Part B. There are several specific areas which warrant particular mention. Firstly, it is essential that all pipework and vessels are thoroughly sterilised and that no "pockets" remain in the systems to harbour microorganisms. In addition to general considerations of sterility, it is important to note that the virus is vulnerable to attack by proteolytic enzymes such as those produced by microorganisms (2). Control of pH and temperature are also critical because of the acid and temperature lability of the virus (3). Thus, temperature should be precisely controlled at 37°C for cell and virus growth and, optionally, inactivation. At other times the temperature should be reduced to 4-6°C when possible. Virus should be maintained at or around pH 7.6 and should never be allowed to drift below pH 7.0.

In general, virus titres reach optimum levels within about 24 hours of the cell culture being infected. The time chosen to "harvest" the culture may be based on a number of assays. Clearly, if all the cells are found to be dead, there is no need to continue the culture. Titre of virus may be assessed by plaque assay, sucrose density gradient (4) or serological techniques. It is preferable to use a method for measuring antigenic mass rather than infectivity, as the two properties need not necessarily coincide. Sucrose density gradient analysis is one such method.

The favoured inactivation procedure is that of ethyleneimine (EI) which is usually prepared as required by dissolving bromoethylamine hydrobromide in sodium hydroxide solution. The EI forms on standing and is added to a virus suspension held at 20 to 37°C to give a final concentration of 0.05%. Inactivation is usually continued for 24 hours and some manufacturers use a second dose of EI for a further 24 hours. To decrease the likelihood of live virus failing to contact the EI, it is a common policy to transfer the vessel contents immediately to a second sterile vessel where inactivation is allowed to go to completion. Samples should be taken regularly for the purpose of measuring the kinetics of inactivation. To monitor inactivation, the treated virus is inoculated into cell cultures of recognised high susceptibility to the virus, e.g. BHK, bovine thyroid cells. Using Roux flasks of BHK cell monolayers, approximately 10 ml per flask can be checked for the presence of live virus. The cell sheet is examined over a period of several days after which the spent medium may be transferred to fresh monolayers and the original monolayers replenished with fresh medium. Freeze-thawing of the original monolayers may be used to release intracellular virus which can be detected by further passage. Using this method, traces of live virus remaining after inactivation can be amplified by the
passage procedure and detected on the basis of the CPE observed. Two to three passages of the original virus preparation are commonly used. Given no detectable virus in the culture, vaccine is formulated and checked for lack of infectivity in cattle (see below).

3. **Batch control**

a) **Safety tests**

Vaccines must be tested in cattle to establish the presence or absence of live virus (5). Each of three healthy seronegative cattle are inoculated intra-dermolingually with 0.1 ml of vaccine at 20 sites. The animals are observed for not less than four days after which three full doses of vaccine are administered subcutaneously to each animal. Should any of the animals develop a lesion(s) the vaccine will fail the safety test. Equally, any undue toxicity attributable to the vaccine should be assessed and may prevent its acceptance.

An additional procedure is used by some laboratories and involves elution of virus from finished vaccine by high molarity phosphate buffers. The eluate is then used to inoculate cell cultures as described above (5).

b) **Potency tests**

The test defined in the European Pharmacopoeia (5) requires the use of 18- to 30-month-old cattle, obtained from areas free from FMD, which have not previously been vaccinated against FMD and are free from antibodies neutralising the different types of FMD virus. Using a suitable buffer solution which does not contain an adjuvant, a series of dilutions of the vaccine at not more than fivefold intervals is prepared in the volume stated on the label as the dose. Three groups, each of at least five cattle, are vaccinated by the route stated on the label, using one dilution per group. Three weeks after vaccination, the vaccinated animals and a control group of two non-vaccinated animals are challenged with a suspension of bovine virus that is fully virulent and appropriate to the virus types in the vaccine under test by inoculating 10,000 ID$_{50}$ intradermally into two sites on the upper surface of the tongue (0.1 ml per site). Animals are observed for 8 days. Unprotected animals show lesions at sites other than the tongue. Control animals must develop lesions on at least three feet. From the number of animals protected in each group, the PD$_{50}$ content of the vaccine is calculated. The vaccine must contain at least 3 PD$_{50}$ per dose for cattle.

Other tests, including measurement of serum neutralising antibodies, may be used to assess the potency of a vaccine provided that a statistical evaluation has established a satisfactory correlation between the results obtained by the test used and the method of the European Pharmacopoeia.

c) **Duration of immunity**

In order to establish a satisfactory level of immunity it is usual to give a primary
course of two inoculations, 2-4 weeks apart, followed by revaccination every 4-12 months. The frequency of revaccination will depend upon the epidemiological situation and the quality of vaccine used.

For calves born of vaccinated dams the first vaccination should be delayed as long as possible to allow decline of maternal antibody, but not beyond four months, since at that time a high proportion can be expected to effectively respond to vaccination. For calves born of non-vaccinated dams the age of the first vaccination is less critical.

d) Stability

The shelf life of FMD vaccines is taken to be one year at 4°C. The vaccine must not be frozen.

REFERENCES


**SUMMARY**

Vesicular stomatitis (VS) is a disease of horses, cattle and pigs caused by VS virus (VSV), a member of the family Rhabdoviridae. Vesicles are produced on the tongue, lips, buccal mucosa, teats, and in the epithelium of the coronary band of the hooves of these animals; many other wild and domestic species and laboratory animals can be infected. The disease is limited to the Western Hemisphere; however, it was once described in France and South Africa. It is enzootic in parts of the Americas.

The mode of transmission is unclear, but the virus has been isolated from sandflies and mosquitoes, suggesting that it could be insect-borne.

Man is susceptible to infection and therefore all manipulations with the virus and pathological materials should be carried out with adequate biohazard containment.

Prompt diagnosis is mandatory since the clinical signs in pigs are indistinguishable from those of foot and mouth disease (FMD), vesicular exanthema of swine and swine vesicular disease. In cattle, the disease is similarly indistinguishable from FMD. Since the clinical signs and histopathological findings are not definitive, a laboratory diagnosis is essential.

**Identification of the agent:** VSV can be readily isolated by various means, namely, by the inoculation of: embryonated chicken eggs, several tissue culture systems, suckling mice, the footpad of guinea pigs, and the tongues of adult chickens, horses and cattle, as well as the snouts of pigs. Viral antigen in infected tissues can be used in complement fixation (CF) tests, the least expensive and most rapid, or it may be used for neutralisation tests in tissue cultures, embryonated chicken eggs or suckling mice.

**Serological tests:** Convalescent animals develop specific antibodies within 4-5 days of infection. These are usually demonstrated by complement fixation or virus neutralisation. Other tests that have been described are agar gel immunodiffusion, counter immunoelectrophoresis and enzyme-linked immunosorbent assay (ELISA).

**Requirements for biological products:** An inactivated virus vaccine has been licensed but is not yet available commercially.

**A. DIAGNOSTIC TECHNIQUES**

Vesicular stomatitis (VS) was described in 1926 (12) and 1927 (8) as a disease of horses, and subsequently of cattle and pigs. Vesicles are produced by the virus
(VSV) on the tongue, lips, buccal mucosa, teats and in the epithelium of the coronary band of the feet of pigs, cattle, horses, and many other species of domestic and wild animals. Many species of laboratory animals are also susceptible.

Man is susceptible to the virus and there is an increase in human illness when VS occurs amongst animals. This indicates that all manipulations involving VSV, including infective materials from animals, should be performed with adequate biohazard containment.

There are two major immunological types of VSV, namely, Indiana and New Jersey. Both viruses are members of the family Rhabdoviridae and have been extensively studied at the molecular level. The New Jersey type is the more important cause of disease in livestock. Several other closely related rhabdoviruses have been isolated during the past decade. These are the Cocal and Argentina and Brazil strains, which are considered to be subtypes II and III, respectively, of the Indiana serotype. The Indiana II virus was first isolated from mites collected from rice rats in Trinidad and Indiana III from a horse in Alagoas, Brazil (1). Both these subtypes are less pathogenic for cattle, pigs and horses than are the classical subtypes Indiana and New Jersey viruses, although of these species the horse is the most susceptible. The disease was once described in France and South Africa. It is enzootic in the Southeastern United States, Mexico, Venezuela, Colombia and Ecuador. At times, epizootics occur as far north as Canada and as far south as Argentina.

Epizootics of the disease in the United States appear between May and October and usually disappear with the first killing frost, leading to the suggestion that it is spread by arthropod vectors. Outbreaks in the Western United States in 1982 were an exception to this rule, as outbreaks continued even after the onset of freezing weather (1). The method of transmission is unclear; but since the virus has also been isolated from sandflies and mosquitoes, this tends to substantiate the idea that it could be insect transmitted. While VS is not diagnosed in livestock in the United States every year, it is considered to be enzootic in wildlife, especially in feral pigs in the southeastern part (3).

1. **Identification of the agent**

An early diagnosis is essential for the control of any vesicular disease (13). Laboratory diagnosis is requisite to determine which infection is present, namely, that of VS, vesicular exanthema of swine (VES), San Miguel sea lion virus (SMSV), foot and mouth disease (FMD) or swine vesicular disease (SVD) (5).

Due to the relatively large size of the VS virus, its characteristic morphology and the large number of virus particles present in vesicular fluid and epithelial tissues, electron microscopy (EM) is a useful diagnostic tool. Immunogold techniques allow identification of the virus serotype involved.

Diagnostic reagents for VSV are not commercially available and each laboratory usually produces its own. The FMD Centre in Brazil (Pan American
Vesicular stomatitis (A2) produces and distributes diagnostic reagents on request. The Foreign Animal Disease Diagnostic Laboratory, Plum Island, New York, responds to similar requests.

**a) Virus isolation**

Strains vary widely in their ability to replicate in different livestock, tissue cultures and laboratory animals (see Table I). It is therefore sometimes necessary to passage virus in a given host or system before it will propagate to high titre. The route of inoculation is important and varies with the species. Suckling mice develop a fatal encephalitis irrespective of the route of inoculation; brain tissue from these is an excellent source of virus. Adult mice resist infection except when the virus is given intracerebrally. In either case, the first signs are hypersensitivity, followed by tremors, ataxia or spastic caudal paralysis and death in 3-5 days.

The most susceptible route for cattle, pigs and horses is intradermalingual administration, following which vesicular lesions may be observed. In cases of extreme doubt, a horse is included in the test series because VSV is the only member of the vesicular group of viruses which routinely replicates in the horse. Pigs are also susceptible when inoculated in the coronary band of the foot or on the snout. Vesicular exanthema virus of pigs has been reported to replicate when inoculated into the horse, but this is extremely rare. Cattle are fully resistant to intramuscular inoculation of VSV, although they will develop both neutralising and complement-fixing antibodies.

**Table I**

Methods for isolation of vesicular stomatitis virus in laboratory hosts

<table>
<thead>
<tr>
<th>Host</th>
<th>Route</th>
<th>Dose</th>
<th>Incubation</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-day chick embryo</td>
<td>allantoic sac</td>
<td>0.1 ml</td>
<td>3-4 days</td>
<td>embryo death</td>
</tr>
<tr>
<td>3-week mouse</td>
<td>intracerebral</td>
<td>0.03 ml</td>
<td>2-3 days</td>
<td>paralysis/death</td>
</tr>
<tr>
<td>Adult chicken</td>
<td>intradermal (tongue)</td>
<td>0.03 ml</td>
<td>1-2 days</td>
<td>vesicle/tongue</td>
</tr>
<tr>
<td>Adult guinea pig</td>
<td>intradermal (foot)</td>
<td>0.03 ml</td>
<td>2-4 days</td>
<td>vesicle/foot</td>
</tr>
</tbody>
</table>

Tissue cultures

<table>
<thead>
<tr>
<th>Host</th>
<th>Route</th>
<th>Dose</th>
<th>Incubation</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chick fibroblasts</td>
<td>direct</td>
<td>0.1 ml</td>
<td>2-3 days</td>
<td>cytopathogenic</td>
</tr>
<tr>
<td>Pig kidney</td>
<td>direct</td>
<td>0.1 ml</td>
<td>2-3 days</td>
<td>cytopathogenic</td>
</tr>
<tr>
<td>BHK-21</td>
<td>direct</td>
<td>0.1 ml</td>
<td>2-3 days</td>
<td>cytopathogenic</td>
</tr>
<tr>
<td>Vero</td>
<td>direct</td>
<td>0.1 ml</td>
<td>1.3 days</td>
<td>cytopathogenic</td>
</tr>
</tbody>
</table>

Adapted from Hanson (11)
In tests used to identify unknown virus, hyperimmune antisera are required. These are prepared by injecting 400- to 500-gram guinea pigs intradermally into the footpads with the desired virus which has been adapted to grow in this species by rapid serial passage. After 6-8 weeks the guinea pigs are inoculated intramuscularly with 1 ml of a 10% suspension of freshly grown virus from infected guinea pig metatarsal footpads. The sera of recipients are tested and, if not up to standard, they are given additional inoculations. The guinea pigs are exsanguinated 10 days later and the sera are processed by centrifugation, filtration and heat inactivation, following which they are lyophilised and stored at 4°C (9). These sera may also be used as positive controls in serological tests.

Neutralisation tests both in vivo or in vitro can be used to identify the virus. The known antiserum is mixed with the unknown virus and inoculated into such test media as a susceptible species of livestock, guinea pigs, mice or tissue cultures. The use of animals for this purpose is decreasing (6). Neutralisation tests are increasingly carried out in microtitre plates using a VERO cell line propagated in Eagle’s minimum essential medium (MEM). A test dose of the virus of about $2 \log_{10} \text{TCID}_{50}$ is mixed with serial doubling dilutions of known antiserum. The mixtures are shaken on a shaker and incubated at 37°C in 5% CO$_2$ with at least 85% humidity. A cell culture suspension is added to all wells and the plates re-incubated. The test may be read in 72 hours, or whenever cytopathic effects (CPE) are well advanced in control cultures. The serum should give the expected titre against the unknown virus if it is homologous.

ELISA has also been used for the identification of virus, but it is not type specific for VSV New Jersey or Indiana because of probable cross-reacting shared antigens between the serotypes. The advantage lies in the ability to distinguish VSV infections from FMD or other vesicular diseases.

b) Viral antigen

The original CF tests for FMD viral antigen (7) were extended to other vesicular diseases, including VS (4). CF remains the method of choice for the rapid routine identification of VSV infection (17). In recent years, CF tests have been done in microtitre plates and can be completed within a few hours. A 20% suspension of the vesicular epithelium or pharyngeal-oesophageal fluid is used as antigen. The antigen is tested with a complement titration, using 3-9 100% haemolytic units, and a 1:10 dilution of reference control sera against all VSV and FMD virus types. The test is incubated for one hour at 37°C and sensitised sheep red blood cells are added. The test is incubated again for 30 minutes at 37°C and centrifuged at 200 g for 5 minutes. A sample is positive if there is no haemolysis, i.e. complete fixation of complement.

A procedure to detect viral antigen by the use of an ELISA test has been described (10). A sandwich indirect ELISA is used with the plates coated with VSV antiserum produced in rabbits. The unknown concentrated and purified antigen is added, VSV antiserum produced in guinea pigs is added, followed by peroxidase-conjugated rabbit anti-guinea pig immunoglobulin and substrate. After each reagent is added there is an incubation period followed by five
washes.

In cases where the original sample is not suitable for use as CF antigen, efforts are made to isolate the virus from the specimen (see Table I) and then to identify it by neutralisation tests, immunofluorescence, CF tests, agar gel immunodiffusion, counter immunoelectrophoresis (CIEP) (15) or ELISA.

2. Serological tests

a) Complement fixation

Variations of the CF test are used to identify VSV humoral antibodies; however, procomplementary substances in pig sera limit its application in that species (2). Frequently, only sera are submitted for diagnosis of VSV infection. These are then examined by CF tests, usually by the microtitration system. In the USA, the New Jersey VSV strain Atlanta, the Indiana VSV strain Chimayo and normal tissue antigen controls are all prepared in embryonating chicken eggs or 1-5 day-old mice inoculated by the intracerebral route. A 25% suspension of brain tissue from infected and normal mice is prepared in veronal buffered saline. Antigen can be prepared in MDBK cells. When 95% of the monolayer is showing CPE (30 to 40 hours post-infection), the cells are removed, ruptured by freezing and thawing or ultrasonication, and centrifuged. The supernatant is stored at -70°C.

The other Indiana subtype strains and related rhabdoviruses used for diagnosis are listed in Table II. Diagnostic laboratories in other countries of the Western Hemisphere (Mexico, Panamá, Brazil, Colombia, Venezuela, Argentina, etc., where VSV is enzootic) may use viral strains that are indigenous locally; particular attention is, however, paid to New Jersey and Indiana 1 serotypes.

Table II
Origin of vesiculovirus serotypes used in diagnosis at the
Foreign Animal Disease Diagnostic Laboratory, Plum Island, New York

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Species of origin</th>
<th>Country of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indiana 1</td>
<td>bovine</td>
<td>USA</td>
</tr>
<tr>
<td>Indiana 2</td>
<td>mites and rodents</td>
<td>Trinidad</td>
</tr>
<tr>
<td>Indiana 3</td>
<td>mule</td>
<td>Brazil</td>
</tr>
<tr>
<td>Piry</td>
<td>opossum</td>
<td>Brazil</td>
</tr>
<tr>
<td>Chandipura</td>
<td>sandflies</td>
<td>India</td>
</tr>
<tr>
<td>Isfahan</td>
<td>bovine</td>
<td>Iran</td>
</tr>
<tr>
<td>New Jersey</td>
<td>bovine</td>
<td>USA</td>
</tr>
</tbody>
</table>

Adapted from Wilks et al. (14)
The commonly used CF procedure for VS is as follows. Test sera and control positive and negative sera are tested against each respective VSV test antigen and normal antigen. For ruminant sera, the modified direct CF test is more sensitive than the direct CF test. Calf serum (5%), which has been tested and found to be suitable as a modifying factor, is included in the complement. No modifying factor is included for equine sera. Swine test sera will usually still be procomplementary after inactivation, and adequate controls of normal swine serum must be included to prevent misinterpretation of the test. Complement, serum and antigen are incubated for 3 hours at 37°C; 1.9 100% haemolytic units of complement are used. After sensitised sheep red blood cells are added, the test is incubated for 30 minutes at 37°C in a waterbath and centrifuged at 200 g for 5 minutes. The titre is the highest dilution in which all the complement is fixed, i.e. no haemolysis is observed. A titre of 1:5 or greater is considered significant.

b) Virus neutralisation (VN)

The VN test is performed using 1,000 TCID$_{50}$. The virus-serum mixture is incubated for one hour at 37°C or overnight at 4°C in microtitre plate wells and then a suspension of VERO cells is added to the wells. The titre is determined by reading for complete lack of cytopathology after two days' incubation. A titre of 1:32 or greater is considered significant. Virus for the test is produced by allowing viral replication in infected monolayers to progress to the stage where cytonecrosis is nearly complete, centrifuging out dead cells and freezing (-70°C) the supernatant fluid in suitably sized aliquots.

c) ELISA

The ELISA test has been described and may be as sensitive as neutralisation tests in detecting antibodies; however, it is not type-specific for VSV New Jersey or Indiana. Cross-reactions are probably due to shared antigens between the serotypes. ELISA has several advantages over other tests for VSV antibodies in cattle. It does not require a cell culture system for assay, it is more rapid, less cumbersome and may be automated (16).

The cell culture antigen, as prepared for the complement fixation test, can be used in an indirect ELISA test. In addition, a normal antigen is prepared from noninfected cell cultures. Plates are coated with both antigens, and sera are tested against both. After the serum is added, the plates are incubated for two hours at 37°C, washed, and anti-species, enzyme-conjugated antibody is added. After incubation at 37°C for one hour and washing, the ABTS substrate is added. An alternative procedure is an indirect sandwich procedure where the antigen is added to plates coated with antiserum against VSV.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

An inactivated virus vaccine has been licensed in the USA but has not yet been used.
REFERENCES


SUMMARY

Swine vesicular disease (SVD) is important because of its clinical similarity to foot and mouth disease, and therefore differential diagnoses of suspected cases of SVD must be obtained urgently. Some strains of SVD virus produce very mild or sub-clinical infections, but the affected pigs will have specific humoral antibodies.

Laboratory diagnosis of SVD is based on the detection of a viral antigen or virus isolation, on the demonstration of a specific antibody, or on a combination of these.

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

Serological tests: An ELISA is available to determine the presence of specific antibodies for diagnosis and for epidemiological purposes. The time taken to obtain results is 3 hours.

A microneutralisation test can also be used, and has the advantage of being sensitive, specific and quantitative. However, it takes at least 2-3 days to complete and is more laborious than the tests described above. It is not well suited to the screening of large numbers of sera; but it is more often used to confirm results of other tests, and to assess the extent and duration as well as the possible origin and likely spread of infection on particular premises.

Requirements for biological products: There is no requirement for SVD vaccines, as their use would complicate diagnosis.

A. DIAGNOSTIC TECHNIQUES

Swine vesicular disease (SVD) can be a sub-clinical, mild or severe vesicular condition according to the strain of virus involved, the route of infection, or the infecting dose (2, 5, 6). Clinically SVD is indistinguishable from foot and mouth disease (FMD) and this is its main importance. It is therefore urgent that cases of SVD be distinguished from FMD by laboratory investigation.
The laboratory diagnosis of SVD depends on either the isolation and characterisation of the virus, or the demonstration of specific antibodies, or on a combination of both. The quantity and quality of lesion material submitted for examination is all too frequently insufficient to allow rapid viral diagnosis by a direct complement fixation (CF) test or ELISA, so that tissue culture is necessary to enhance the virus titre. In SVD, the animal excretes virus at least 48 hours before the appearance of lesions. Even in outbreaks where only unruptured and recently ruptured vesicles, which suggest early disease, are described, antibody in high titre will be found in many cases. However, the infection can develop in an inapparent or mild form showing only a transitory decline in the general appearance of an animal and the presence of circulating antibodies. In all cases when SVD is suspected, clinicians should submit not only vesicular epithelium from lesions, but also serum or blood from suspect pigs and their immediate contacts.

1. **Identification of the agent**

Any vesicular condition in pigs may be FMD. If FMD has been eliminated, the diagnosis of SVD requires the facilities of a specialised laboratory. Countries that lack such a facility should send samples for investigation to the World Reference Laboratory for Foot and Mouth Disease, Institute for Animal Health, Pirbright, Woking, Surrey GU24 ONF, UK. In the Americas, parallel testing for vesicular stomatitis viral antigen may also be conducted.

The investigation should start with the examination of a suspension of lesion material in direct CF tests or ELISA against specific antisera to SVD and FMD viruses. This suspension should also be inoculated onto monolayers of IB-RS-2 cells (or suitably susceptible porcine cells), primary calf thyroid cells, and primary (or secondary) calf kidney cells. FMD virus will grow in all 3 tissue culture systems; SVD will grow only in cells of porcine origin.

An analysis of isolates of SVD virus by polyacrylamide gel electrophoresis can be useful in epidemiological investigations of field outbreaks.

a) **Direct CF test**

This is the same test as that used for FMD diagnosis. However, this test is no longer used in the World Reference Laboratory (WRL), Pirbright, although it is still in use in other laboratories. It has been replaced at WRL by an ELISA, which is more sensitive.

Equal reagent volumes are used in the test, which is performed in round-bottomed microtitre plates (1). Clarified 1:10 and 1:30 suspensions of ground vesicular epithelium are used as antigen against reference antisera for SVD and all 7 serotypes of FMD (or those considered appropriate in that country). Normal fixation is for 1 hour at 37°C. A more sensitive test using standard optimal dilutions of antisera against a dilution range of antigen can be used with fixation times of either 4 hours at 37°C or overnight at 4°C. The time taken to obtain a result may therefore vary. If the sample is adequate in
quantity of ruptured vesicle, results of the standard test may be obtained in as little as 2.5 hours after arrival of the specimen in the laboratory.

Veronal buffer diluent (VBD) is readily available commercially (e.g. at Oxoid Ltd., Wade Road, Basingstoke, Hants., RG24 OAN, UK). Alternatively, a 5-fold concentrated solution can be prepared: sodium chloride 85 g, diethyl barbituric acid 5.75 g, sodium barbitone 3.75 g, magnesium chloride 6H2O 1.68 g, and anhydrous calcium chloride 0.28 g. Dissolve the diethyl barbituric acid in 500 ml of single distilled water heated to almost boiling. When cool, add the remaining constituents dissolved in 100 ml of cold distilled water. Make up to a total volume of 2 litres. Separate into smaller bottles and sterilise at 1 atmosphere for 15 minutes. Dilute 1:5 with distilled water before use.

The haemolytic system consists of 2.5 ml packed and washed red cells of sheep added to 97.5 ml VBD to make a 2.5 % suspension, together with an equal volume of rabbit anti-sheep haemolysin diluted to the recommended optimal sensitising dose and added to the red cell suspension to produce a final 1.25% suspension. This is incubated at 37°C for 15 minutes before use.

Reference antisera are currently produced in guinea pigs using a double inoculation of inactivated 160S antigen at intervals of 4 weeks between injections. Appropriate adjuvants may be incorporated. The guinea pigs are exsanguinated 15 days after the final inoculation. Hyperimmune guinea pig sera are also adequate for diagnosis. All sera are inactivated at 56°C for 30 minutes before use.

To perform the test:

i) Remove as much transport medium as possible from the samples, using sterile absorbent paper. Weigh, and chop finely with scissors into a mortar and grind with sterile sand and 0.04 M phosphate buffer to produce a 1:10 (w/v) suspension. Clarify by centrifugation at 1,000 g for 5-10 minutes.

ii) Using 50 µl dropping pipettes and 25 µl loops, prepare a 1.5-fold dilution series of the reference antisera in VBD across the plate commencing at 1:16 (single row of 6 wells/serum).

iii) Add 0.025 ml complement (C') containing 3 haemolytic units (HU) to all test wells.

iv) Add 0.025 ml epithelial suspension (antigen) to all test wells.

v) Include in each test serum controls (no antigen), antigen controls (no serum), C' controls (no serum or antigen). Three wells for each control receive 3, 2 and 1 HU of C' respectively. A cell control is the haemolytic system only. Make up the volumes of the control wells to the test well volume with VBD.

vi) Cover and incubate at 37°C for 1 hour.
vii) Add 0.025 ml haemolytic system to each well. Cover plate with pressure-sensitive tape and incubate at 37°C for 30 minutes, shaking at intervals to keep cells in suspension.

viii) Centrifuge plate at 500 g for 5 minutes or allow cells to settle.

ix) Read results visually by the degree of lysis represented by the size of the button of deposited cells. A 50% inhibition or more of lysis with respect to the controls represents a positive result.

Field samples at 1:10 suspension are frequently anti-complementary. Results may sometimes be clarified by setting up a simultaneous duplicate test using the antigen at 1:30.

b) ELISA

This test is the same as that used for FMD diagnosis, a sandwich-type test. Duplicate rows in multiwell ELISA plates are coated with rabbit antiserum to SVD virus. This is the trapping serum. Test sample suspensions are added to each of the rows. Appropriate controls are also included. Guinea pig detection serum is added at the next stage followed by rabbit anti-guinea pig serum conjugated to enzyme. Extensive washing is carried out between each stage to remove unbound reagents. A positive reaction is indicated if, on the addition of enzyme substrate, there is a colour reaction. With strong positive reactions this will be evident to the naked eye, but results can also be read spectrophotometrically at 492 nm, in which case an optical density reading greater than or equal to 0.1 above background indicates a positive reaction.

c) Tissue cultures

A portion of the clarified epithelial suspension is inoculated onto monolayers of IB-RS-2 cells or other susceptible porcine cells. For differential diagnosis (e.g. FMD) bovine cell culture systems should also be employed. A culture medium found to be satisfactory is 50:50 Eagle's complete medium-yeast hydrolysate (LYH). For cell growth, add 10% bovine serum; for maintenance, add 3% bovine serum; and for virus isolation, add no serum, only antibiotics.

Cultures are examined twice daily. When a cytopathic effect (CPE) is complete in the positive tubes, the supernatant fluid is harvested and used as antigen in the CF test or ELISA for virus identification. Negative cultures are blind-passaged at 48 hours.

2. Serological tests

Sera may be examined for specific antibodies by ELISA or virus neutralisation (VN). Use of ELISA will give results within a few hours, whereas VN tests may take up to 3 days.
a) ELISA

A qualitative indirect ELISA (4) is used routinely in conjunction with counter immunoelectrophoresis (CIEP) by the World Reference Laboratory for the rapid serological confirmation of SVD. It is specific, economical and suitable for large-scale epidemiological surveillance. It is unaffected by haemolysis or the presence of red blood cells.

The antigen is virus propagated on monolayers of IB-RS-2 cells, concentrated by ammonium sulphate precipitation and purified by ultracentrifugation on a 15-45% sucrose density gradient. Peak fractions (160S) are pooled and inactivated with a final concentration of 0.05% N-acetylatedlyleneimine (AEI) for 48 hours at 26°C. Aliquots are stored at -70°C in silicone-treated glass vials.

The coating buffer is 0.05 M carbonate-bicarbonate buffer, pH 9.6, the washing buffer is phosphate buffered saline (PBS), and the diluting buffer 0.05% Tween 20, 5% bovine serum and 2% sodium chloride in PBS. The conjugate is rabbit anti-pig immunoglobulin conjugated with horseradish peroxidase; the substrate is orthophenyleneamine. The tests are conducted in U-bottomed plates:

i) Add antigen diluted in coating buffer (2 mg/ml) to each well on the plate and air-dry (solid phase). Wash 5 times with PBS.

ii) Add a single dilution (1:60) of each test sample to a pair of wells and incubate for 30 minutes at 37°C with shaking. Wash 5 times with PBS.

iii) Add diluted conjugate (1:1,000) to each well. Incubate for 15 minutes at 37°C with shaking. Wash 5 times with PBS and add substrate.

iv) Positive and negative controls are included on each plate; the reaction is stopped with 1.25 M sulphuric acid when the positive controls have a visually estimated optical density reading of about 1.0 and when the negative controls are still colourless.

v) The results are read visually, or more accurately by spectrophotometry at 492 nm.

b) Virus neutralisation

The quantitative VN microtest for SVD (3) is performed with IB-RS-2 cells in flat-bottomed tissue culture grade microtitre plates. Although specific and very sensitive, it takes up to 3 days to produce results and is therefore more suited to epidemiological investigations and the corroborations of results of other tests than to rapid diagnosis.

Virus is grown in IB-RS-2 cell monolayers and stored at -20°C after the addition of 50% glycerol. SVD virus has been found to be stable under these conditions for at least 1 year. The sera are inactivated at 56°C for 30 minutes before testing. For ELISA tests sera should not be inactivated, as this may give
rise to non-specific reactions. The control reference serum is a 21-day convalescent pig serum. A suitable medium is Eagle's complete medium-LYH with antibiotics.

The test is an equal volume test in 0.05 ml amounts:

i) Starting from a 1:4 dilution, sera are diluted in a 2-fold dilution series across the plate, 2 rows of wells/serum.

ii) Previously titrated virus is added; each 0.05 ml unit volume of virus suspension contains about 100 TCID$_{50}$.

iii) Controls include a reference antiserum of known titre, a cell control, a medium control and a virus titration used to calculate the actual virus titre used in the test.

iv) Incubate at 37°C for 1-2 hours with the plates covered.

v) A cell suspension at 10$^6$ cells/ml is made up in medium containing 10% bovine serum throughout the test for cell growth. 0.05 ml of cell suspension is added to each well.

vi) Plates are sealed with pressure-sensitive tape and incubated at 37°C for 2-3 days. Alternatively, the plates may be covered with loosely fitting lids and incubated in an atmosphere of 5% carbon dioxide at 37°C for 2-3 days.

vii) Microscopical readings may be feasible after 48 hours; the plates are finally fixed and stained routinely on the third day. Fixation is effected with 10% formol-saline for 30 minutes; staining is done by immersion in 0.05% methylene blue in 10% formalin for 30 minutes. The plates are rinsed in tapwater.

viii) Positive results are blue-stained cell sheets; the negative wells are empty. Titres are expressed as the final dilution of serum present in the serum-virus mixture at the 50% end-point. The test is considered valid when the amount of virus actually used per well is between 10$^{1.5}$ and 10$^{2.5}$ TCID$_{50}$, and the positive reference serum is within 2-fold of its expected titre.

ix) Interpretation: VN of 1:45 or more may be regarded as positive. Titres of 1:16 to 1:32 should be considered doubtful, and further serum samples should be obtained for testing.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

There is no requirement for SVD vaccines. The use of such products would complicate the interpretation of serological surveys and diagnostic tests.
REFERENCES

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SUMMARY

Rinderpest is an acute, highly fatal disease of domestic cattle and water buffalo caused by a Morbillivirus. Sheep, goats, pigs and game animal species may also be affected. It occurs in Egypt, in sub-Saharan Africa as far south as Tanzania, on the Arabian peninsula and in India and its neighbours.

Clinically the disease is characterised by pyrexia, the progressive development of shallow erosions on the gums, tongue, cheeks and hard palate, together with serous or mucopurulent ocular and nasal discharges. Alimentary tract involvement is marked by the development of severe diarrhoea.

Identification of the agent: Laboratory confirmation is principally based on the demonstration of precipitating antigens in the spleen, lymph nodes, or ocular or nasal secretions of acutely infected animals. Agar gel immunodiffusion or counter immunoelectrophoresis tests are used. The virus must be isolated in cell culture from lymphoid tissue or from blood leucocytes if a geographic extension or a significant zoosanitary deterioration has occurred.

Post-mortem examinations should pay particular attention to the abomasum, which may be highly engorged or show a grey discolouration, to the Peyer's patches which may show lymphoid necrosis, and to the development of linear engorgement and blackening of the crests of the folds of the caecum, colon and rectum. The principal differential diagnosis is in small ruminants where peste des petits ruminants (PPR) may be confused with rinderpest unless distinguished by laboratory methods. In cattle, differentiation from bovine virus diarrhoea is required, using the appropriate laboratory methods.

Serological tests: Neutralising antibody estimations should be used to detect the presence of convalescent animals in field investigations. For surveys of post-vaccination herd immunity levels an ELISA test is used.

Requirements for biological products: An attenuated vaccine can be manufactured in cell culture and confers life-long immunity following a single inoculation of susceptible cattle. The vaccine can also be safely used in sheep, goats and various game animal species.

A. DIAGNOSTIC TECHNIQUES

Classic rinderpest is a highly lethal viral disease of domesticated cattle and water buffalo caused by a Morbillivirus in the family Paramyxoviridae. In addition, the virus affects sheep, goats and Asiatic pigs together with a large variety of free-living species within the order Artiodactyla, although not always in a clinically apparent form.
Historically the virus has had a wide distribution, including Europe and Asia. Geographically the virus has never established itself in the Americas or in the Antipodes. In Africa the virus is broadly distributed south of parallel 16°N, except for the additional inclusion of northern Egypt. The southern distribution is limited by the forest belt in Gabon, Congo and Zaire but on the eastern seaboard infection is recorded as far south as Tanzania. In recent years the disease has been found in most countries of the Arabian Peninsula and has extended northwards into Syria and Iraq. The virus is endemic in southern India and has also been recorded in Sri Lanka, Bhutan, Nepal and Pakistan.

Following an incubation period of one to two weeks the ensuing clinical episode is characterised by an acute febrile attack within which prodromal and erosive phases can be distinguished. The prodromal period lasts approximately three days during which time the developing pyrexia reaches temperatures of 40 to 41.5°C and affected animals show partial anorexia, constipation, congestion of visible mucosae, serous ocular and nasal discharges, depression and drying of the muzzle. However, it is not until the onset of the erosive phase, and the development of necrotic mouth lesions, that a specific clinical diagnosis can be made. Still at the height of fever, small pin-head sized flecks of raised, whitish, necrotic epithelium appear on the lower lip and gum. In rapid succession such lesions may also appear on the margin between the upper gum and dental pad, on the underside of the tongue, on the cheeks and cheek papillae and on the hard palate. Through the extension of existing lesions and the development of subsequent foci, in the next two- to three-day period the extent of oral necrosis can increase dramatically. Much of the necrotic material works loose leaving shallow, non-haemorrhagic mucosal erosions. Diarrhoea is another characteristic feature of rinderpest and develops one or two days after the onset of mouth lesions. The diarrhoea is usually copious and watery at first but later may contain mucus, blood and shreds of epithelium and be accompanied by tenesmus. During the erosive phase necrosis may be seen in the nares, in the vulva and vagina and on the preputial sheath. Anorexia becomes absolute, the muzzle dries out completely, the animal is profoundly depressed, and mucopurulent ocular and nasal discharges develop. The breath is foetid and terminally the animal may go down for 24 to 48 hours; during this period a characteristic checked expiration may be observed accompanied by a grunted exhalation.

The pyrexia may remit slightly in the middle of the erosive period and then, two or three days later, rapidly return to normal at which time mouth lesions resolve quickly and diarrhoea ceases. Alternatively animals may die while showing severe lesions, high fever and severe diarrhoea or may die while severely affected but after a precipitous fall in body temperature, often to subnormal values.

Most virus circulating in Africa or Asia produces a disease picture that, in general, conforms to the above description. On the Arabian peninsula, however, and in association with highly susceptible imported cattle, peracute disease has been seen in which deaths occur at the end of the prodromal period. Mild strains which, by virtue of the fleeting and greatly reduced nature of the lesions that develop, may be difficult to diagnose clinically, possibly still occur in parts of Africa.
Typically the carcass is dehydrated, emaciated, soiled and foetid. The nose and cheeks bear evidence of mucopurulent discharges, the eye is sunken and the conjunctiva congested. In the oral cavity there is often extensive desquamation of the necrotic epithelium, which always appears sharply demarcated from adjacent areas of healthy mucosa; the lesions frequently extend onto the soft palate and may also involve the pharynx and upper portion of the oesophagus. The rumen, reticulum and omasum are usually spared, although necrotic plaques are occasionally encountered on the pillars of the rumen. The abomasum is severely affected, especially the pyloric region, and shows severe congestion, petechiation and oedema of the submucosa. Epithelial necrosis gives the mucous membrane a slate-like colour. The small intestine is not commonly involved except for striking changes in the Peyer's patches where lymphoid necrosis and sloughing leaves the supporting architecture engorged or blackened. In the large intestine changes involve the ileocaecal valve, the caecal tonsil and the crests of the longitudinal folds of the caecal, colonic and rectal mucosae. The folds appear highly engorged in acute deaths or darkly discoloured in older cases; in either event the lesions are known as zebra stripes.

1. **Identification of the agent**

As a routine step in the provision of a confirmed diagnosis, samples from clinical and post-mortem examinations should be submitted for laboratory tests appropriate to the demonstration of virus, virus-specific antigens or humoral antibodies. The successful isolation of rinderpest virus from sterile whole blood depends on preserving the leucocyte fraction by the use of anticoagulants such as heparin or EDTA at final concentrations of 10 IU/ml or 0.5 mg/ml respectively. Samples should be thoroughly mixed and transferred to the laboratory on ice, but not frozen. Virus or antigen can be demonstrated in samples obtained at necropsy from the spleen, prescapular or mesenteric lymph nodes of dead animals; these samples may be chilled to sub-zero temperatures. Precipitinogens can be demonstrated in the ocular secretions of infected animals during either the prodromal or erosive phases. Secretions should be collected using cotton wool buds and manoeuvring the swab beneath the upper and lower eyelids in the process. Clean, sterile serum should be collected from numbered individuals during the acute disease and again two weeks after remission of clinical signs. Paired samples are examined for rising levels of neutralising antibody.

Uncoagulated blood is centrifuged at 2,500 g for 15 minutes to produce a buffy coat layer at the boundary between the plasma and the red cells. This is removed as cleanly as possible, mixed in 20 ml physiological saline and recentrifuged in a washing procedure designed to remove any early neutralising antibody present in the plasma. The resulting cell pellet is suspended in cell culture maintenance medium and 2 ml aliquots are distributed onto established roller tube monolayers of primary calf kidney or VERO cells. Tubes should be refed periodically and observed microscopically for the development of cytopathic effects characterised by refractility, cell rounding, cell retraction with elongated cytoplasmic bridges (stellate cells) or giant cell formation. Isolates of virus can be identified by the demonstration of precipitinogens in infected cell debris, by immunoperoxidase (IPX) staining or by neutralisation with specific antisera.
Alternatively, 20% suspensions (w/v) of lymph nodes or spleen may be used for virus isolation. These are made by macerating the solid tissue in serum-free culture maintenance medium using standard grinding or shearing techniques.

Agar gel immunodiffusion (AGID) tests may be conducted in plastic petri dishes or on glass microscope slides (9). In either instance the surface should be covered with agar to a depth of about 4 mm using a 1% (w/v) aqueous solution of any high quality agar or agarose. Wells are usually cut in a hexagonal pattern of six peripheral wells around a single central well. For slides, wells should be 3 mm in diameter and 2 mm periphery to periphery. For petri dishes the wells can be increased to 4 mm in diameter and the distance between wells to 3 mm. The closer wells are placed the shorter will be the reaction time. Tests are better developed at 4°C or at low ambient temperatures. Using a Pasteur pipette, rinderpest hyperimmune rabbit serum should be placed in the central well. Similarly, control positive antigen prepared from rinderpest infected cell debris or from macerated mesenteric lymph nodes of a positively infected ox should be placed in peripheral wells one, three and five. Negative control antigen is placed in well four. Test antigens are obtained as exudates from the cut surface of spleen or lymph nodes submitted for testing; if no exudate can be obtained a small portion of the sample should be ground with sand and a minimum of saline. Ocular exudates can usually be squeezed directly from the swabs or, alternatively, may be squeezed out by placing the swab in the barrel of a syringe. Test samples are added to wells two and six. Tests should be inspected after two hours for the appearance of clean, sharp lines of precipitation. The result is not acceptable unless precipitation reactions are obtained with the control positive antigen preparations.

Counter immunoelectrophoresis tests may yield a positive reaction slightly ahead of the AGID test (3). Reactions are carried out on standard microscope slides coated to a depth of 2.0 mm with 1% agar or agarose in 0.025% veronal-acetate buffer (pH 8.6). Wells are cut in pairs at an interperipheral distance of 6 mm, the left hand being anodal, and filled with rinderpest hyperimmune serum; the right hand cathodal wells are filled with test samples or positive and negative antigen preparations. Samples are run for 40-60 minutes at a constant current of 10 mA/slide, after which the reaction area is examined for precipitation.

Of the many other methods available for demonstrating the same rinderpest antigens, the most worthwhile at present are the direct and indirect immunoperoxidase tests (9, 10).

Special mention must be given to the inability of clinical observations or group antigen detection tests to clearly differentiate between rinderpest and peste des petits ruminants when either of these morbilliviruses causes overt disease in sheep and goats. Although such differentiation can be based on epidemiological considerations or can follow virus isolation (11), it can be undertaken more rapidly and conveniently using cDNA probes specific for the mRNA of the nucleocapsid protein of each virus (2).
2. **Sero logical tests**

a) **Virus neutralisation**

The standard neutralisation test is undertaken in roller-tube cultures of calf kidney or VERO cells. Inactivated sera are diluted at intervals of 1 in 2 or 1 in 10 and mixed with a stock virus suspension containing approximately \(10^{3.0}\) TCID<sub>50</sub>/ml. Mixtures containing equal volumes of virus and serum are held overnight at 4°C, after which 0.2 ml of the mixture is inoculated into each of the five roller tubes, immediately followed by 1 ml of dispersed indicator cells suspended in growth medium at a rate of \(2 \times 10^5\) per ml. Tubes are sloped for 3 days at 37°C after which those showing virus-specific cytopathology are discarded; the medium in the remaining tubes is replaced with a maintenance formulation and the tubes are rolled pending a final examination at seven days. For calculating end points the virus challenge dose is regarded as satisfactory if it falls within the range \(10^{1.8}\) to \(10^{2.8}\) TCID<sub>50</sub>/tube; serum dilutions are considered to double after mixing with virus. In the past any detectable antibody, even at a dilution of 1:2, was considered positive. This criterion can still be used for qualifying cattle as susceptible for vaccine testing and for international trade purposes. However, for tests of post-vaccinal immunity a final dilution of 1:8 should be used as the starting point.

Virus neutralisation tests in microtitre plates are also available (8, 12). Sterile flat-bottomed 96-well tissue culture plates are used and sera are diluted across the plate using a multichannel pipette in a 2-fold dilution series (25 µl volumes). This is followed by the addition of 25 µl of stock rinderpest virus to give 100 TCID<sub>50</sub> per well. Following incubation at 4°C overnight, 150 µl of bovine kidney or VERO cells are added at \(1 \times 10^{3.6}\)/ml to each well. The plates are sealed with sellotape, incubated at 37°C and read microscopically over seven days. The criteria for acceptance limits for virus input and positive/negative thresholds are the same as for the tube test.

This test can be used for screening post-vaccinal cattle sera. However, it is not recommended for testing sheep and goat sera due to the presence of non-specific viral inhibitors in such sera.

b) **ELISA**

For large-scale post-vaccinal serosurveillance work with cattle, a solid phase, indirect micro-ELISA test has been developed (1). Antigen is prepared from cells infected with the RBOK vaccine strain (see below) maintained in serum-free media and can generally be used at a predetermined working dilution in phosphate buffered saline (PBS) (generally approximately 1:100); binding is carried out at 37°C for 60 minutes using an orbital shaker. Following washing and drying routines duplicate 60 µl samples of test sera are added, by diluting 15 µl neat serum with 45 µl of freshly prepared blocking buffer containing 100 µl Tween 20 (0.1%) and 5 g skimmed milk powder per 100 ml of PBS. This dilution step can be carried out in the test wells. Again the plates are
incubated at 37°C for 60 minutes and then washed. Antibody binding is detected using a horse radish peroxidase labelled rabbit anti-bovine IgG conjugate at a predetermined working dilution (generally approximately 1:10,000), followed after further washing, by the addition of orthophenyl-diamine (OPD) as the substrate. Reactions are stopped using sulphuric or trichloracetic acid and measured with an ELISA reader at 492 nm. Negative cut-off values must be independently established for the population under consideration.

c) Competitive ELISA

The competitive ELISA is based on the competition between a rinderpest-specific anti-rinderpest monoclonal antibody and antibodies in the serum sample for binding to the solid-phase rinderpest antigen. The presence of antibodies to rinderpest virus in the serum sample will block binding of the monoclonal antibody, resulting in a reduction in expected colour following the addition of enzyme-labelled anti-mouse IgG conjugate and substrate/chromogen solution. As this is a solid-phase assay, wash steps are required between each step to ensure the removal of unreacted reagents.

ELISA antigen is prepared from Madin-Darby bovine kidney cell cultures infected with the attenuated Kabete "O" strain of rinderpest virus. The antigen can be prepared either by ammonium sulphate precipitation or by a combination of sonication and centrifugation. The monoclonal antibody should be rinderpest-specific (showing no cross-reactions with PPR). PBS pH 7.6 is used for antigen adsorption and all other reagents are added in PBS supplemented with 0.1% (v/v) Tween-20 detergent and 0.3% (v/v) normal bovine serum (blocking buffer). Volumes of 50 μl are used throughout and all stages are incubated for one hour at 37°C on an orbital shaker.

Plates are coated with antigen at a pre-determined optimal dilution (generally 1.100) in PBS, and following incubation the plates are washed three times in PBS diluted 1:5 (washing solution) to remove unbound antigen. Test sera are then added by diluting 10 μl of neat serum with 40 μl of blocking buffer. Appropriate controls of strong positive, weak positive and reference negative serum are also included on each plate. This is immediately followed by the addition of 50 μl of monoclonal antibody at a pre-determined dilution (generally 1:100) in blocking buffer. Following further incubation and washing steps, 50 μl of horseradish peroxidase-labelled rabbit anti-mouse conjugate is added at a pre-determined dilution (generally 1:1,000). After a final incubation the plates are washed and 50 μl of substrate/chromogen solution (hydrogen peroxide/orthophenylene diamine) are added and the colour allowed to develop for 10 minutes. Colour development is stopped by the addition of 1 M sulphuric acid and the plates are read on an ELISA reader at 492 nm. A monoclonal antibody control consisting of antigen, monoclonal antibody and enzyme-conjugate in the absence of test serum is included on each plate and is used to calculate the percentage inhibition (PI) values for each serum. Sera giving PI values greater than 50% (i.e. 50% reduction in expected colour) are considered positive.
This test has largely replaced the indirect ELISA and the virus neutralisation test for sero-monitoring following rinderpest vaccination campaigns.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Susceptible animals may be successfully immunised against rinderpest with live attenuated vaccines. At different times the virus has been attenuated in rabbits, goats and cell culture. The induced immunity is generally life-long. Rinderpest cell culture vaccine (TCRV) was developed at the East African Veterinary Research Organisation using a modification of the virulent Kabete "O" strain. It possesses many advantages over lapinised and caprinised vaccines and is the only rinderpest vaccine which the OIE recommends. It may be used in zoological collections and on cattle, buffalo, sheep and goats.

1. Seed management

a) Characteristics

Strains used for the production of seed lots must be identifiable by written historical records which must include information on the origin of the strain and on its subsequent manipulation. Such strains must have been shown to yield a cell-culture vaccine that is safe and that confers an immunity in cattle lasting at least five years. In addition the virus must have shown stable attenuation during no fewer than five back passages in cattle and lack the ability to spread by contact. Effectively, only the attenuated rinderpest, bovine, "Old Kabete" (RBOK) virus fulfills these criteria.

b) Culture

Vaccine seed must be maintained in a seed lot system between passage levels 90 and 120. Seed lot virus must be preserved at temperatures at or lower than -20°C and in a freeze-dried state. The virus must be cultured in primary or serially cultivated kidney cells derived from normal bovine fetuses or very young calves. Serially cultivated cells may not be more than 10 passages removed from the primary cultivation.

c) Validation as a vaccine

Seed lots must be shown to be:

i) Pure, i.e., free from adventitious viruses and from contamination with bacteria, fungi or mycoplasmas.

ii) Safe, i.e., inducing no abnormal clinical reaction on inoculation into rinderpest-susceptible cattle.

iii) Efficacious, i.e., inducing an immunity to rinderpest on inoculation into rinderpest-susceptible cattle.
The necessary tests are described in section 4 below.

2. **Manufacture**

Individual vaccine batches are prepared by infecting cell cultures and, after an appropriate incubation period, harvesting the overlying media into which large numbers of live virus particles have been released. To facilitate long-term storage and cold-chain distribution this fluid is mixed with a cryoprotectant consisting of 5% lactalbumin hydrolysate plus 10% sucrose, and freeze dried. Virus may be grown in primary kidney cells from bovine embryos or calves, provided each batch relates to production in cells of an individual calf or embryo. Alternatively, cells derived in a homogeneous manner by serial cultivation from either of these sources may be used. Additionally, vaccine may be grown in approved cell lines, where their non-permissiveness to BVDV makes VERO cells especially attractive. To constitute a batch, infected cultures must have been inoculated with the same seed virus and incubated and harvested together. Two harvests are permissible from the same set of cultures and may be pooled to form a bulk suspension. Written records must accompany all stages in vaccine manufacture.

3. **In-process control**

Cells: Primary or serially cultivated cells must be derived from normal looking calves or embryos and must retain a normal morphology during cultivation, and they must also be shown to be free of contamination with adventitious viruses, particularly bovine virus diarrhoea virus (BVDV). Where primary cells are committed for vaccine production, uninfected control cultures must be maintained using the same media and incubation conditions as for the rinderpest infected cells. They must be subjected to frequent microscopic observations. Following harvesting the control cultures are washed to remove ox serum and reincubated for 10 days in media containing ox serum substitutes. They are again subject to frequent microscopic observations for evidence of cytopathic change. At the same time a sample of the cultures must be examined for the presence of non-cytopathic BVDV using immunofluorescence or immunoperoxidase techniques.

A preferable system is to grow primary cells in stationary cultures and to qualify the cells before using them for vaccine production. In this routine they can also be screened for bacterial sterility before use.

Serum fractions used in culture media must previously have demonstrated a lack of neutralising effects on rinderpest virus.

Virus: A virus titration must be undertaken on the seed lot using 10-fold virus dilutions in a microplate or roller tube system employing 10 replicates per dilution. A similar titration must be undertaken on the final bulk. Virus may not be harvested more than 10 days after the date of inoculation. The harvest should be clarified by low-speed centrifugation before mixing with a cryoprotectant. It may be held for not more than 5 days at 4°C but for considerably longer if frozen at -20° to -60°C. The final bulk must be tested for freedom from bacteria, fungi and mycoplasmas. As adventitious viral contamination may arise during manufacturer's
manipulations or from the use of contaminated media, rabbit hyperimmune serum should be used to neutralise the rinderpest content of the bulk suspension after which the mixture should be used to infect calf kidney cells which are handled as described above.

4. Batch control

Samples should be taken from each batch and tested following reconstitution to the original concentration of the final bulk.

a) Sterility

Tests for sterility and freedom of contamination of biological materials may be found in the chapter on General Information.

b) Safety

Using rinderpest susceptible cattle, the contents of five randomly selected vials are pooled and used to inoculate one ox with a volume equivalent to 100 cattle field doses and one with a volume equivalent to 1/10th of a cattle field dose. These animals are closely maintained with an uninoculated ox for the following three weeks. During this period they are subject to daily temperature recording and frequent clinical inspections. At the end of this period the cattle are examined for rinderpest neutralising antibodies and challenged with a strain of rinderpest capable of inducing a pyrexia. The vaccine is considered safe and efficacious if it induces no abnormal clinical reaction, if both animals receiving vaccine are protected and if there is no evidence that the vaccine virus has been transmitted. Each vaccine lot must be tested for innocuity in small animals.

c) Potency

The close relationship between immunising potency and total infectivity allows the latter to be used as the basis for potency estimations. Three infectivity titrations are undertaken using cells of an approved continuous line or cells grown from each of three different bovine calf or embryonic kidneys. In the first instance the vial pool established for cattle safety testing can be used. The second and third estimates are made on further pools, each of three final containers. The sensitivity of the cells used in each working session must be measured using a standard laboratory preparation. The final titre is the geometric mean of the three estimations, each undertaken using 10-fold dilutions and ten observations per dilution.

d) Duration of immunity

It is unnecessary to routinely establish the duration of immunity to TCRV. The reported results (4, 5) indicate that life-long immunity can be expected following vaccination of cattle free of all vestiges of maternal immunity.
e) Stability

The vaccine is highly stable in the freeze-dried form and will keep for over four years at either +4°C or -20°C, if carefully conserved (7). Representative samples should be tested for residual moisture and for stability at 37°C. Following reconstitution the virus undergoes rapid thermal degradation, limiting the period for field use to not more than one working day (6).

REFERENCES


PESTE DES PETITS RUMINANTS
(A5)

SUMMARY

Peste des petits ruminants (PPR), otherwise known as kata, pseudo-rinderpest, pneumoenteritis, or stomatitis-pneumoenteritis complex, is an acute contagious disease caused by a Morbillivirus of the family Paramyxoviridae. It affects small ruminants such as sheep but especially goats. It can also infect American white-tailed deer. It occurs in Africa, the Arabian Peninsula and the Middle East.

The clinical disease resembles rinderpest in cattle. It is usually acute and characterised by serous ocular and nasal discharges. There is severe pyrexia which can last for 3-5 days. Erosive lesions occur in the mouth. There is diarrhoea and pneumonia.

At necropsy, characteristic zebra markings occur in the large intestine. The main lesions occur in the lungs where there is a bronchopneumonia.

Identification of the agent: The collection of the samples at the correct time is important to achieve a diagnosis by virus isolation. Samples should be obtained in the acute phase of the disease when clinical signs are still apparent. Swabs can be made of the conjunctival sac, nasal secretions, and buccal and rectal mucosae. Unclotted blood is also taken.

Rapid diagnosis can be made by counter immunoelectrophoresis. Agar gel immunodiffusion, indirect fluorescent antibody tests, complement fixation, and enzyme-linked immunosorbent assay (ELISA) may also be employed. The disease must be differentiated from rinderpest, bluetongue, foot and mouth disease and other exanthemous conditions.

Serological tests: There is a haemagglutination test which takes advantage of an antigenic relationship between measles and PPR viruses. Other serological tests that may be used include virus neutralisation, ELISA, counter immunoelectrophoresis, indirect fluorescent antibody tests, agar gel immunodiffusion and a precipitinogen inhibition test.

Requirements for biological products: Vaccination is carried out using rinderpest tissue culture vaccine based on the principle that an antigenic relationship exists between PPR and rinderpest viruses. A homologous vaccine has been prepared.
A. DIAGNOSTIC TECHNIQUES

Peste des petits ruminants (PPR), synonyms kata, pseudorinderpest, pneumoenteritis, stomatitis-pneumoenteritis complex, is an acute viral disease of small ruminants characterised by fever, occulo-nasal discharges, stomatitis, diarrhoea and pneumonia. The virus is of the genus *Morbillivirus* of the family *Paramyxoviridae* on the basis of its similarities to the viruses of rinderpest, canine distemper and measles (9). It is transmitted by aerosols.

The natural disease has only been reported in goats and sheep, but it is usually more severe in goats and only occasionally so in sheep. Some convalescing goats develop orf-like lesions on the lips (14). The American white-tailed deer *Odocoleus virginianus* has been infected experimentally (10). PPR was first described in Côte d’Ivoire (8), and it now occurs in Africa, the Arabian Peninsula and the Middle East. It causes heavy losses in sheep and goats in Nigeria (11).

Infected animals present clinical signs similar to those of rinderpest in cattle. For this reason PPR used to be regarded as a form of rinderpest of sheep and goats, until the two diseases were recognised as having a separate aetiology and epidemiology (16, 18). The disease must be differentiated from rinderpest of cattle. PPR virus causes clinical disease in sheep and goats but, whereas it will infect cattle, it produces no clinical picture. Where cattle are affected with a rinderpest-like disease, rinderpest must be suspected; if such a disease is limited to sheep and goats where cattle are not vaccinated against rinderpest, PPR is to be suspected. If cattle are inoculated with suspect rinderpest material, rinderpest virus will produce the classical disease whereas it is only mild in sheep and goats.

The incubation period is 4–5 days but may range between 3–10 days. The clinical disease is acute, with pyrexia up to 41°C which can last for 3–5 days. The occlonal nasal discharges become mucopurulent and, if death does not ensue, persist for about 14 days. Within four days of the onset of fever the mouth becomes hyperaemic, progressing to extensive erosions of the buccal mucosa and excessive salivation. These lesions may become necrotic. A watery blood-stained diarrhoea is common in the later stage. There is a pneumonia, coughing, pleural rales and abdominal breathing. The morbidity rate can be up to 100%, and, in severe outbreaks, with 100% mortality. In milder outbreaks, the mortality rate may not exceed 50%. A tentative diagnosis of PPR is made on these clinical signs, but where rinderpest exists in cattle, laboratory confirmation is required. There is a reduction in plasma volume and increased plasma cell volume, and a leucocytosis followed by a leucopenia. The diarrhoea can lead to imbalance of such electrolytes as sodium and potassium.

At necropsy, the lesions are very similar to those observed in cattle affected with rinderpest. Erosive lesions may extend from the mouth to the reticulo-rumen junction. Characteristic linear haemorrhages or zebra stripes occur in the large intestine, commonly at the caeco-colic junction. Lymph nodes are enlarged, the spleen may show necrotic lesion, and there is an apical pneumonia.
1. Identification of the agent

Swabs are made of the conjunctival discharges from under the eyelid, and from the nasal, buccal and rectal mucosae. Whole blood is collected into an anticoagulant, such as heparin, for virus isolation and haematology. The blood and anticoagulant should be mixed gently. A serum sample should also be obtained for serology: this is stored at -20°C until required.

In general, samples should be transported under refrigeration, for example in vacuum flasks with ice. Samples of clotted blood for serum must not be frozen but kept cool. Samples are also collected aseptically post-mortem for virus isolation; for histopathology, tissues are placed in 10% formalin. Representative samples of tissues such as lymph nodes, especially the mesenteric and bronchial nodes, spleen, large intestine and lungs are required.

a) Counter immunoelectrophoresis (CIEP)

This is the most rapid test for viral antigen detection (13). It is carried out on a horizontal surface, using a suitable electrophoresis bath which consists of 2 compartments connected through a bridge. The apparatus is connected to a high-voltage source. Agar or agarose (1-2%, w/v) dissolved in 0.025 M barbitone acetate buffer is dispensed onto microscope slides in 3 ml amounts. Up to 6-9 pairs of wells are punched in the solidified agar.

Standard PPR viral antigen is prepared from the mesenteric and bronchial lymph nodes ground with sterile sand as 1:3 suspensions in buffered saline. These are centrifuged at 500 g for 10-20 minutes and the supernatant fluids stored in aliquots at -20°C. They may be retained for at least 1-3 years. Some antigens prepared from spleen or lung material have also proved to be potent. Control antigens are prepared similarly from normal tissues.

Standard antiserum is made by hyperimmunising sheep with 5 ml of PPR virus with a titre of $10^4$ TCID$_{50}$/ml given at weekly intervals for 4 weeks. The animals are bled 5-7 days after the last injection. Standard rinderpest antiserum is also effective in detecting PPR antigen.

The electrophoresis bath is filled with 0.5 M barbitone acetate buffer. The pairs of wells in the agar are filled with the reactants, sera in the anodal wells and antigen in the cathodal wells. The slide is placed on the connecting bridge and the ends connected to the buffer in the troughs by wetted porous paper. The apparatus is covered, and a current of 10-12 milliamps per slide is applied for 30-60 minutes. The current is removed and the slides viewed by intense light: the presence of 1-3 precipitation lines between pairs of wells is a positive reaction. There should be no reactions between wells containing the negative controls.

b) Agar gel immunodiffusion

A 1% agar in normal saline, containing thiomersal or sodium azide as a
bacteriostatic agent, is dispensed into petri dishes (6 ml per 5 cm dish). A pattern of wells is punched in the agar. The reagents are the same as for the CIEP test. A central well is filled with positive antiserum, 3 peripheral wells with positive antigen, and 1 well with negative antigen. The remaining wells are filled with test antigen. Usually 1-3 lines will develop between the serum and antigens within 18-24 hours (5, 17). These are intensified by washing the agar with 5% glacial acetic acid for 5 minutes; this procedure should be carried out with all apparently negative tests before recording a negative result.

c) **Indirect fluorescent antibody test**

This has been applied to detect PPR viral antigen in tissues of affected goats (4). A fluorescein-labelled anti-sheep IgG antiserum is obtained commercially. Standard PPR antiserum is prepared as before in sheep. Tissues obtained post-mortem are sectioned on a cryostat set at -40°C. These are placed on microscope slides and air-dried or fixed in acetone. Control slides are made of PPR-infected tissue culture cells. All preparations are covered with PPR antiserum at a dilution of 1:10-1:20 and incubated at 37°C for 30-45 minutes. The sections are washed with phosphate buffered saline (PBS) 3-4 times for 2-4 minutes each. After drying, the conjugate is applied, at a dilution previously determined as being optimal for 30-40 minutes; again washed with PBS, dried and mounted in 90% glycerol phosphate. Positive reactions appear as intracellular fluorescence, often as crystals.

d) **Enzyme-linked immunosorbent assay (ELISA)**

The test is done in microtitre plates as solid state assays. 50 µl of test antigen dilution is added to each well, incubated at 4°C overnight (or rocked at 37°C for 45 minutes), discarded and the plates washed with PBS containing Tween 20 (PBS-Tween) 4-5 times. 50 µl of a pre-determined dilution of standard antiserum is added to each well and the plate rocked for 1 hour at 37°C. Control wells are filled with normal sheep serum. The tests are preferably performed in duplicate. After rewashing, 50 µl peroxidase conjugate of anti-species antiserum or Protein A is added to each well and the plate rocked for another 45 minutes at 37°C.

Excess conjugate is discarded, the plates washed, and 50 µl of orthophenylene-diamine in hydrogen peroxide added. The reactions are stopped with 1 M sulphuric acid, and the optical densities of the colour reactions read on a spectrophotometer. These are compared with the colour changes in the wells containing normal serum. The mean density of the duplicate tests minus the mean of the control negative wells is taken as the actual density of the sample.

e) **Complement fixation (CF) test**

This is not employed routinely and is of doubtful use.
f) Electron microscopy (EM)

A drop of virus suspension in the form of ocular swab fluid or the supernatant fluid of homogenised lymph node material is applied to activated EM grids and absorbed for a few minutes. A drop of phosphotungstic acid or methylamine tungstate is added to stain for a few seconds. The grid is examined by EM for virus particles. These are roughly spherical with a diameter of 130-390 nm. Filamentous forms occur (7).

g) Isolation methods

This method of diagnosis is not widely applied to PPR virus because it is time-consuming. The virus can be isolated from field samples in tissue cultures or in seronegative sheep or goats.

Four to six sheep or goats are separately housed as uninoculated controls, whilst another 4-6 animals are inoculated with 2 ml of a 10% suspension of homogenised lymph nodes, spleen or other tissue mixed with 100 IU/ml neomycin, 100 IU/ml polymixin and 5 IU/ml gentamycin. The inocula may be given orally, intranasally or subcutaneously. When fever develops some animals should be sacrificed and the lymph nodes and spleens harvested and tested for viral antigen. The control animals should remain healthy.

PPR virus may be isolated in primary lamb kidney or in VERO cell tissue cultures. Monolayer cultures are inoculated with suspect material and examined daily for evidence of cytopathic effect (CPE). The CPE produced by PPR virus can develop within 5 days and consists of cell rounding and aggregation culminating in syncytia formation. The latter is characterised by a circular arrangement of nuclei giving a "clock face" appearance. Coverslip cultures may give a CPE earlier than the fifth day. There are also intracytoplasmic and intranuclear inclusions. Some cells are vacuolated. Similar cellular changes may be seen in stained histopathological sections of infected tissues.

2. Serological tests

Goats and sheep infected with PPR virus develop antibodies which may be demonstrated to support a diagnosis by the antigen detection tests. Paired serum samples are taken where possible but this takes 2-3 weeks before results can be obtained. Four-fold rises in antibody titre are significant. Tests that may be employed include CIEP, ELISA, agar gel immunodiffusion, precipitinogen inhibition, indirect fluorescent antibody tests, virus neutralisation, and a measles haemagglutination inhibition test.

The CIEP (6), ELISA, immunodiffusion and indirect fluorescent antibody tests are conducted as described above in the tests for antigen. Precipitating antibodies to PPR virus may be detected in 37-62% of infected animals depending on the interval between onset of disease and sampling (2). Convalescent rinderpest antibodies do not precipitate with PPR antigen (3).
a) Precipitinogen inhibition test

Humoral antibodies to PPR virus inhibit viral precipitinogen in a normal immunodiffusion test. Doubling dilutions of PPR viral antigen are made in test serum in tubes containing 0.2 ml serum per tube. Control normal serum is also tested. Antigen and antisera are allowed to react for 30-45 minutes at room temperature or at 4°C overnight. Each dilution of viral antigen in serum is then tested against standard hyperimmune antiserum in immunodiffusion tests. For a positive result the antigen titre should be at least 4-fold less than the titre of antigen diluted in normal serum (19).

b) Virus neutralisation test

This is sensitive, specific and used routinely (15), but is time-consuming. The standard neutralisation test is carried out in roller-tube cultures of VERO cells. Inactivated sera are diluted in a 2-fold dilution series and mixed with a stock virus suspension containing approximately $10^{3.0}$ TCID$_{50}$/ml. The virus-serum mixtures are held overnight at 4°C, after which 0.2 ml of the mixture is inoculated into each of five roller tubes, followed immediately by 1 ml of VERO cell suspension in growth medium at a rate of $2 \times 10^5$ cells/ml. Tubes are sloped for three days at 37°C, after which those showing virus-specific cytopathic effect are discarded; the medium in the remaining tubes is replaced with maintenance medium and the tubes rolled for a further seven days. The virus challenge dose is acceptable if it falls between $10^{1.8}$ to $10^{2.8}$ TCID$_{50}$/tube. Any detectable antibody at a dilution of 1:8 is considered positive.

Due to the high levels of non-specific viral inhibitors present in sheep and goat sera, the microtitre virus neutralisation test is not recommended for use with such sera.

c) Measles haemagglutination inhibition test

This is based on the antigenic relationship between measles and PPR viruses. PPR antibodies will inhibit measles virus haemagglutination reactions. Monkey red cells are used. The measles virus haemagglutinin (MVH) is available commercially. This is titrated against a 0.5-0.8% suspension of monkey red cells to determine the haemagglutinating unit (HAU): the lowest antigen dilution which causes 50% agglutination of the red cells represents 1 HAU. Serial dilutions of the test sera are reacted against 4 HAU of MVH. Tests are incubated at 37°C for 1 hour, and the titres calculated (12). Suitable negative controls must be incorporated in each test.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Sheep and goats that recover from PPR develop an active immunity against the disease. Antibodies have been demonstrated four years after infection (6). Vaccination against PPR is carried out by the use of the rinderpest tissue culture vaccine based on the principle of an antigenic relationship between PPR and
rinderpest viruses. The vaccine has been successfully used in the control of PPR in some parts of West Africa and the OIE has endorsed its use since 1977. A homologous vaccine has been produced, although original trials gave inconclusive results (1).

The methods and details of vaccine production are therefore to be found in the chapter on rinderpest (Chapter 4).

REFERENCES


Contagious bovine pleuropneumonia (CBPP) is a disease of cattle caused by Mycoplasma mycoides subspecies mycoides SC (bovine biotype). The main problems for control or eradication are the frequent occurrence of subacute or symptomless infections and the persistence of chronic carriers after the clinical phase.

Identification of the agent: The samples to be taken from live animals are nasal swabs and/or broncho-alveolar washings or pleural fluid obtained by puncture. Samples to be taken at necropsy are lung lesions, lymph nodes and pleural fluid. Direct examination of the exudate or smears is possible, but requires great skill.

For cultivation of the pathogen, the tissues are ground in medium with antibiotics and inoculated into special media that contain inhibitors to prevent the growth of contaminating bacteria. As the growth of M. m. subsp. m. SC takes several days, desiccation of the solid medium should be limited by closing the petri dishes hermetically or by using controlled humidity incubators.

In broth, growth is visible in three to ten days as a homogeneous cloudiness with whirls when shaken; on agar, small colonies develop, 1 mm in diameter, with the classical "fried egg" appearance. The biochemical characteristics to be checked are the following: sensitivity to digitonin, reduction of tetrazolium salts, breakdown of glucose, absence of arginine hydrolysis, and the lack of phosphatase and proteolytic activities. Special media have been described that are recommended for these tests. Diagnosis is confirmed by serological tests such as the growth inhibition and immunofluorescence tests (both use hyperimmune sera).

Serological tests: For retrospective diagnosis, the Campbell and Turner complement fixation test remains the recommended procedure, but an ELISA test has been described for testing large numbers of animals. An agglutination test that detects recently infected animals can be performed, but is only reliable for herd diagnosis.

Requirements for biological products: Two attenuated strains are widely used for vaccine production: the T4-44 and the KH3 J strains. The former should be used only in zebu cattle, as post-vaccine reactions are not infrequent when this strain is inoculated into sensitive breeds such as taurindicus breeds.

The minimal recommended titre is $10^7$ viable mycoplasmas per vaccine dose. Streptomycin resistance of the two attenuated strains has been developed to serve as a marker of attenuation in order to simplify safety tests during vaccine production.
A. DIAGNOSTIC TECHNIQUES

Contagious bovine pleuropneumonia (CBPP) is a contagious disease caused by Mycoplasma mycoides subsp. mycoides SC (bovine biotype), which in natural conditions affects only the ruminants of the Bos genus, i.e. mainly bovine and zebu cattle and, in some areas, domestic buffaloes (Bubalus bubalis). Among wild animals, only yaks and American buffaloes (Bison bison) are susceptible, not African buffaloes (Syncerus caffer) or other wild ruminants.

Diagnosis is possible on the basis of clinical signs but it must be kept in mind that a high proportion of infected animals in a herd develop subacute or symptomless forms. Fortunately in all cases showing pathological change the gross lesions are very characteristic.

1. Identification of the agent

a) Samples

The following samples should be taken:

In live animals:

- Nasal swabs or nasal discharges, broncho-alveolar lavage or transtracheal washing. The presence of pathogens varies greatly with the development of the lesions, and a negative result is not conclusive.

- Pleural fluid collected aseptically by puncture made in the lower part of the thoracic cavity between the seventh and eighth ribs.

- Blood may be cultured (5).

After necropsy:

- Lungs with lesions.
- Pleural fluid ("lymph").
- Lymph nodes of the broncho-pulmonary tract.
- Joint fluid from calves.

When dispatching the samples to the laboratory, it is advisable to use a transport medium that will protect the mycoplasmas and prevent proliferation of other bacteria (heart infusion broth without peptone and glucose, 10% yeast extract, 20% serum, 0.3% agar, 500 IU/ml penicillin, thallium acetate 1:7,000).

The samples must be kept under cold conditions: +4°C for a few days or frozen at or below -25°C for a longer period. For laboratory to laboratory transfer, lung fragments or pleural fluid can also be freeze-dried. Samples should be frozen in protein-rich transport medium if they cannot be processed the same day.
b) Direct diagnosis

Direct diagnosis can be performed on the exudate or on lung smears, either without staining under a phase-contrast microscope or after May-Grünwald-Giemsa (MGG) staining. This direct examination requires great skill and should be considered indicative only. An indirect fluorescent antibody test (IFAT) can also be performed on the smears using hyperimmune serum against *Mycoplasma mycoides* subsp. *mycoides* SC and labelled anti-bovine IgG. The test is satisfactory when applied to pleural fluid smears but less so with lung smears due to considerable nonspecific fluorescence. However, good results can be obtained using lung smears counterstained with Eriochrome black.

An agar gel test can detect the specific circulating antigen (galactan) present at the surface of *Mycoplasma mycoides* subsp. *mycoides* SC (4). Pleural fluid, ground lung fragments or even sequestra are tested against a hyperimmune serum in two wells cut 5 mm apart in the gel. The gel is composed of 12 g Noble agar (Difco) and 0.2 g sodium merthiolate in 1,000 ml of phosphate buffered saline (PBS), pH 7.2. The results are better at 37°C and can be read within 24 hours but only positive reactions should be taken into account.

A simpler field test has been developed using impregnated paper discs instead of wells (8). Even quicker is the interfacial precipitation test in liquid medium based on the same principle. However, in this test the samples must be centrifuged in order to be absolutely transparent (11).

c) Cultivation of the agent

*Mycoplasma mycoides* subsp. *mycoides* SC is not difficult to grow as long as adequate media are used (9).

The media should contain:

- A basic medium based on heart infusion or peptone (Difco tryptose, Tryptone Difco or Oxoid);
- Difco yeast extract to provide group B vitamins (1%);
- Antibody-free horse serum, 10%.

Several other components can be added, such as glucose, glycerol, DNA, L-cysteine, fatty acids, but the effects vary with the strains.

To avoid growth of other bacteria, inhibitors are necessary: penicillin, colistine or thallium acetate. The media can be used as broth or solid medium with 1.0 to 1.2% of agar.

After grinding in broth with antibiotics, the lung samples are inoculated into five tubes, diluted tenfold to eliminate contaminating bacteria, and on agar.
The pleural fluid is inoculated directly. Hermetic sealing of the petri dishes or the use of incubators with controlled humidity atmosphere (CO₂ incubators are not necessary) are recommended in order to avoid desiccation. The tubes and petri dishes are monitored every day for 10 days. In fluid medium a homogeneous cloudiness usually appears within 3 to 5 days, frequently with a silky, fragile filament called a "comet", which is quite characteristic of *Mycoplasma mycoides* subsp. *mycoides* SC (or the F38 strain). During the following days a uniform opacity develops, with whirls when shaken. On agar media, the colonies are small (diameter: 1 mm) and have the classical appearance of "fried eggs" with a dense centre. At this stage MGG staining, Dienes' staining or the indirect immunofluorescence test can be performed.

d) **Identification of the agent**

After two or three subcultures, antibiotics should be omitted from the medium to check if the isolate is a mycoplasma or an L-form of a bacterium that will regain its original shape in the medium without inhibitors.

Once this test is done and after cloning (at least three colonies should be selected), the organism can be identified using biochemical as well as serological tests (1).

*Mycoplasma mycoides* subsp. *mycoides* SC is sensitive to digitonin (like all members of the order Mycoplasmateles), does not produce "films and spots", breaks down glucose, reduces tetrazolium salts (aerobically or anaerobically), does not hydrolyse arginine, has no phosphatase activity, and has no or weak proteolytic properties.

For these tests, special media have been developed by the former FAO/WHO Collaborating Centre for Animal Mycoplasmas (Aarhus, Denmark). All these media have the same basic ingredients (heart infusion broth, horse serum, 25% yeast extract solution, 0.2% DNA solution), to which is added 1% of a 50% glucose solution for glucose hydrolysis, 4% of a 38% arginine HCl solution for arginine hydrolysis, and 1% of a 2% triphenyl tetrazolium chloride for tetrazolium reduction.

Once the biochemical characteristics have been checked, two serological tests are necessary to confirm the diagnosis. Both the growth inhibition test and direct immunofluorescence test should be used, since results may vary due to differences in sensitivity and specificity.

i) **The growth inhibition test (GIT)**

This test is based on the direct inhibition of the growth of the agent on a solid medium by a specific hyperimmune serum. It is a very specific and simple test to perform but some results will need a certain experience to be interpreted: small inhibition zones (less than 2 mm wide), partial inhibition with "break-through colonies", false negative and false positive reactions (very rare).
ii) The direct or indirect fluorescent antibody test (FAT/IFAT)

The direct immunofluorescence test is commonly used, although it is slightly less specific than the indirect one.

The FAT can be performed:

- From a broth culture: place 2 drops on a microscope slide, fix for 15 minutes with methyl alcohol, leave in contact with the labelled hyperimmune serum for 30 minutes at 37°C in a humid chamber, rinse 3 times with PBS and read under an epifluorescence microscope.

- From colonies grown on solid medium: cut a block of agar supporting a number of young colonies and put it on a slide with the colonies upwards, place 1 or 2 drops of the labelled hyperimmune serum on the block and leave it in a humid chamber for 30 minutes, put the block into a tube and wash twice for 10 minutes with PBS, put the block on a slide with the colonies upwards and read as before.

- In a petri dish: the gel should not be too thick (no more than 3 mm) and should contain as little horse serum as possible. Rinse the gel 3 times with PBS, flood the surface with 1 ml of labelled serum and incubate 30 minutes in a humid chamber, rinse 4 times with PBS and read directly under the microscope (10 x 8). The FAT in a petri dish is used mainly just after isolation and before cloning, as it is very useful in the case of mixed infection with several mycoplasma species.

In the FAT with broth culture the mycoplasmas appear bright green on a dark background. However, experience is required for the FAT carried out on colonies on agar, since the background appears dark green.

2. Serological tests

a) Complement fixation test

Since the Campbell and Turner technique has been described the complement fixation test remains the recommended procedure (although the current method is slightly different from the original one), and it is widely used in all countries where infection occurs (10). The modified CFT requires:

- 0.25 ml of a haemolytic system, i.e. a mixture of haemolytic serum (12 HD$_{50}$) and 6% sheep red blood cells;
- 2.5 units of complement;
- 2 units of antigen (in the original technique 5 units were used). A checkerboard titration gives one antigen unit as the highest dilution of antigen that gives a positive complement fixation with the highest dilution of serum.
- A short fixation period: 30 minutes at 37°C.
Before performing the test, the titres of the haemolytic system, the antigen and the complement may have to be checked according to the classical techniques if the reagents are not kept in good conditions.

The sera to be tested have to be decomplemented at 56°C for 30 min and diluted (1 in 2) in saline starting at dilution 1/10 up to 1/1,280 or more. To 0.25 ml of each dilution of the serum, are added 0.25 ml of antigen (2 units) and 0.25 ml of complement (2.5 units). The tubes are incubated for half an hour at 37°C before adding 0.25 ml containing the haemolytic serum and 6% sheep red blood cells. The tubes are once more incubated at 37°C for 30 min with agitation every 10 min. Serum and antigen control tubes have to be incorporated.

This test is highly specific and false positive results are rare (although they may occur in areas which are free of disease); however, since the test is not very sensitive, false negative results are possible, especially in the chronic stages of the disease. Strongly positive results (4+ at 1:10) are due to classical disease (clinical or symptomless) in its early stages or to chronic cases with sequestra. Doubtful results (2+ at 1:10) are due to very recent infections.

b) ELISA test

An ELISA test has also been developed (6, 7) and may be used when a large number of sera are to be tested. However, it is not yet standardised.

c) Agglutination test

A rapid field slide agglutination test with either whole blood or serum (12) has been developed to detect specific agglutinins: the antigen is a dense suspension of coloured mycoplasmas that is mixed with a drop of blood or serum. Due to a lack of sensitivity, the test detects only animals in the early stages of the disease and therefore should be used only on a herd basis.

Conclusion

Generally speaking, the isolation and identification of the CBPP agent is not very difficult as long as the appropriate procedures and media are used. When possible, classical bacteriology laboratories should set up a special section for handling only *Mycoplasma mycoides* subsp. *mycoides* SC and other mycoplasmas.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Since the beginning of the 20th century, many vaccines have been described (e.g. killed vaccines, heterologous vaccines) but none of them have proven really satisfactory. Today, the only vaccines commonly used are produced with attenuated *M. mycoides* subsp. *mycoides* SC strains.
1. **Vaccinal strains**

Two types of strains are recommended and widely used:

a) **T<sub>1</sub> strains: T<sub>1</sub>-44 and T<sub>1</sub>-SR**

The T<sub>1</sub> strain is a naturally mild strain isolated in 1951 by Sheriff and Piercy in Tanzania. The 44th egg-passage is sufficiently attenuated to protect zebu cattle without post-vaccinal reactions. However, more susceptible breeds such as the *Bos taurus* breeds may still exhibit severe reactions, and they should not be vaccinated with this strain.

The T<sub>1</sub>-44 strain induces at least 12 months' protection. A variant has been obtained by passages in broth cultures with streptomycin to make it streptomycin-resistant and even streptomycin-dependent (it grows in the presence of 1 mg of streptomycin per ml).

The T<sub>1</sub>-SR strain presents several advantages:

- It may be used to produce combined vaccines (e.g. rinderpest).
- Its resistance to streptomycin may be used as a marker when testing the vaccine for purity.

b) **KH<sub>3</sub>J strains**

The history of the KH<sub>3</sub>J strain is not well known. After its isolation in Sudan, it was passaged several times in broth culture before being inoculated into embryonated eggs. The vaccinal strain obtained after 4 to 5 egg passages is now at the 88th passage. The KH<sub>3</sub>J strain does not have any residual pathogenic effect and it may be safely used in all breeds; however, immunity after vaccination is not absolute and does not last more than 6 to 8 months. A streptomycin-resistant variant has also been developed with the same advantages as for T<sub>1</sub>-SR.

2. **Seed bank**

The vaccinal strains (the 44th egg passage for the T<sub>1</sub> strain or the 88th passage for the KH<sub>3</sub>J strain) are kept in freeze-dried form at -20°C.

A seed bank for a year's production is prepared by using two broth cultures, distributed in vials and kept frozen.

3. **Manufacture**

The media used for vaccine production are either Gourlay's medium or F66 medium: they both contain the same basic ingredients but F66 medium is more complex (9). They are filtered for sterilisation and the pH must be 7.8-8.0. The
media should be used in the days immediately after preparation. They should be distributed in tubes, 1 litre flasks (500 ml per flask), and 10 litre flasks (5 litres per flask) and incubated at 37°C for 48 hours to check sterility before use.

To obtain high titres, the vaccine should be harvested by the end of the logarithmic growth phase before the peak is reached, as the number of viable mycoplasmas will decrease quickly afterwards. Therefore, a kinetic curve should be drawn in each production laboratory. The preparation of the medium is a very important step and all the medium batches should be similar, as the curve may change with the quality of the medium.

The frozen vials are thawed and inoculated in tubes (1 in 10 dilution). The last tubes where growth occurs are used to inoculate 1 litre flasks. The 10 litre flasks are heavily inoculated with a 48-hour-old culture of the 1 litre flasks (10 ml per litre of medium) and incubated at 37°C. After 24 hours of incubation, slow magnetic stirring is recommended. Just before the peak is reached (between 60 to 70 hours after inoculation), the flasks are examined (Gram staining and microscopical examination) and prepared for freeze-drying. If freeze-drying cannot be performed immediately, the flasks can be cooled rapidly to 4°C and stored for 2 or 3 days at most. All the following steps should be carried out on melting ice:

- Addition of the freeze-drying protective medium: skimmed milk (4.5% i.e. 225 g per 5 litres).
- Distribution into vials (the volume depends on the number of doses required per vial and on the principles of lyophilisation).

For freeze-drying, shelf freeze-driers are recommended so that the different steps can be controlled and monitored. Immediately after freeze-drying, the vials must be sealed to avoid leakage.

4. In-process control

Microscopical examination of Gram-stained preparations of the broth or blood agar plate cultures of the broth must be performed at every step to check for the absence of bacterial contamination.

Prior to freeze-drying, a rapid examination should also be carried out to check for the presence of Mycoplasma mycoides subspecies mycoides SC by phase-contrast microscopical examination of the harvested medium, FAT examination with a labelled hyperimmune antiserum, or an interfacial precipitation test in tubes.

5. Batch control

a) Titration

The minimum vaccinal dose for cattle is $10^7$ viable mycoplasmas. Titration is performed after reconstitution of the freeze-dried vaccine in the diluent recommended for vaccination.
Dilutions (1 in 10) are made in Gourlay's medium. The use of an agitator (vortex type) is useful to homogenise the suspension in each tube. Five ml of the tubes $10^{-6}$ to $10^{-12}$ are inoculated in five further tubes of medium (1 ml per tube – 5 tubes per dilution). All the tubes are incubated for 4 days at 37°C and growth is detected by colour change of the indicator. The titre is obtained using MacCrady's "Most Probable Number" table or the Reed and Muench method.

b) Sterility

Classic aerobic and anaerobic broths are inoculated, together with blood agar; they are incubated at 37°C. All media should remain sterile.

c) Identity

Samples of the freeze-dried vaccine are inoculated onto mycoplasma agar or broth to carry out a growth inhibition test (3).

When streptomycin-resistant strains (KH$_3$J-SR or T$_1$-SR) are used this characteristic should be checked as a marker for the attenuation of the mycoplasma.

d) Safety

Five vials are reconstituted in cold buffer and pooled before inoculation into two mice subcutaneously, two mice intraperitoneally, and two male guinea pigs intraperitoneally. None of the animals should die within the following month and the guinea pigs should not show signs of orchitis. Safety tests should also be carried out on cattle: two zebu cattle are inoculated with 10 doses each of T$_1$ strain or two taurindicus cattle with 10 doses each of KH$_3$J strain. This test may be omitted if streptomycin-resistant strains are used and checked.

e) Potency

Because CBPP cannot easily be reproduced experimentally, potency tests may be performed once a year only on the seed bank.

Five susceptible cattle are inoculated with a virulent strain by the endobronchial route (2) and used as a source of contact infection for four vaccinated cattle (3 weeks before) and two unvaccinated ones. All the animals should be kept in close contact and monitored daily. The two unvaccinated controls should show signs of the disease or the presence of lesions when slaughtered after two months while the four vaccinated ones should not.

The complement fixation test should be performed for the examination of all the batches of vaccine. Young, serologically negative cattle should show antibody response (at least 4+ at 1:10) 3 weeks after vaccination.
REFERENCES


SUMMARY

Lumpy skin disease (LSD) is a sporadic or epidemic disease of cattle, caused by a capripox virus, and characterised by local or generalised skin thickenings of 0.5-5.0 mm diameter. The epidermis and dermis are affected and the hair stands erect over the lesion, which is usually irregularly round. The regional lymph nodes are involved and affected limbs are often oedematous. There may also be lesions of mucous membranes, such as in the oesophagus, rumen, etc.

Identification of the agent: The excision of an early skin lesion is necessary for diagnosis. Lesions may also be obtained from the subcutis and oropharynx of dead animals. For virus isolation the tissues are homogenised and the resulting suspensions sonicated or frozen and thawed. These are used to inoculate tissue cultures. Cultures of lamb or calf testis, calf kidney or sheep choroid plexus are recommended. Capripox-type cytopathic effects may usually be distinguished from those of herpesviruses which are frequently found in skin tissue. Specific identification may be made by immunofluorescent staining of infected cultures in flying coverslips. Electron microscopy of the tissues readily identifies a poxvirus.

Serological tests: Indirect fluorescent antibody (IFA) and virus neutralisation (VN) tests have been used to investigate LSD outbreaks. These tests have a broad level of cross-reaction amongst all capripox viruses, as well as low IFA cross-relationships with cowpox virus. Both IFA and VN tests are useful in establishing a diagnosis of LSD where clinical signs are evident.

Requirements for biological products: Modified live virus vaccines are available.

A. DIAGNOSTIC TECHNIQUES

Lumpy skin disease (LSD) is a disease of cattle caused by a virus of the capripox type. It is characterised by erect hair over the lesions in the dermis and epidermis. These lesions may be irregularly round, the regional lymph nodes may be involved and the limbs oedematous. There may be lesions of the mucosae of the oesophagus and rumen.

1. Identification of the agent

A skin biopsy must be taken from an early lesion of a suspected clinical case of LSD. The virus can be isolated for up to 4 weeks after the first appearance of the skin lesions. An elliptical portion of skin is removed under local anaesthesia to
include a small area of adjacent normal skin. A portion is fixed for histopathological examination and another used for virus isolation or electron microscopy. Samples from lesions may also be removed from the skin, subcutis, or oropharynx of dead animals.

Tissue should be dispatched in a transport medium consisting of a tissue culture medium containing penicillin 500 IU/ml, streptomycin sulphate 500 µg/ml and mycostatin 50 units/ml or fungizone 2.5 µg/ml and kept cold. If a cool temperature cannot be maintained, then a buffered glycerol medium should be used. The tissues are minced with scissors into a pestle and mortar, and ground thoroughly with sterile sand. This is made up to approximately a 50:50 w/v suspension with transport medium, and sonicated or frozen and thawed 3 times in dry ice and alcohol. The supernatant fluid is diluted to give a 10% w/v suspension of the original tissue in phosphate buffered saline (PBS) or Hanks' salt solution. This is used to inoculate cell cultures.

Primary cell cultures of lamb or calf testis are the most sensitive for primary isolation of LSD virus. There are considerable differences in the sensitivity of cells derived from different breeds of sheep. Those from wool breeds of sheep of European origin are often 1,000-fold more sensitive than those obtained from some African hair sheep. Testis cells are usually 10-fold more sensitive than kidney cells, and ovine cells often better than those of bovine origin. Fetal muscle, thyroid or other cell types may also be suitable for diagnosis.

Cell cultures infected with the tissue supernatant fluid from a positive LSD lesion can be expected to show cytopathic effects (CPE) within 2-10 days. An increased refractivity in groups of cells is accompanied by cell rounding and ultimate detachment from the monolayer. New foci of CPE develop progressively to involve much of the monolayer. The rate at which these changes take place varies according to the sensitivity of the cells used.

Flying coverslips can be stained by haematoxylin and eosin to show the predominantly acidophilic intracytoplasmic inclusions of the poxvirus type. Direct immunofluorescent staining with a conjugated hyperimmune antiserum will identify the agent as a capripox virus. This can also be achieved by a virus neutralisation (VN) test. Low titre cross-fluorescence occurs with cowpox virus in indirect fluorescent antibody (IFA) tests but no relationship can be detected by cross-neutralisation.

The virus of LSD will grow on the chorio-allantoic membrane (CAM) of embryonated hens' eggs. Optimal conditions include an incubation temperature of 33.5-35°C using 7-9 day embryos. Lesions may or may not develop in the CAM, but virus can be demonstrated in sections of the CAM by staining with haematoxylin and eosin, by electron microscopy or immunofluorescence. Adaptation of the virus is necessary to attain titres of $10^4$ TCID$_{50}$/ml, and the method is not recommended for primary virus isolation unless no cell cultures are available.

Differential diagnosis is necessary from bovine herpesvirus type 2 infections, which produce very superficial lesions in cattle skin. The CPE of this herpesvirus is quite
distinct, with the formation of syncytia and intranuclear inclusions; also, in contrast to that of LSD, it does not multiply in chick embryos. The virus particles are also readily differentiated on electron microscopy. Other orphan herpesviruses are frequently isolated from skin.

2. **Serological tests**

IFA and VN tests have been those most widely employed in LSD diagnosis.

In IFA, LSD infected cells are grown on coverslips or in multiwell slides to act as antigens; various cell types may be used to prepare these. A 10-20% cell infection rate is optimal, and fixation in ice cold acetone allows them to be stored at -20°C or 4°C for long periods. Test sera are compared with known positive and negative controls using the same anti-species conjugate. Convalescent serum titres may be up to 1:5,000 after recent LSD infections. The test is specific for capripox virus antibody, with low titre cross-relationships with cowpox.

A VN test using the constant virus-varying serum dilution method may be performed in tube or microtitre cultures. A less sensitive culture has proved more satisfactory than lamb testis for this purpose. Sera are inactivated at 56°C for 30 minutes and neutralised with the virus at the various dilutions for 2 hours at 37°C. End points are taken where approximately 50% of the viral CPE is suppressed.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

1. **Seed management**

   a) **Characteristics**

   The strains of LSD virus employed as vaccines have been prepared from 2 different live virus seeds, namely:

   - a modified live LSD virus: 60 passages in lamb kidney cell cultures followed by 20 passages in embryonated hens' eggs.

   - a low passage level of a Kenyan sheep and goat pox virus.

   Both strains are avirulent, immunogenic and stable in the lyophilised form. Seed lots are prepared free from any contaminating virus or other agent. A minimum protective dose for each seed used in production should be established.

   b) **Culture**

   Primary cultures or early passages of lamb testis, kidney or fetal muscle are usually employed for production. The RK13 rabbit kidney cell line has also been used. All cultures used in the preparation of a seed stock should be screened by appropriate tests for adventitious agents. For non-cytopathogenic
viral agents, haemadsorption and IFA tests should be performed. The cultures should also be maintained and observed for the development of a CPE due to a contaminating virus present in the original cells or serum.

c) Control methods

The cell cultures should be examined before use for the presence of cytopathic or noncytopathic agents such as bovine pestivirus (virus diarrhoea). Haemadsorption and immunofluorescence tests should be performed. Uninoculated control cultures should be maintained during the production process.

2. Manufacture

Flat or roller bottle cultures may be used. Seed virus is adsorbed onto the monolayer cell cultures used for vaccine production, at a high input multiplicity such as undiluted or at a dilution of 1:10 of the seed stock. The cultures are examined daily for the appearance of a CPE, and harvested when 40-70% of the cell monolayer is affected. The bottles are either ultrasonicated, or frozen and thawed 3 times to release any intracellular virus. After centrifugation to remove cell debris, the supernatant fluid is mixed with an equal volume of diluent; 2.5% lactose or sucrose with 5% peptone is satisfactory. This is then lyophilised. The identity of the virus is confirmed by IFA and VN tests.

3. In-process control

Control cultures are maintained under the same conditions and observed for a period of 14 days for any CPE due to an adventitious virus. Tests for bovine pestivirus should be carried out on these cultures by fluorescent antigen or other appropriate tests to exclude such viruses.

4. Batch control

a) Sterility tests

Tests for sterility and freedom from contamination of biological materials can be found in the chapter on General Information.

b) Safety tests

Two animals are inoculated subcutaneously with the field immunising dose and 2 with 100-fold this amount. The animals are observed daily for 3 weeks for any local or systemic effects which might be related to the vaccine. Any untoward reactions should result in the rejection of the vaccine batch with an investigation of the possible causes.

c) Potency tests

The virus content of the product is determined by titration. The animals used in
tests for safety can serve for the tests of potency. A minimum immunising dose of $10^{3.5}$ TCID$_{50}$ per animal is required. A minimum protective dose for each isolate used in production should be established before production.

The antibody responses to LSD vaccine, with appropriate tests to exclude other viruses, should be determined by IFA or VN. If the vaccinated animals have developed antibodies, the batch is acceptable.

d) Duration of immunity

The modified live virus vaccine produces antibodies which have been shown to persist for three years. The Kenya sheep and goat pox strain produces antibodies persisting for at least two years. Immunity is likely to be lifelong.

e) Stability

Freeze-dried virus ampoules and reconstituted vaccine are sensitive to exposure to direct sunlight. The virus titre remains stable at 4°C and -20°C if ampoules are kept in the dark. The vaccine is reconstituted in 1% peptone in a phosphate buffer.

REFERENCES


SUMMARY

Rift Valley Fever (RVF) is an acute, febrile, contagious arthropod-borne zoonotic disease caused by a Bunyavirus of the genus Phlebovirus. It causes high rates of abortion and neonatal mortality in sheep, goats, and cattle. Other species are susceptible to a much lesser extent. Susceptibility to infection and to disease decreases with age; a high mortality rate of 95-100% may occur in lambs and kids less than one week old. The disease has a short incubation period, the clinical course is acute, and typical necrotic lesions may be found in the liver at necropsy. The disease is confined essentially to Africa in association with dense populations of vector mosquito species.

Man is susceptible and can be infected by direct contact. Laboratory infections have been recorded in some parts of the world, so that all manipulations with infective materials must be carried out with biohazard containment.

Identification of the agent: RVF virus can be isolated from samples obtained during the febrile stage of the disease. These samples include whole blood, plasma, spleen and liver tissue. Primary isolations are made in mice or hamsters, but lambs aged 1-2 days old, embryonated chicken eggs and tissue cultures of various types may be employed. Rapid diagnosis can be achieved by a combination of cell culture and immunofluorescence. The latter technique is used to identify the viral antigen directly in cryostat sections or in impression smears of liver, spleen or brain from animals which have been sacrificed in the febrile stage of the disease. Antigen may be detected in blood taken during the febrile stage of the disease by immunodiffusion or enzyme immunoassay. The presence of characteristic histopathological changes in the liver assists in diagnosis.

Serological tests: Infected animals develop specific antibodies which can be demonstrated by enzyme-linked immunosorbent assay (ELISA), or by virus neutralisation (VN), immunofluorescence, haemagglutination inhibition or complement fixation (CF) tests. A serological response can be detected within 3 days of infection by plaque reduction neutralisation. Other tests will demonstrate antibodies within 6-7 days of infection, but the CF test later than that. VN tests are performed with live virus and are not recommended outside an endemic area. The ELISA is recommended as a reliable and sensitive method to detect either infection or vaccine-induced antibodies.

Requirements for biological products: Live virus vaccines are prepared from highly-adapted neurotropic or other mutagenised, attenuated strains of RVF virus. Inactivated virus vaccines are made from pantropic RVF virus grown in cell cultures, inactivated with formaldehyde or β-propiolactone. They are used with an adjuvant.
A. DIAGNOSTIC TECHNIQUES

Rift Valley fever (RVF) is an acute, febrile, arthropod-borne viral zoonotic disease. The virus is classified in the family Bunyaviridae, genus Phlebovirus, and causes high rates of abortion and neonatal mortality, primarily in sheep, goats and cattle. Other species of animal are also susceptible but to a much lesser extent. Susceptibility to infection and to disease significantly decreases with age, with the highest mortality rate of 95-100% being recorded in lambs and kids less than one week old. The disease is characterised by a short incubation period and an acute course; typical necrotic foci may be seen in the liver at necropsy.

Man is also susceptible, and may be infected by direct contact with sick animals, their fetuses, excretions or infected tissues. Four clinical syndromes have been described in infected individuals: undifferentiated fever, encephalitis, retinitis with blindness, or severe haemorrhagic fever and death. It is therefore essential to perform all manipulations with infective materials in conditions of biohazard containment.

The disease has been recognised exclusively in African countries, with an underlying association with high rainfall and dense populations of vector mosquitoes. The only epizootic outbreak of RVF outside sub-Saharan Africa was recorded in animals and man in Egypt in 1977-78. Laboratory infections have been recorded in other parts of the world.

1. Identification of the agent

RVF virus is present in the blood and organs of infected animals. It may be isolated from specimens collected during the febrile period of the disease, including whole blood, serum, spleen and liver tissues. Primary isolations can be carried out in mice or hamsters, but 1- to 2-day-old lambs, embryonated chicken eggs and various cell cultures may be used. A combination of cell culture and immunofluorescence is recommended for a rapid (overnight) diagnosis.

Approximately 5 ml of blood are collected without preservative during the acute phase of the disease; or, approximately 5 g of liver, spleen or brain are collected after death. Specimens should be kept at 4°C during transit or on dry ice or in liquid nitrogen.

Homogenised specimens are suspended 1:10 in a suitable medium, e.g. cell culture medium or buffered saline (pH 7.5) containing 10% serum that is negative to RVF, with antibiotics. The suspension is centrifuged at 1,000 g for 10 minutes and the supernatant fluid injected intracerebrally into 2- to 5-day-old mice, or intraperitoneally into adult mice. Infant mice will either die or be obviously ill by the second day. Adult mice are affected 1-3 days later. Although mice are the animals of choice, hamsters and lambs may also be used. Cell cultures of VERO, CER and primary calf kidney, calf testis or lamb testis cells can be employed. These are then observed microscopically for 5-6 days. RVF virus induces a cytopathic effect (CPE) characterised by slight rounding of the cells; thereafter, the whole cell sheet
becomes affected within 12-24 hours.

The virus can be identified by neutralisation tests with known positive antiserum. Infant mice are injected intraperitoneally. The constant serum-variable virus technique is usually employed. If facilities permit, a plaque reduction neutralisation test using VERO or CER cells can be performed. The virus neutralisation test in roller tubes is not recommended.

Identification of the virus may also be made by direct or indirect immunofluorescence, carried out directly on cryostat sections of liver, spleen or brain of animals killed during the febrile stage of the disease. It can also be used to detect viral antigen directly in impression smears of liver or fetal brain. A rapid diagnosis can be made by demonstrating viral antigen in the sera of febrile animals by agar gel immunodiffusion tests or by enzyme-linked immunosorbent assay (ELISA).

Histopathological examination of liver sections of affected animals will reveal characteristic inclusions of RVF virus infection.

2. Serological tests

The following serological tests have been used to detect RVF antibodies both in man and in domesticated or wild animals: complement fixation (CF), agar gel immunodiffusion (ID), haemagglutination inhibition (HI), micro virus neutralisation (VN), immunofluorescence (IF), plaque reduction neutralisation (PRNT), mouse neutralisation (MN), ELISA, enzyme-linked immunofluorescent assay (ELFA), and radio-immunoassay (RIA).

The earliest antibody response may be detected within 3 days of infection by PRNT. The other techniques, such as HI, ID, ELISA, RIA and IF will detect antibodies later; infected animals develop HI, ELISA, VN, IF, and CF antibodies within the first week of infection. The VN and PRNT tests are specific and will record the earliest response and high titres; but these tests may only be performed with live virus and cannot be recommended for use outside endemic areas. In other tests, cross-reactions occur between RVF virus and other viruses of the phlebotomus fever serogroup. The order of specificity is PRNT, CF, HI, and IF. The ELISA is a reliable, sensitive test which may be employed with both sheep and cattle sera to detect antibody responses to either active infection or vaccination.

The HI test is the test of choice in non-endemic areas. However, sera from individuals who have had prior infections with viruses of the phlebotomus fever serogroup other than RVF may react with RVF antigen to titres as high as 1:40, and rarely to titres of 1:320. In view of this, Reference Centre assistance will be required to carry out PRNT tests for specificity on a proportion of these sera.

a) Haemagglutination inhibition

Stocks of antigen are prepared and stored either frozen or, if lyophilised, at 4°C. An inactivated mouse liver antigen is used. This is diluted so that the
amount used in each haemagglutinating unit (HAU) is 4-8 times that which agglutinates 50% of the red cells in the test system. Sera are first inactivated at 56°C for 30 minutes. Kaolin or acetone treatment may be used to remove non-specific serum inhibitors. The red cells should be derived from normal white male geese. The cells are washed 3 times by centrifugation, and a 0.3% suspension prepared in isotonic saline. Sera should be absorbed before use by incubation with an equal volume of packed red cells for 1 hour at 0°C.

Positive and negative control sera are incorporated into each test. A test is considered valid only if the control sera give the expected results. Sera with titres below 1:40 are considered negative; titres between 1:40 and 1:320 are considered doubtful. Titres above 1:320 are positive. In countries where other viruses of the phlebotomus serogroup do not occur, HI titres below 1:10 are considered negative, titres of 1:10 to 1:20 are suspect, and titres above 1:20 are positive.

b) Plaque reduction neutralisation

One of several cell lines (BHK, VERO, or CER) and stock RVF virus which produces reproducible, clearly visualised plaques are used. A solid or semi-solid overlay is used. A fresh aliquot of virus is thawed for each test, and diluted to contain 30-100 plaque-forming units (PFUs) per volume of the antigen to be used in the test. Sera are inactivated at 56°C for 30 minutes, diluted 2-fold, mixed with diluted virus, and incubated at 37°C for 1 hour. After inoculation of the virus-serum mixtures onto cell cultures, solid or semi-solid overlay medium is added and the cultures incubated for a time previously determined to provide optimal plaque formation. A control titration of the virus is done with each test. Known positive and negative control sera are included with each test. Following the incubation period, all plaques are enumerated. The titre is expressed as the highest dilution of serum which causes a 50% or greater reduction in the number of PFUs contained in the control units.

c) ELISA

Micro-ELISA polystyrene plates (for example Dynatech, Immunolon II or Nunc) are used in a solid-phase immunoassay (1). Constant volumes (100 µl) are employed throughout. The wells are coated at 4°C for 14 hours with a 1:1,000 mixture of anti-RVF monoclonal antiserum in phosphate buffered saline (PBS), pH 7.4 (available from the WHO/FAO Centre for Arbovirus Research and Reference, Yale University Arbovirus Research Unit (YARU)). The plates are washed once with PBS containing 0.25% Tween-20 (PBS-Tween); this fluid is used for all washes throughout the test.

All test reagents are diluted in PBS containing 0.5% Tween-20 and 500 µg/ml of dextran sulphate. The RVF-positive antigen, which is first inactivated with β-propiolactone (supplied to YARU by the US Army Medical Research Institute for Infectious Diseases), is a sucrose extract of infected mouse liver infected with the Entebbe strain of RVF virus. This is diluted 1:50. A control
antigen is prepared in the same way but is not inactivated. Both antigens are added to the previously coated plate, which is then incubated at 37°C for 1 hour and then washed 3 times. The test sera are added to duplicate wells, one of which is coated with the positive antigen and the other with the control. After incubation for 1 hour at 37°C, the plates are washed 3 times. Rabbit anti-sheep IgG (H and L chains) peroxidase conjugate (Zymed Laboratories, South San Francisco, USA) diluted 1:3,000 or rabbit anti-bovine IgG (H and L chains) peroxidase conjugate diluted 1:2,500 are added. The plates are finally incubated at 37°C for 1 hour, washed 3 times and the substrate, 2,2-azino-di-(3-ethyl-benzthiazoline-sulphonic acid) (Kirkegaard & Perry Labs, Inc., Gaithersburg, Maryland, USA) is added.

The plates are incubated at 37°C for 30 minutes and the reaction read in a spectrophotometer at a wavelength of 410 nm. The specific activity of each dilution of the test sera is calculated by subtracting the non-specific background optical density in the wells with the control antigen from those with the positive antigen. A positive value is one at least 3 standard deviations greater than the mean of the corresponding dilutions with the known negative serum. A titre of 1:50 or more is considered positive (1).

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

RVF vaccines may be prepared either as attenuated live virus vaccines or as inactivated virus vaccines. A new mutagen-attenuated large-plaque vaccine (ZH-548-M12 or MV P12) has recently been developed in the USA (4). It was found to be immunogenic and non-abortogenic for ewes. Its field trials were recommended by the FAO/OIE/WHO meeting held in Dakar in March 1988.

1. **Seed management**

a) **Characteristics**

Live vaccine
The stock antigen for live virus vaccine production is derived from Smithburn's original neurotropic strain. It is maintained at -18°C as a lyophilised 5% mouse brain suspension. The adapted virus differs from the virulent form in that it is not lethal to adult mice, as are virulent strains, when inoculated intraperitoneally.

The recently developed MV P12 vaccine strain is a mutagenised, large-plaque clone of RVFV, derived from the Egyptian ZH-548 strain, originally isolated from the serum of a human being with nonfatal RVF in 1977. It has been found to be highly immunogenic, non-pathogenic, and non-abortogenic in sheep.

Inactivated vaccine
Inactivated RVF vaccines are prepared from cell cultures that have been infected with pantropic RVF virus, and then inactivated with formaldehyde or
β-propiolactone. They may be used in combination with an adjuvant. Pantropic strains are more immunogenic than the attenuated neutropic virus strains.

b) Culture

The seed live virus is pooled mouse brain suspension, and is derived by serial passage in adult mice. The seed virus must be shown to be free of contamination with other infectious agents.

c) Validation as a vaccine

Live vaccine

The virus is identified serologically by means of neutralisation tests in suckling mice. Specific RVF antiserum is tested against the virus seed by inoculating the mice with 0.2 ml of the serum-virus mixtures intraperitoneally. To confirm that the virus remains attenuated it is also inoculated at a dilution of 1:100 intraperitoneally into 12 adult mice, whereupon no mice should die.

The seed virus pool is titrated intracerebrally in infant mice and should have a titre of at least $6 \times 10^6$ mouse LD$_{50}$ per ml.

Two susceptible lambs are each inoculated subcutaneously with 1 ml of seed virus containing at least $4 \times 10^4$ mouse LD$_{50}$ per ml. These animals are kept under observation for 3 weeks and should remain healthy throughout. They are bled for serum assayed in infant mice for the presence of neutralising antibodies.

The vaccine should be subjected to an occasional potency test carried out in sheep to determine that it continues to induce an adequate antibody response.

2. Manufacture

Live vaccine

A vial of lyophilised seed virus is reconstituted with sterile Eagle's medium. Confluent BHK cell sheets are seeded at a dilution of 1:1,000. As soon as cytopathic effects (CPE) are observed, both the medium and cells are harvested. This harvested material is lyophilised immediately.

Inactivated vaccine

A high infectivity titre is required for the seed virus. The original strain should have a titre of $10^{7.5}$ mouse LD$_{50}$ per ml or more.

The most susceptible cells for cultivating the pantropic strain are CER or BHK-21 cells. For maximum virus yield a pH of 7.4-7.8 is optimal. The in-put multiplicity of the strain passaged in tissue cultures should be 0.001-0.01 mouse LD$_{50}$ per cell. The cultures are harvested after 2-5 days when more than 50% of the cell sheet should show degenerative changes. The titre of the harvests must be at least $10^{7.5}$ mouse LD$_{50}$ per ml.
The inactivation process is carried out with formaldehyde at a final concentration of 0.2% for 12 hours at 37°C. The formalin is neutralised by the addition of sodium bisulphite. The concentration of residual formalin must be less than 1:5,000.

If β-propiolactone is employed for inactivation, it should be used at a dilution of 1:2,300 for 2 hours at 37°C.

3. In-process control

Live vaccine
A vial of lyophilised stock antigen is reconstituted with sterile Eagle’s medium and subjected to control tests for bacterial and fungal sterility, and freedom from mycoplasmas.

Freedom from adventitious agents is demonstrated by inoculation of guinea pigs, mice and embryonated eggs, both before and after neutralisation with specific antisera.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

b) Safety

Live vaccine
Two susceptible sheep are inoculated with 1 field dose of vaccine and kept under clinical observation for 3 weeks. They must remain healthy during this period. A suitable test with mice should also be carried out.

To assess freedom from bacterial and fungal contamination, at least 10 final vials are selected at random and the contents of each are reconstituted with 1 ml sterile distilled water. The contents of each vial are inoculated into suitable culture media and observed for 14 days.

Inactivated vaccine
Tests are conducted in laboratory animals, sheep, and tissue cultures.

Two families of suckling mice are inoculated intracerebrally with 0.02 ml per mouse; 10 weanling mice are inoculated intraperitoneally with 0.1 ml per mouse; and 10 weanling hamsters are inoculated intraperitoneally with 0.1 ml per hamster. All mice and hamsters are observed daily for 21 days and must survive the test.

Two lambs, 1 month old, are each inoculated subcutaneously with 5 ml from each lot of vaccine. Their temperatures are taken daily and blood samples are collected for virus isolation for 10 days. Both lambs must remain healthy.
without obvious clinical abnormality throughout.

Tests for proper inactivation of the virus are also made in CER or VERO cell cultures. These cells are highly susceptible to RVF virus. They are inoculated with inactivated vaccine virus and observed daily for any evidence of CPE. These cells must remain free from any signs of CPE specific for RVF virus.

c) Potency

Live vaccine
(Smithburn neurotropic strain) The lyophilised vaccine obtained from at least 2 vials of the final product is reconstituted, pooled and titrated intracerebrally in infant mice. The vaccine should contain at least 10⁷ mouse LD₅₀ per ml.

Two vials of the final vaccine product are incubated at 37°C for 1 week, then reconstituted and titrated as before. After this the vaccine should contain at least 10⁶ mouse LD₅₀ per ml.

Inactivated vaccine
The in vivo potency test to be performed on the final product consists of a two-stage, adult mouse antigen extinction test. Serial 5-fold dilutions of vaccine are prepared and a single dose of 0.5 ml inoculated intra-peritoneally into 10 mice per dilution. The mice should be at least 42 days old. They will be challenged 14 days later with approximately 10⁵-10⁶ mouse LD₅₀ of an appropriate challenge strain subcutaneously. This challenge strain should be one that differs from the vaccine strain.

Following the results of this preliminary testing, a second, single inoculation assay is performed using serial 2-fold dilutions of vaccine over a dilution range designed to include adequately the anticipated 50% end-point of the test. These data are plotted on probit paper and the confidence limits of the assay determined.

REFERENCES


SUMMARY

Bluetongue (BT) is a subclinical to acute, febrile, non-contagious arthropod-borne disease of ruminants caused by a virus of the family Reoviridae, genus Orbivirus. The disease is characterized by inflammation and congestion leading to cyanosis, haemorrhages, and ulceration of the mucous membranes. Other clinical signs include laminitis and sloughing of the hoof, myositis causing an early stiffness and later muscle degeneration, oedema of the head and neck, and various fetal abnormalities when animals are infected early in pregnancy. In sheep the disease is characterised by high morbidity and low mortality. In cattle the disease is primarily inapparent and has low morbidity and mortality. In goats the disease is usually inapparent but may occasionally cause some morbidity and mortality similar to that observed in either sheep or cattle. In non-domestic ruminants the disease can vary from an acute haemorrhagic disease with high mortality as observed in the white-tailed deer (Odocoileus virginianus) to an inapparent disease as seen in the North American elk (Cervus canadensis).

Identification of the agent: Bluetongue virus (BTV) can be isolated from blood collected from affected animals during the febrile stage of the disease and often several weeks afterwards. Whole blood collected in an anticoagulant such as heparin or sodium citrate, from which the blood cells are washed and resuspended in sterile buffered lactose peptone, phosphate buffered saline or sodium chloride, is the preferred laboratory sample. Samples may also be collected in oxalate-phenol-glycerin (OPG) preservative solution if refrigeration is not available. Primary isolations are made by intravenous inoculation of embryonating chicken eggs, inoculation of susceptible sheep, or directly on cell culture. Isolates are passed to cell culture and adapted for identification by immunofluorescence or a peroxidase-antiperoxidase (PAP) staining procedure or other enzyme immunoassays.

Serological tests: Both group-specific and type-specific serological tests are available. The group-specific tests will differentiate BTV or epizootic haemorrhagic disease virus (EHDV) antibodies from antibodies against other Orbivirus groups but will not differentiate between serotypes of BTV or EHDV. The group-specific tests include agar gel immunodiffusion (AGID), complement fixation (CF), and enzyme linked immunosorbent assay (ELISA). The serotype-specific tests will differentiate between antibodies produced by specific BTV or EHDV serotypes but may be negative or have low reactivity to antibody of a different serotype and may fail to detect BTV or EHDV-specific antibody if the wrong antigen is used. Serotype-specific tests include micro- and macrovirus neutralisation and plaque neutralisation (PN). Only the AGID, CF and ELISA tests are in common use in diagnostic laboratories, whereas PN ELISA tests are done in a few specialised laboratories. Serological responses in ruminants can be expected 7 to 14 days
after exposure with the ID and the PN tests and 14 to 21 days with the CF test. The PN titre and ID reaction will usually peak in 2 to 4 weeks, plateau and last for years whereas the CF titre will usually peak in 4 to 6 weeks and last for 4 to 18 months.

Requirements for biological products: Live virus vaccines are prepared from different serotypes attenuated by passage in embryonating chicken eggs or cell cultures. Vaccines should only be used in sheep and should be of the same serotype as that causing the disease in the area. Pregnant sheep should not be vaccinated during the first half of pregnancy as this may cause fetal death and deformities. Vaccines are not recommended for cattle since the infection has such a low morbidity and mortality. Vaccines may also cause hypersensitivity in cattle that could increase the incidence and severity of the disease. Some of the vaccinated white-tailed deer may also develop the disease.

Multivalent vaccines are used in some areas where multiple serotypes are enzootic but live multivalent vaccines should be avoided because of the risk of reassortment and recombination. A vaccine should not produce a viral titre of such height and duration that it could be transmitted by insects.

A. DIAGNOSTIC TECHNIQUES

Seasonal appearance of the typical clinical signs in sheep in a BT enzootic area is sufficient to make a presumptive diagnosis of BT. In epizootic areas, or areas where the disease has never been previously diagnosed, it should be confirmed by virus isolation. In cattle, the disease cannot be clinically differentiated from other diseases, and should always be confirmed by virus isolation. Epizootics of haemorrhagic diseases in wild ruminants should always be differentiated by virus isolation because they may be due to BTV or other agents. In epizootics, viral isolation and identification should be done to identify the serotype of BTV involved.

Clinical signs include characteristic inflammation, cyanosis and ulceration of the mucous membranes, and laminitis, sloughing of the hoof, myositis and oedema of the head. In sheep morbidity in fully susceptible flocks may be 80 to 100%. Mortality varies widely and may range from 0 to 50%.

When sheep die during the acute viral infection there will not usually be extensive pneumonia but interalveolar hyperaemia and alveolar oedema may be observed. Haemorrhages are often seen on the serosal and visceral surfaces of the internal organs with the most consistent finding being haemorrhage around the base of the pulmonary artery and apex of the heart (12).

BT must be differentiated from vesicular diseases, photosensitisation, pneumonia, contagious ecthyma, polyarthritis, foot rot, sheep bots, scabies, white muscle disease, plant poisonings, border disease, malignant catarrhal fever, mycotic stomatitis, actinomycosis, and actinobacillosis.
1. **Identification of the agent**

a) **Samples for viral isolation**

Whole blood collected in an anticoagulant such as heparin or sodium citrate, from which the blood cells are washed and resuspended in sterile phosphate buffered saline, isotonic sodium chloride, or buffered lactose peptone (BLP), is the preferred laboratory sample (4, 18). Washed samples may be stored at refrigerator temperatures (4°C). Samples should not be frozen unless they are placed in buffered lactose peptone (BLP) and frozen at -70°C or colder. Samples may also be collected in an oxalate-phenol-glycerin (OPG) preservative solution (11) if refrigeration is not available. From an acutely affected viraemic animal 10 to 20 ml blood is usually more than adequate to isolate BTV. If an animal is suspected of being chronically infected or a carrier then 50 to 500 ml of blood should be collected.

Liver, red bone marrow, spleen, selected lymph nodes, or brain tissue are the tissues of choice from animals that have died. To isolate BTV the tissues should be refrigerated and taken fresh immediately to the laboratory. Dry ice (frozen carbon dioxide) should not be used to freeze samples as it will lower the pH and destroy BTV which is inactivated at pH 6.0 or lower. It should also be noted that BTV in solution or suspension (i.e. after the tissue has been processed) is unstable at approximately -20°C, but stable at -70°C or lower temperatures. To identify viral antigens by genetic probes the tissues should be quickly frozen to -70°C and sent to a laboratory that can perform this test.

b) **Sheep inoculation**

Identification of BTV by inoculation of susceptible sheep was originally described by Spreull (31) and, until the development of the procedure for intravenous inoculation of embryonating chicken eggs, was the only satisfactory method for isolating BTV. From chronically infected or carrier animals or samples that may have low concentrations of virus it is still the best method of isolation as 200 to 300 ml of sonicated blood can be inoculated into a sheep.

c) **Inoculation of embryonating chicken eggs**

Initial culture of BTV in embryonating chicken eggs (ECE) was done by the yolk sac route (22). Reducing the temperature of incubation from 37°C to 33.5°C was found to assist in the propagation of BTV in the chicken embryo (2). Intravenous inoculation of chicken embryos for direct isolation of BTV from the blood of infected animals demonstrated that intravenous (IV) inoculation of embryonating chicken eggs is several times more sensitive than yolk sac inoculation and equal in sensitivity to inoculation of sheep from animals with a measurable viraemia (14, 13). For low titred blood samples or chronically infected or carrier animals sheep may still be more sensitive, probably because of the larger inoculum that can be injected into them.
The IV procedure is difficult to perform and requires some practice. Washed blood cells, disrupted in distilled water and/or sonicated, are used as the inoculum. Each of 12 prepared 10- or 11-day-old chick embryos is inoculated IV with 0.1 ml of material. The eggs are incubated in a humid chamber at 33.5 ± 1.0°C. The eggs must be candled daily and any deaths before 48 hours discarded, as non-specific. Embryos which die between 3-7 days are retained under refrigeration, remaining embryos are killed after 7 days.

When good mortality rates occur in inoculated ECE either on initial inoculation or subpassage the harvested supernatant should be inoculated directly onto cell cultures for further identification, or chorio-allantoic membranes may be tested directly with the indirect peroxidase-antiperoxidase (PAP) test.

d) Cell culture

Direct isolation of BTV on various vertebrate and invertebrate cell lines has been used but is generally less satisfactory than either inoculation of sheep or embryonating chicken eggs. New cell lines and cell culture procedures are constantly being investigated for potential as a more rapid, sensitive, efficient and less laborious procedure for direct isolation of BTV. In order to identify BTV or to serotype the virus after isolation, it is necessary to adapt the virus to cell culture from ECE. Baby hamster kidney (BHK₂₁), African green monkey kidney (VERO), and mouse L-cells have been most commonly used.

Virulent high titred BTV and EHDV can usually be isolated by inoculation of BHK₂₁, VERO or mouse L-cells. Diluted ECE suspensions, whole blood, washed blood cells, or macerated and clarified suspensions of tissue specimens with minimal toxicity may be inoculated onto monolayered cell cultures.

An inoculum should be prepared from first or second passage ECE suspension which has been tested for bacteriological sterility and mixed with equal parts of BLP at pH 7.0 or above and stored at -70°C, or 0.5% phenol should be added and the inoculum stored at 4°C. The entire first passage inoculum must be subpassaged onto an initial cell monolayer. The monolayers are harvested when about 75% cytopathic effect is observed.

Samples are considered as negative for BTV after 2 passages (one subpassage) in cell culture. Virulent BTV will usually be positive by late first or second subpassage. The identity of BTV may be confirmed by immunofluorescence, serotyping, or PAP staining procedures.

e) Fluorescent antibody identification of BTV

Direct immunofluorescence (IF) tests have been described in baby hamster kidney (BHK) cells (27) and in bovine fetal kidney cells (30). The indirect fluorescent antibody (IFA) procedure is now the preferred method, particularly when monoclonal antibody is used. The test will readily
differentiate between BTV and EHDV but is a group-specific test and will not differentiate serotypes.

Viruses isolated in sheep or ECE must be adapted to cell cultures before they can be identified with the IFA test.

f) **Indirect peroxidase-antiperoxidase (PAP) identification of BTV**

This test may be used to identify BTV directly in infected ECE without adaptation to cell cultures. The test is group-specific, so a primary monospecific antibody to any serotype or mixed antibody to several types would give a positive test for any serotype of BTV.

Chorio-allantoic membrane sections are collected from ECE which die between 2 and 7 days after inoculation. The chorio-allantoic membrane is fixed in formalin, embedded in paraffin, cut into sections and mounted on slides. The mounted sections are incubated with BTV-specific serum, then with a linking rabbit anti-goat antibody. The tissues are stained with a peroxidase-antiperoxidase substrate and counterstained with haematoxylin.

BTV positive stained tissues will appear as a red staining of the cytoplasm from the product of the 3-amino-O-ethyl carbazole substrate. The nucleus appears blue in negative samples from the acid haematoxylin stain (6).

g) **Avidin-biotin complex identification of BTV**

This procedure is used to detect the presence of BTV antigen in tissue samples collected from infected animals. It has not yet been adapted for routine diagnostic testing, but has been used primarily to test fetal samples from animals infected in utero. It provides some advantage over embryonating chicken eggs or sheep inoculation of tissue from autolysed samples, which may be toxic to ECE or in which the virus has been killed by the autolysis, but the antigen remains in the tissue specimen. Recently the test has been adapted to detection of BTV and EHDV in infected insect vectors (25).

Tissues from brain, thymus, lung, liver, spleen, lymph nodes, or kidney should be collected from suspected fetuses and snap frozen in cold freon or stored at -70°C in a freezer until ready to section. Frozen sections are cut and mounted on slides. The prepared sections are then incubated with normal goat serum followed by BTV-specific rabbit antiserum and with a biotinylated goat anti-rabbit antiserum.

The sections are stained with an avidin, biotin, horseradish peroxidase stain followed by 3,3-diaminobenzidine and hydrogen peroxide. The sections are counterstained with haematoxylin.

Sections are observed under a light microscope. The staining will appear similar to that seen with the peroxidase-antiperoxidase method described above.
h) Viral genome probes

In recent years several probes using complementary DNA (cDNA) to viral RNA have been described for direct identification of BTV blood samples or cultured cells (29). Further refinement of the technique and development of probes to different genome segments has allowed the procedure to be used for identification of serotypic differences (8) and genetic differences within serotypes (10) of BTV.

Washed red blood cells or cultured cells are fixed on glass coverslips or nitrocellulose and subjected to *in situ* hybridisation with a nick-translated, biotin-labeled DNA probe. To detect hybridised BTV genome the samples are treated with biotinylated horseradish peroxidase and streptavidin then hydrogen peroxide. Samples are examined under a phase contrast microscope. In positive samples the infected cells stain brown.

2. **Serological tests**

Orbivirus genus includes 12 serogroups with over 100 individual virus species. The bluetongue subgroup contains 24 different serotypes. A major concern with the BTV serogroup of Orbivirus is the number of other viruses in the genus that have some serological relatedness and some degree of cross-reaction with BTV.

Immunologically mature animals that become infected with BTV develop antibody 7 to 21 days following exposure depending on the test used. These animals eventually overcome the virus infection but may retain antibody titres for months or years afterwards. Both group-specific and serotype-specific antibodies are produced following a BTV experience. If the animal has not been previously exposed to BTV only the serotype-specific antibody to the infecting virus appears. If, however, the animal is later infected with a different serotype of BTV, serotype-specific antibody to the infecting virus appears and there is also anamnestic appearance of antibody to previous virus exposures, as well as serotype-specific antibody to serotypes with which the animal has had no experience. This can make interpretation of the serotype-specific reaction in a single animal virtually impossible.

a) Virus neutralisation (VN)

A virus neutralisation test for BTV was first reported in suckling mice (17). Neutralisation of BTV on cell cultures of primary lamb kidney cells (or on cell lines such as BHK, VERO and L929) has been used to analyse the immunological response of sheep. The technique was further refined and procedures developed to use cell culture plaque reduction test procedures to differentiate isolates of BTV into serotypes. A microtitre VN test has been developed in Australia and been recognised as an official standard test in that country (24). The test is conducted in flat-bottom 96-well microtitre plates.

The VN tests are type-specific for the currently recognised 24 serotypes of BTV. The test may be used to type virus isolates or as a differential serotyping
procedure to identify specific antibody to a virus type.

b) Complement fixation (CF)

A complement fixation test for BT using antigen prepared from brains and spinal cords of BTV infected suckling mice (32) was modified by addition of normal calf serum and became the standard modified direct complement fixation (MDCF) test which was widely used to test ruminants for international movement until 1982, when it was largely replaced by the agar gel immunodiffusion test.

c) Agar gel immunodiffusion (AGID)

The immunodiffusion test for BT was first described as a micro-gel diffusion test. That test has since been modified (26). Since 1982 the test has been the standard testing procedure for international movement of ruminants.

The antigen used in the test is a purified soluble antigen prepared on BTV-infected cell cultures using a single serotype. The antigen can be purified by ultracentrifugation and concentrated by ultrafiltration. The currently used standard antigen is a proprietary product licensed by the United States Department of Agriculture under Veterinary license number 336.

The test is done in petri dishes or trays in which a 7-well pattern is used with a centre well and six wells in a circle around it. The wells are 2.4 mm apart and 4 mm in diameter. The agar is a 0.9% solution of agarose agar prepared in physiological saline (0.85% NaCl in distilled water). Six ml of agar is used in the 60 x 15 mm plate or 15 ml in the 100 x 15 mm plate. The agar should be 2.8 mm thick. The agar should be cut the same day it is poured and used the same day it is cut. Antigen is placed into the centre well and 3 positive control serum samples and test samples are placed in alternate wells surrounding the centre well. Test sera or plasma are placed in the 3 remaining alternate wells surrounding the centre well. The plates are incubated at room temperature (20-25°C) in a closed chamber for 24 hours.

For negative samples the precipitin lines will continue into the test sample wells without bending toward the antigen well. In positive samples the control lines join with the test well line and form a continuous line. In weak positive samples the control lines bend toward the antigen well and away from the test sample well but may not form a continuous line between the control test wells. All weak positive samples and other samples that produce questionable results should be repeated using 5.3 mm wells 2.4 mm apart.

d) Indirect enzyme linked immunosorbent assay (ELISA)

Indirect ELISA tests have the advantage of high specificity and sensitivity and do not cross-react with EHDV, which is a problem with both the ID and the MDCF testing procedures.
Purified BTV antigen prepared in cell culture is adsorbed to polystyrene plates. Specific binding of anti-BTV antibody is detected by adding a specific anti-species antibody prepared in rabbits and conjugated to horseradish peroxidase. A substrate is added and a colour reaction occurs in the test serum when it has combined with the specific BTV antigen (9, 15, 19, 28).

e) Competitive ELISA

The blocking and competitive ELISA tests were developed to counteract the problems of purification of ELISA antigen which may give rise to false positive reactions against host cell antigens. For BTV-specific antibody, the competitive ELISA is reported to have better specificity than the indirect ELISA and to be equal or greater in sensitivity to the AGID, MDCF and virus neutralisation tests in detecting early antibody (1).

A procedure for the competitive ELISA (C-ELISA) has recently been standardised after comparative studies were carried out in several institutes. The C-ELISA, for establishing group-specific antibodies to bluetongue virus, is carried out as follows.

Flat-bottomed 96-well microtitre plates are coated with antigen (either the tissue-derived sonicated cell culture antigen (5) or the yeast-expressed viral protein (21)), using 100 μl per well and incubating overnight at +4°C or for one hour at 37°C.

The plates are then washed 5 times in wash/diluent buffer (0.01 M phosphate buffered saline plus 0.05% Tween 20 pH 7.2) before the addition of control sera in 50 μl amounts at a 1:5 dilution. Monoclonal antibody control wells will contain diluent buffer in place of serum.

Test sera are then added (50 μl per well) at a 1:5 dilution (final dilution 1:10) in duplicates of columns 3-12 of the plate. Immediately afterwards the anti-bluetongue virus monoclonal antibody (either MAb 3-17-A3 (5) or MAb 20EA (21)) is added to all wells in 50 μl amounts at a predetermined dilution. Plates are then incubated for one hour at 37°C with continuous shaking.

The plates are washed as previously and the enzyme conjugate (anti-mouse immunoglobulin, H + L, labelled with horseradish peroxidase) is added in 100 μl amounts to all wells, and the plates are incubated for a further one hour at 37°C with shaking.

Once again the plates are washed 5 times before the addition of the substrate/chromogen (H₂O₂ + ABTS system) in 100 μl amounts. The plates are then held at room temperature with shaking for 10 minutes before the reaction is stopped by the addition of 50 μl per well of the stopping solution (sodium azide).

After blanking the ELISA reader on a separate plate using the first column of 8 wells containing substrate plus stopper, the optical density (OD) of the test
plates is read – optimally at 414 nm, but it may be read at 405 nm.

The results are calculated using the following formula:

\[
% \text{ inhibition} = 100 - \left[ \frac{\text{Mean OD of test serum}}{\text{Mean OD of Mab control}} \right] \times 100
\]

Percentage inhibition values greater than 50% are considered positive. The results of the duplicates of test sera can vary as long as they do not lie either side of the 50% inhibition value. On each plate strong and weak positive and a negative serum should be included. The weak positive serum should give 60-80% inhibition and the negative serum 0-5% inhibition. The bluetongue antibody status of these sera should be predetermined by the AGID and VN tests. All controls should fall into the range of expected inhibition values and the results of a plate should be disregarded if this does not occur. However, with controls done in quadruplicate one outlier may be allowed and disregarded.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

There are several attenuated live BT vaccines used throughout the world. It is important that the serotypes of virus in the vaccines match the serotypes found in the area or region where they are to be used. Most of the vaccines available for use have been attenuated by serial passage through embryonating chicken eggs (3).

There has been a great deal of interest in developing an inactivated BT vaccine in recent years. Development of a bioengineered recombinant vaccine may have some future promise but a product is not yet available.

1. Seed management

a) Characteristics

Master seed virus must be free of contaminating bacteria, viruses, fungi, and mycoplasmas. Each lot of master seed virus must also be tested for transmissibility and reversion to virulence before it is used in the manufacture of a vaccine.

b) Culture

The first BT vaccines were propagated in chicken embryos. After adaptation to chicken embryos the virus could be readily propagated by yolk sac inoculation (3, 23).

Adaptation of BTV to primary bovine embryo kidney cells (16), primary lamb kidney cells or fetal lamb kidney cells allowed the production of vaccine on these cells. Because of the possible presence of oncogenic viruses, genes or
other contaminants in continuous cell lines, they should not be used for master
seed virus culture.

c) Validation as a vaccine

To be used as a vaccine a product must be pure, safe, potent and efficacious. It
should be shown to be stable with no tendency to revert to virulence and should
not be transmissible by insect vectors from a vaccinated animal to other
animals. A vaccine that meets the above criteria for purity, safety, potency,
stability and non-transmissibility and is the same serotype as existent virulent
field strains of BTV in the area, region or nation is a valid vaccine.

Each lot of master seed virus should be tested for immunogenicity before use in
manufacture of vaccine. A suitable procedure is as follows:-

i) Use 25 lambs susceptible to the BTV serotype contained in the vaccine as
test animals (20 vaccinates and 5 controls).

To test for susceptibility draw blood samples from each lamb and test the
collected serum with a virus neutralisation test using a constant
virus/varying serum method. There should not be any neutralisation at a 1:2
final serum dilution with 60 to 300 TCID\textsubscript{50} of BTV of the same type as the
vaccine.

ii) Establish a geometric mean titre of the vaccine produced from the highest
passage from the master seed before the immunogenicity test is conducted.
Five replicate virus titrations should be conducted on a sample of the
vaccine to be used.

iii) Administer a predetermined quantity of the vaccine to each of the 20 test
lambs.

iv) Collect individual serum samples from each of the vaccinates at least once
14 to 18 days post-vaccination. Test for virus neutralising antibody using 60
to 300 TCID\textsubscript{50} of BTV of the same serotype as the vaccine. If at least 19 of
the 20 vaccinates do not have BTV neutralising antibody titres of 1:4 final
serum dilution or higher the master seed virus is unsatisfactory.

v) Challenge the immunity of the 20 vaccinates and 5 control lambs 21 to 28
days post-vaccination using a fully virulent BTV of the same serotype as in
the vaccine.

vi) Observe the 25 lambs for 14 days after challenge. Take and record the
rectal temperature for 17 consecutive days beginning 3 days pre-challenge.
Record presence or absence of lesions or other clinical signs of BT each
day for 14 days post-challenge.

vii) At least 4 of the 5 controls must show clinical signs of BT and have a
temperature rise of 1.7°C or higher over the pre-challenge mean
temperature. Otherwise the test is inconclusive and should be repeated using a different strain of virulent BTV of the same serotype as the vaccine.

If more than one of the vaccinates shows a temperature rise of 1.7°C or higher than its pre-challenge mean temperature for 2 or more days, or if more than one of the vaccinates exhibits clinical signs of BT, the master seed virus is unsatisfactory.

The master seed virus lot should be retested for immunogenicity at least once each 3 years using 5 vaccinated and 5 control susceptible lambs.

2. **Manufacture**

Continuous live cell lines such as the monkey kidney derived cells (LLC-MK₂) or baby hamster kidney cell lines (BHK₂₁) are used to propagate the vaccine (7). Continuous cell lines must be chosen carefully to be certain that they do not contain viral contaminants.

3. **In-process control**

All ingredients of animal origin, including serum, primary cells or cell lines, must be tested for presence of viable bacteria, viruses, fungi, or mycoplasmas according to the same protocol recommended for testing master seed virus. If cell lines are used they should be examined for characteristics determined to be normal to the cell line.

4. **Batch control**

a) **Sterility**

Each batch (serial) of vaccine should be tested for presence of viable bacteria, extraneous virus, fungi, or mycoplasmas according to the same protocol recommended for testing master seed virus.

b) **Safety**

In addition to the tests for sterility a mouse safety test and sheep safety test should be done.

To conduct the mouse safety test 8 adult mice should be inoculated intracerebrally with 0.03 ml and 8 inoculated intraperitoneally with 0.5 ml of the final vaccine. Both groups should be observed for 7 days. If unfavourable reactions attributable to the product occur in 2 or more mice in either group the batch must be considered unsatisfactory and discarded. If unfavourable reactions occur in 2 or more mice which are not attributable to the vaccine the test is inconclusive and must be repeated.

To conduct the sheep safety test inject 2 lambs at least 6 months of age with the equivalent of 10 doses of the vaccine and observe the sheep for 21 days. If an unfavourable reaction attributable to the vaccine occurs in either sheep during
the observation period, the batch of vaccine must be considered unsatisfactory and discarded. If unfavourable reactions not attributable to the vaccine occur the test is inconclusive and must be repeated.

c) Potency

Final container samples of finished vaccine should be tested for virus titre and should be greater than the titre of virus used to test the immunogenicity of the master seed virus. The vaccine should maintain a titre of $10^{0.7}$ greater than that used for the immunogenicity test until the end of the expiration period.

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SUMMARY

Sheep pox and goat pox are highly contagious skin diseases of small ruminants caused by capripox viruses. These diseases are endemic in most of Africa, the Middle East and Asia. The level of host specificity appears to vary in different parts of the world. A clinical diagnosis may often be made on the basis of the generalised skin eruptions. A confirmatory laboratory diagnosis is required, particularly when differentiation from parapox virus infection is necessary.

Identification of the agent: Full skin thickness biopsies should be taken for virus isolation, preferably within one week of the first appearance of the lesions. Lung lesions may also be used. The tissue should be homogenised and ultrasonicated, or frozen and thawed to release intracellular virus.

Cell cultures prepared from lamb testis, goat testis or kidney may be used to culture the virus. Characteristic intracytoplasmic pox virus inclusions become evident within a few days of inoculation. These may be specifically identified by direct immunofluorescence. However, differentiation from lumpy skin disease is not possible by serological methods.

Differentiation from contagious pustular dermatitis, caused by a parapox virus, may be made by immunodiffusion tests, or by electron microscopy, when the parapox and capripox virions are readily distinguishable. The size of the capripox virus is 300-400 nm, whereas that of parapox is approximately 200-250 nm.

Serological tests: Indirect fluorescent antibody, agar gel immunodiffusion and virus neutralisation tests have been used most frequently for the investigation of capripox virus infections.

Requirements for biological products: There is a wide range of live virus vaccines and inactivated vaccines for the prophylaxis of these diseases. Most, if not all, strains of sheep and goat pox viruses will cross-immunise.

A. DIAGNOSTIC TECHNIQUES

Sheep pox and goat pox (SGP) are highly contagious skin diseases of small ruminants caused by capripox viruses. They are endemic in most of Africa, the Middle East and Asia. A diagnosis may be based on the appearance and distribution of the generalised skin eruptions that occur on affected animals. A confirmatory diagnosis is however preferably made in the laboratory, particularly when there may be some need to differentiate the origin of the lesions from those of parapox virus infection, the cause of contagious pustular dermatitis (CPD) or
contagious ecthyma.

1. **Identification of the agent**

Virus isolation and identification are required, especially where there is a possibility of confusion with CPD. Various cultures may be used. Lamb testis cells are probably the best, although varying levels of sensitivity to SGP virus have been found in cells originating from different breeds of sheep. Lamb kidney, calf kidney or testis, and ovine or bovine fetal muscle skin, lung or other cells can also be used. Continuous cell lines such as VERO or BHK21-C13 have not proved to be of value for the primary isolation of capripox viruses.

Tissue samples from skin biopsies of early lesions, or from lesions in the lung, may be used for virus isolation. They are minced with sterile scissors and then ground in sterile sand with a pestle and mortar. This is mixed with an equal amount (w/v) of a transport medium containing penicillin 500 IU/ml, streptomycin sulphate 5 μg/ml and mycostatin 50 units/ml or fungizone 2.5 μg/ml. The mixture is left at 25°C for one hour and then ultrasonicated, or frozen and thawed 3 times using dry ice and alcohol. Virus particles, identifiable as capripox virus, may be seen by electron microscopy of this tissue suspension. It is made up to an approximate 10% suspension with Hanks' medium or phosphate buffered saline (PBS), and centrifuged at 600 g for 10 minutes. The supernatant fluid is used as the inoculum for virus isolation.

The cultures, including some containing flying coverslips, are inoculated with the supernatant fluid which is allowed to adsorb for 1 hour. They are observed daily and cytopathic effects (CPE) may become evident after 4-14 days. Initially the CPE manifests as an increase in refractile cells, rounding into small groups. This becomes more obvious and involves most of the monolayer after a further 4-9 days. The infected cells gradually become detached. Identification of viral antigens may be made by haematoxylin and eosin staining of coverslips to show the eosinophilic intracytoplasmic inclusions that are characteristic of pox viruses.

It is possible to identify capripox viral antigen by immunofluorescence tests. The coverslips are washed, air dried and fixed in cold methanol for 10 minutes, then stained by direct or indirect immunofluorescence methods. A direct conjugate can be made from antisera of hyperimmunised rabbits or from experimentally infected sheep or goats. The serum immunoglobulins are precipitated with fluorescein isothiocyanate (FITC) at a ratio of 1 mg FITC to 50 mg of precipitated protein. This furnishes a highly satisfactory reagent. Coverslips infected with material from the suspected case, together with positive and negative controls, are stained and examined. This procedure allows a firm diagnosis to be made in 2-4 days.

The growth of CPD virus occurs in similar cell types and it is recommended, where cases prove to be negative for capripox virus, that they be examined with a fluorescent conjugate prepared against this virus. Alternatively, the original tissue preparation, before dilution with Hanks' medium or PBS, may be tested by immunodiffusion against a control CPD-positive antiserum in parallel with positive
Sheep pox and goat pox (A10)

and negative controls. Similar tests may be carried out to identify the SGP viral antigen. It must be remembered that there are considerable cross-reactions between members of the pox virus group in agar gel immunodiffusion tests.

2. **Serological tests**

Indirect fluorescent antibody (IFA) tests and virus neutralisation (VN) tests have been used most widely for the investigation of sheep and goat pox.

Tissue cultures are grown on coverslips or in multichamber teflon coated slides, and the infected cells which contain SGP viral antigen are used for IFA tests. Control positive and negative sera are examined in parallel with the test sera. Anti-sheep or anti-goat immunoglobulin conjugates can be obtained commercially or prepared in the laboratory. Positive sera have titres up to 1:500 to 1:5,000 in the 2-3 months following infection. These are specific for capripox viruses.

Neutralisation tests can be performed in tubes or in a microtitre system. A 2-hour neutralisation period is usual. The constant virus-varying serum method is recommended, using a serum dilution range of 1:5 to 1:500, although higher neutralising titres may be obtained. End-points are determined where there is a 50% reduction in CPE. Neutralisation tests using lamb testis cells frequently give problems of a late 'breakthrough' of virus growth and accompanying CPE, which distorts the earlier neutralisation pattern. This has been overcome by using cultures of the less sensitive fetal muscle cells.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

A wide range of vaccines, of the inactivated and the live virus types, are used for prophylaxis. Serological and cross-immunity tests show that most, if not all, strains of sheep- or goat-adapted capripox viruses will cross-immunise.

1. **Characteristics**

Virus strains used for production must be adequately characterised as to their pathogenicity, immunogenicity, and freedom from adventitious virus or other agents. Their pathogenicity must be adequately tested in the breeds of animal which are to be vaccinated. A good deal of variation in the susceptibility of different breeds to the same virus strains can be found.

Identification of the virus is carried out by the inoculation of cultures and examining them by direct immunofluorescence.

a) **Culture**

Cell cultures of the lamb or bovine tissues used for virus isolation will be suitable for vaccine production. Their sensitivity to the seed virus must be established from earlier studies. Rabbit kidney cells have also been used for
vaccine production.

b) **Control methods**

The cell cultures should be carefully examined, before use, for the presence of any adventitious agents. Uninoculated control cultures should be maintained, and examined for non-cytopathogenic viral agents such as the Pestiviruses, Border disease, and bovine viral diarrhoea. Haemadsorption and immunofluorescence tests should be performed. Only scrapie-free flocks should be used for the preparation of primary cell cultures.

2. **Manufacture**

Monolayers prepared in stationary or roller bottles should be used, the latter being rolled at approximately 6-8 revolutions per hour. A wide multiplicity of input gives a good yield of virus with a rapid CPE. This is most convenient for production. Cultures should be harvested when the CPE involves 50-70% of the monolayers. Ultrasonication, or freezing and thawing, is necessary to release cell-associated virus. Clarification by centrifugation at 600 g for 10 minutes is recommended. The harvests are pooled to make a single batch. A sugar and protein stabiliser may be incorporated during lyophilisation, for which sucrose, lactose, peptone, and lactalbumin have all proved satisfactory.

Inactivated vaccines are prepared from cell cultures infected with a pathogenic virus strain of low passage history. These are harvested when 40-60% CPE is observed, sonicated or frozen and thawed three times and clarified by centrifugation. The resultant supernatant is titrated and adjusted by volume with phosphate buffered saline to give 1,000-2,000 TCID$_{50}$/ml. An equal volume of alhydrogel is added to the virus-saline mixture and stirred for 48 hours at 4°C. Merthiolate at 1/10,000 and formalin at 0.03% are then added and mixing continued for a further 48 hours at 4°C. The vaccine is then dispensed and stored at 4°C.

3. **In-process control**

Inoculated and uninoculated tissue culture vessels should be observed for any signs of nonspecific degeneration of the cell monolayer. The pH should be maintained at 7.2-7.4. Bottles showing degeneration or a rapid pH change are rejected.

Pooling of individual harvests may be delayed until all are shown to be free from bacterial contamination by overnight incubation in a suitable broth culture.

4. **Batch control**

a) **Sterility tests**

Tests for sterility and freedom from contamination of biological materials can be found in the chapter on General Information.
b) Safety tests

The vaccine is inoculated subcutaneously into 4 sheep or goats. Two animals receive a field vaccine dose and 2 others a 100-fold of this amount. They are examined daily for any febrile reaction, clinical signs or other untoward reaction. A very mild clinical reaction can be accepted.

c) Potency tests

The minimum protective titre should be established for the seed virus used to manufacture vaccine. Alternatively, a host animal protection test can be used. Titration of the virus in cell cultures, or the intradermal inoculation of sheep or goats is used to determine vaccine potency.

Comparison of the reduction in the intradermal virus titre in vaccinated and unvaccinated animals is most commonly used to assess the potency of sheep and goat pox vaccines. The test itself is done by shaving vaccinated and unvaccinated control sheep closely on one flank from the shoulder to the proctal area. The challenge virus strain is then inoculated intradermally in 0.1 ml volumes; 4 separate inoculations are made in a vertical line for each dilution 10^{-1} to 10^{-6}. Usually, the inoculations are made from the anterior to the posterior of the animal, i.e. 10^{-1} to 10^{-6}.

In unvaccinated animals, necrotic lesions with oedema and swelling occur at all the inoculation sites up to the 10^{-5} or 10^{-6} dilutions. In the vaccinated animals, initial hypersensitivity reactions with erythema and slight oedema may occur at some inoculation sites, but this rapidly disappears. Limited areas of necrosis may develop at the 10^{-1} or 10^{-2} inoculation sites. A reduction in the intradermal titre greater than 2.5 \log_{10} is considered satisfactory. This has been correlated with the antibody responses in vaccinated animals and with efficacy of vaccine batches in the field.

Pre- and post-inoculation sera are examined for evidence of an adequate response.

d) Duration of immunity

The modified live virus vaccines have been shown in the laboratory to produce an immunity which lasts for at least one year. Field observations in Kenya suggest that the immunity persists for at least two years, and may be lifelong.

Breakdowns in the immunity produced by inactivated vaccines have occurred from 6 months after vaccination. A period of 9 months is considered all that can be expected.

e) Stability

Most lyophilised vaccines are stable for 2-4 years when stored at 4°C or longer if stored at -20°C.
Aluminium hydroxide gel-adsorbed vaccines may suffer some loss in potency if exposed to repeated changes in temperature. If they are maintained at 4°C their shelf life is about one year. It is important not to freeze such vaccines.

REFERENCES


AFRICAN HORSE SICKNESS
(A11)

SUMMARY

African horse sickness (AHS) is a Culicoides-borne viral disease of Equidae. The causal agent is a member of the genus Orbivirus of the family Reoviridae and 9 distinct serotypes are known to date. The disease is often fatal in horses and mules. It is characterised by clinical signs and lesions associated with impaired respiratory and circulatory functions.

AHS is essentially a disease which occurs in tropical Africa, from where it spreads regularly to Southern Africa and occasionally to North Africa. A few outbreaks have occurred outside Africa, such as in the Middle East (1959-63) and in Spain (1966, 1987-1989).

Identification of the agent: Although the clinical signs and lesions are fairly characteristic, the disease can nevertheless be confused with other conditions, which will obviously necessitate laboratory confirmation of a tentative diagnosis of AHS. Isolation of the causal virus and subsequent serotyping is absolutely essential whenever outbreaks occur outside of the enzootic regions.

AHS virus (AHSV) can be isolated quite readily from blood collected during the early febrile stage (preferably in heparin but alternatively in other anticoagulants) as well as from lung, spleen and lymph nodes collected at autopsy. Virus isolation can be achieved in cell cultures (BHK21, VERO) as well as by the intracerebral inoculation of newborn mice or by intravascular inoculation of 10-12 day old embryonated eggs. It is always preferable to attempt virus isolation in at least two of the host systems mentioned.

Virus isolates can be identified by group-specific serological tests (e.g. complement fixation, ELISA, immunofluorescence) and serotyped by type-specific tests (such as plaque reduction or plaque inhibition neutralisation or haemagglutination inhibition) using known positive and negative antisera.

Serological tests: Convalescent animals develop specific antibodies within 10-14 days which reach a peak 21 days after infection. These may be demonstrated by group-specific serological tests (ELISA, complement fixation, immunodiffusion) or by type-specific tests (plaque inhibition or plaque reduction neutralisation, haemagglutination inhibition). In enzootic regions it would be imperative to use paired (acute and convalescent phase) serum samples.

Requirements for biological products: Monovalent and polyvalent live attenuated vaccines are used to induce effective and durable protection in horses, mules and donkeys. An inactivated vaccine is currently being developed and should soon be commercially available.
A. DIAGNOSTIC TECHNIQUES

African horse sickness (AHS) is an infectious (non-contagious), insect-borne viral disease of horses and other Equidae.

As its name indicates, AHS is a truly African disease which is endemic in the central tropical regions of the continent, from where it spreads regularly to Southern Africa and occasionally to North Africa. A few outbreaks have occurred outside Africa, such as in the Near and Middle East (1959-63) and in Spain (1966, 1987-1989).

The aetiological agent has been characterised as a typical orbivirus (family Reoviridae). Nine distinct serotypes are known, the last of which was isolated in 1960. AHSV is serologically unrelated to other known orbiviruses.

AHS is a non-contagious disease. It can, however, readily be transmitted by the parenteral injection of infective blood or organ emulsions. In nature the virus is biologically transmitted by midges of the genus Culicoides. In view of this, the disease has a seasonal incidence in more temperate zones and its prevalence is directly influenced by climatic conditions favouring insect breeding. The role of other blood-sucking insects as biological or mechanical vectors has not been proved unequivocally.

Members of the family Equidae constitute the main hosts. Horses are the most susceptible and the mortality rate in this species can be as high as 95%. The figure for mules varies between 50 and 70%. Donkeys in the endemic regions of Africa are very resistant and experience a subclinical infection. However, European and Asian donkeys are moderately susceptible and a mortality rate of about 10% may be observed. Zebra are also markedly resistant and, apart from a mild febrile reaction, show no clinical signs of infection.

The susceptibility of the dog to AHS has been known since 1907 but its role in the epizootiology of the disease is probably minimal. Natural outbreaks of AHS (with mortality rates of up to 30%) have been described in dogs that were fed virus-infected horsemeat and there is also evidence of transmission to dogs by means of biting insects. It could be postulated that the true natural reservoir host of AHSV is still unknown and, in all likelihood, restricted to tropical Africa.

Virus replication occurs mainly in the lungs, spleen and lymph nodes. Viraemia generally parallels the febrile curve in horses and mules. The average duration of viraemia in these species is about 4-8 days and very rarely as long as 18 days. The viraemic period in donkeys and zebra can be as long as 4 weeks. Virus replication results in selective increased permeability of blood vessels in specific organs or regions of the body. Clinically the disease varies from peracute to subacute in horses and mules and subclinical in African donkeys and zebra. The peracute form is characterised by alveolar, subpleural and interstitial oedema of the lungs and occasionally by a severe hydrothorax. In the subacute form oedema of the head (particularly the supraorbital fossae and eyelids), neck and shoulders as well as hydropéricardium is commonly observed. Oedema of the lower limbs is never seen,
African horse sickness (A11) 93

which distinguishes AHS from other conditions such as equine viral arteritis and purpura haemorrhagica.

1. Identification of the agent

Despite the fact that the clinical signs and gross lesions of AHS are fairly characteristic, the condition is nevertheless often misdiagnosed and necessitates laboratory confirmation. In outbreaks occurring outside the enzootic regions isolation of the causal virus and subsequent serotyping is essential.

Nucleic acid probes are in the process of development but to date their sensitivity is insufficient for the reliable demonstration of AHSV in horse blood and organs.

AHSV can be isolated quite readily from blood collected during the early febrile stage. It is imperative to collect the blood in anticoagulant (preferably heparin, 10 IU/ml) as it is virtually impossible to isolate virus from serum or from a macerated clot. Small pieces (2-5 gram) of spleen, lung and lymph node should be collected at autopsy and should be kept at 4°C or collected into glycerol-saline. A 10% tissue suspension is prepared in phosphate buffered saline (PBS) or cell culture medium containing relevant antibiotics before inoculation into the host systems for virus isolation.

The various host systems are very similar in their susceptibility to AHSV but it is nevertheless advisable to use more than one of these systems, particularly when dealing with important samples.

Virus isolates can be identified by group-specific serological tests such as capture ELISA, complement fixation (CF) or immunofluorescence. Serotyping can be done by plaque reduction or plaque inhibition tests on VERO or MS cell cultures using hyperimmune guinea pig antisera.

a) Cell cultures

About 6-12 early confluent roller tube cultures of BHK₂₁ or VERO cells should be seeded with each sample. Blood samples collected in heparin can be used undiluted whereas samples collected in chelating agents should be diluted 5- to 10-fold to prevent detachment of cells. The cultures are washed after 10-30 minutes adsorption time and refed with maintenance medium.

Cytopathic effects may appear 3-7 days post-infection and occasionally only become conspicuous in the second subculture. After 3 passages advanced cytopathic effects will develop within 2-4 days.

b) Newborn mice

Each sample is inoculated intracerebrally into 2 families of 1-3 day old mice. After about 4-10 days one or more mice may develop nervous signs. The brains from sick mice should be collected, emulsified and subinoculated into newborn
mice. In the second passage the incubation period should be shortened to 3-5 days and 100% infection rate should be achieved.

c) Embryonated eggs

At least 6 10- to 12-day-old embryonated chicken eggs are inoculated intravenously with each test sample. Following inoculation the eggs should be incubated at 33°C and should be candled daily. Specific mortality occurs 3-7 days after infection and virus-infected embryos usually appear vividly red.

d) ELISA

A group-specific indirect sandwich ELISA has now been developed which is capable of detecting AHSV antigens in original field samples as well as in laboratory adapted viruses (5, 7). The assay has been shown to be sensitive and specific and provides confirmation of AHSV within four hours.

Fifty \( \mu l \) per well of the optimal dilution of previously titrated AHSV specific hyperimmune rabbit antisera, diluted in 0.05 M carbonate/bicarbonate buffer pH 9.6, is passively coated onto the solid phase of ELISA plates. Plates are incubated overnight at 4°C in a humidity chamber. Coated ELISA plates are washed five times with PBS pH 7.6 and residual buffer removed by blotting the plates. Up to ten samples are diluted in single columns (100 \( \mu l \)/well) across the ELISA plate in a twofold dilution series prepared in PBS containing 0.05% Tween-20 (PBST). Duplicate wells in columns 11 and 12 receive a twofold dilution series of known AHSV as a positive control.

Plates are incubated at 37°C for one hour on an orbital shaker. After washing, 50 \( \mu l \)/well of the optimal dilution of AHSV specific immune guinea pig antisera diluted in PBST containing 5% skimmed milk powder (PBST-M) is added and the plates incubated at 37°C for one hour on an orbital shaker. Plates are washed as before and 50 \( \mu l \)/well of the optimal dilution of previously titrated rabbit anti-guinea pig immunoglobulins conjugated to horse radish peroxidase, diluted in PBST-M, is added. The plates are again incubated at 37°C for one hour on an orbital shaker. After washing, 50 \( \mu l \) of chromogen/substrate (one 30 mg orthophenylene-diamine dihydrochloride tablet dissolved in 75 ml distilled water plus 0.05% \( H_2O_2 \)) is added to each well. Colour development is stopped after 15 minutes by the addition of 50 \( \mu l \)/well of 1.0 M \( H_2SO_4 \).

Clear positive reactions can be read by eye although a more accurate interpretation of results may be obtained by spectrophotometric analysis at 492 nm. Samples giving optical density (OD) values less than 0.1 are considered negative, samples giving OD values between 0.1 and 0.15 should be confirmed by virus isolation, and samples giving OD values greater than 0.15 are considered positive.

A capture ELISA has also been described in ref. 2.
African horse sickness (A11)

2. **Serological tests**

a) **Complement fixation (CF) test**

The CF test is frequently used for the demonstration of group-specific antibodies against AHSV. A sucrose-acetone mouse brain extract is commonly used as antigen.

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 normal mouse brain similarly extracted and diluted.

Horse serum should be inactivated at 56°C, zebra serum at 60°C and donkey serum at 62°C for 30 minutes.

Sera are diluted 1:10 and inactivated. Titrations of positive sera may be performed using additional 2-fold dilutions. The CF antigen and control antigen (normal mouse brain) are diluted the same, with the CF containing 4-8 antigen units in veronal buffered saline containing 1% gelatin (VBSG); guinea pig complement is diluted in VBSG to contain 4 complement haemolytic units-50% (CH₅₀). Sera, complement and antigen are reacted in 96-well round-bottom microtitre plates, or in tubes if the macro- technique is used, at 4°C for 18 hours. Haemolysin is diluted to contain 2 haemolytic units and used to sensitise washed sheep erythrocytes (SRBCs). The SRBCs are standardised to 3% concentration and 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: 1) serum and complement; 2) serum and SRBCs; 3) CF antigen and control antigen each with 4 CH₅₀, 2 CH₅₀, and 1 CH₅₀ of complement; 4) CF antigen and SRBCs; 5) control antigen and SRBCs; 6) complement dilutions of 4 CH₅₀, 2 CH₅₀, and 1 CH₅₀; and 7) SRBCs. The inverse of the highest dilution of serum specifically fixing complement with the CF antigen is called the titre. A titre of 1:10 is positive, smaller than 1:10 is negative.

To avoid anti-complementary effects, sera should be separated from the blood as soon as possible, in particular sera from asses. Positive and negative control sera should be used in the test.

b) **ELISA**

A group-specific competitive ELISA for the detection of AHSV antibodies has been developed using methods similar to those described for the detection of antibodies against bluetongue virus (1). The assay shows a good agreement with the virus neutralisation test but is more sensitive and specific than the agar gel immunodiffusion and complement fixation tests (4, 6), and the test can more readily be automated for the testing of large numbers of sera. Results can be obtained within three hours.
Previously determined amounts of concentrated AHSV antigen, diluted in 0.05 M carbonate/bicarbonate buffer pH 9.6, are passively adsorbed onto the solid phase of the ELISA plates. Plates are incubated overnight at 4°C in a humidity chamber. Coated ELISA plates are washed five times with PBS pH 7.6 and blotted to remove residual waning buffer. A 1 in 5 dilution of each test serum, prepared in PBS containing 0.05% Tween-20, 1% adult normal bovine serum and 5% dried skimmed milk powder (PBST-BM) is added to duplicate wells (50 μl/well) in columns 1 to 10 (40 sera/plate) of U-bottom microplates (transfer plates). Positive and negative serum controls are included in column 11 of each plate. Duplicate wells in column 11 receive dilutions (50 μl/well) of known AHSV positive antiserum to give strong positive, weak positive and high negative reactions, and a 1 in 5 dilution of known negative horse serum. Each well of column 12 receives 50 μl of PBST-BM but no test serum. Fifty microlitres of the optimal dilution of AHSV specific immune guinea pig antiserum diluted in PBST-BM is added simultaneously to each well of the transfer plate.

The mixtures are transferred onto the corresponding wells of the washed ELISA plate, which is then incubated for one hour at 37°C on an orbital shaker. Plates are washed as before and 50 μl/well of previously titrated rabbit anti-guinea pig immunoglobulins conjugated to horse radish peroxidase, diluted in PBST-BM, is added and the plates are incubated at 37°C for one hour on an orbital shaker. After washing, 50 μl of chromogenic/substrate (one 30 mg orthophenylene-diamine dihydrochloride tablet dissolved in 75 ml distilled water plus 0.05% H₂O₂) is added to each well. The reaction is stopped after 10 minutes by the addition of 50 μl/well of 1.0 M H₂SO₄.

The value for no competition (maximum colour) is obtained from the mean of the OD₄₉₂ values obtained in column 12, and measures the interaction between guinea pig antiserum and AHS virus (AHS antigen control). ELISA positive reactions are recorded when test samples record greater than 50% inhibition of colour compared to the mean OD value recorded in column 12.

An ELISA test for the detection of antibodies to AHSV has also been described in ref. 9.

c) Other tests

The agar gel immunodiffusion test is a useful alternative test, particularly when dealing with anti-complementary serum samples. Concentrated cell culture material is commonly used as antigen. The specificity of precipitin lines should be established by the use of positive control sera.

The plaque reduction neutralisation test is the test of choice for the detection of type-specific AHS antibodies. Although antigen preparation is a lot more complicated, the haemagglutination inhibition test can also be employed for this purpose.
B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

There are presently no international standards for viruses, antisera and other diagnostic reagents, nor is there a standardised methodology for AHSV and antibody detection.

A live attenuated vaccine, modified by serial intracerebral passage in adult mice, has been commercially available since 1935 and is still produced in a few countries. These strains have been further passaged in MS (monkey stable) cell line cultures and used as the seed viruses for the production of a polyvalent or monovalent tissue culture vaccine. The details of the requirements for these cell culture vaccines have been published by Ozawa et al. (8). However, these neurotropic vaccines have been shown to produce encephalitis in a low percentage of vaccinated donkeys and occasionally also in horses. To eliminate post-vaccination reactions a new polyvalent live vaccine based on the selection in VERO cell cultures of avirulent, genetically-stable macroplaques has recently been developed by Erasmus at the Veterinary Research Institute, Onderstepoort, South Africa (3). Requirements for the new VERO cell culture vaccine are summarised below.

1. **Seed management**

   a) **Characteristics**

   The seed virus is prepared by the selection in VERO cells of genetically stable large plaques from low passage levels of AHSV. Such plaque mutants are then further multiplied by 3 passages in VERO cells. A large quantity of this antigen is lyophilised and stored at -20°C as seed stock antigen.

   b) **Culture**

   The seed virus is grown in roller cultures of VERO cells.

   c) **Control methods**

   The seed virus must be shown to be free of contaminating viruses, bacteria and mycoplasmas by the appropriate techniques. The serotype identity of the seed virus is confirmed by means of a plaque inhibition neutralisation test.

2. **Manufacture**

At the onset of a production run working antigens are produced from the seed stock antigens in either BHK21 or VERO cell cultures. The working antigens are tested for sterility, purity and identity and should contain at least $1 \times 10^6$ PFU/ml of infective virus.

Roller bottle cultures of VERO or BHK21 cells are grown using gamma-irradiated bovine serum in the growth medium. Once the cultures are confluent the medium is poured off and the cells are seeded with the working antigens. One hour later
maintenance medium is added to the cultures. Incubation is continued at 37°C for two to three days. Once cytopathic changes are advanced both cells and supernatant medium are harvested and harvests from the same serotype are pooled and stored at 4°C.

3. **In-process control**

The pooled harvests of the individual serotypes are tested for sterility and assayed for infectivity by plaque titration on VERO cell cultures. The minimum acceptable titre is $1 \times 10^6$ PFU/ml.

Finally two quadrivalent vaccines are constituted by mixing equal volumes of serotypes 1, 3, 4, 5 and 2, 6, 7, 8 respectively. After addition of a suitable stabiliser, the vaccine is distributed in 1.0 ml volumes into glass vials and freeze-dried (constituting single doses).

4. **Batch control**

a) **Safety tests**

Following lyophilisation 5 bottles of vaccine are selected at random and tested for sterility by internationally accepted methods. Innocuity of the vaccine is determined by the inoculation of reconstituted vaccine into mice (0.25 ml i.p.), guinea-pigs (1.0 ml i.p.) and a horse (5.0 ml s.c.). All the animals are observed daily for 14 days. The rectal temperature of the horse is taken twice daily for 14 days and should never exceed 39°C.

b) **Potency**

Potency is largely based on virus concentration in the vaccine. The minimum immunising dose for each serotype is about $1 \times 10^3$ PFU/dose. The infectivity titre of the final product is assayed by plaque titration in VERO cell cultures and should contain at least $1 \times 10^5$ PFU/dose. The horse used for the safety testing is also used for determining the immunogenicity of the vaccine. Serum samples are collected on the day of vaccination and 21 days later and tested for neutralising antibodies against each serotype by a plaque reduction test using two-fold serum dilutions and about 100 PFU of virus. The horse should develop a neutralising antibody titre of at least 20 against at least 3 of the 4 serotypes in the quadrivalent vaccine.

c) **Duration of immunity**

Duration of immunity is not assessed with every batch of vaccine but is known to persist for at least 4 years. However, in the light of possible interference between the individual serotypes in each quadrivalent vaccine, annual revaccination is advocated in enzootic regions. Vaccination with monovalent vaccine stimulates a practically life-long immunity.
d) Stability

In the lyophilised state the vaccine is known to retain its potency for many years when stored at 4-8°C. However, for practical reasons, an expiry date of 2 years is normally given.

The stability of the final product is determined by an accelerated stability test. The vaccine is incubated at 37°C for 7 days, reconstituted and titrated in VERO cell cultures. To be passed, the vaccine should have a residual infectivity titre of at least $3 \times 10^4$ PFU/dose.

REFERENCES


SUMMARY

African swine fever (ASF) is caused by a virus which belongs to an unnamed family separate from the Iridoviridae.

Laboratory diagnostic procedures for African swine fever fall into two groups; the first contains the tests for virus isolation and antigen detection, while the second contains the tests for antibody detection. The selection of the tests to be carried out depends on the disease situation in the area or country.

In countries free from ASF but suspecting its presence, the laboratory diagnosis must be directed towards isolation of the virus by simultaneously carrying out the inoculation of pigs and pig leucocyte or bone marrow cultures and detection of antigen in smears or cryostat sections of tissues by direct immunofluorescence. However, the detection of antibodies in tissue fluids by the indirect fluorescent antibody test should also be carried out simultaneously.

Identification of the agent: Isolation of virus in pigs requires the inoculation of two groups of animals: one unvaccinated group and one group vaccinated against classical swine fever (hog cholera). The pigs are monitored daily by recording rectal temperatures and collection of blood for the preparation of leucocyte cultures in which haemadsorption may be observed. When the animals develop a fever of more than 40°C, they may be sacrificed and tissues collected for examination.

Tissues submitted from suspected pigs in the field and from pigs inoculated at the laboratory should be examined for specific antigen by the direct immunofluorescence test on smears or cryostat sections and for the presence of virus by inoculation of primary pig leucocyte cultures, which are examined daily for haemadsorption and cytopathic effects. The cells from negative cultures are examined for antigen by direct immunofluorescence and sub-inoculation into fresh leucocyte cultures.

In doubtful cases the material is passaged by repeating the above procedures.

Serological tests: Where the disease is endemic or where a primary outbreak is caused by a strain of low virulence, the investigation of new outbreaks should start with the detection, using the ELISA, of specific antibodies in serum or extracts of the tissues submitted.

Requirements for biological products: At present, there is no vaccine for ASF.
A. DIAGNOSTIC TECHNIQUES

African swine fever (ASF) virus has been classified as a member of the family *Iridoviridae* for a number of years, but recent work has shown that the structure of the genome and the replication strategy of the virus have many features in common with members of the *Poxviridae* (10). In 1984, at the 6th meeting of the ICTV in Sendai (Japan), the proposal was accepted that ASF virus be placed in a family separate from the *Iridoviridae*. ASF virus is the only known member of this family.

ASF viruses produce a range of syndromes varying from peracute disease to chronic and apparently healthy virus carriers. The more virulent strains produce peracute or acute disease characterised by high fever, loss of appetite, haemorrhages in the skin and internal organs and death in 2-10 days. Mortality rates may be as high as 100%. Less virulent strains produce mild clinical signs – slight fever, reduced appetite and depression – which can be readily confused with many other conditions in pigs and may not lead to suspicion of ASF.

ASF cannot be differentiated from classical swine fever (hog cholera) by either clinical or post-mortem examinations and both diseases should be considered in the differential diagnosis of any acute febrile haemorrhagic syndrome of pigs. Laboratory tests are essential to distinguish between the two diseases.

In countries free from ASF but suspecting its presence, the laboratory diagnosis must be directed towards isolation of the virus by simultaneously carrying out the inoculation of pigs and pig leucocyte or bone marrow cultures and detection of antigen in smears or cryostat sections of tissues by direct immunofluorescence. However, the detection of antibodies in tissue fluids by the indirect fluorescent antibody test should also be carried out at the same time in order to avoid a delay in detecting the antigen due to unexpected causes.

1. Identification of the agent

Where ASF is suspected, the following samples should be sent to the laboratory: blood in anticoagulant (heparin or EDTA), spleen, tonsil, kidney, lymph nodes. These should be kept as cold as possible, without freezing, during transit.

Suspensions are prepared by grinding up the tissues in buffered salt solution or tissue culture medium containing antibiotics, clarified by centrifugation and the supernatant used for pig and tissue culture inoculation.

a) Pig inoculation

A pool is made with aliquots of each tissue suspension and 2 ml inoculated intramuscularly into each of four pigs: two of these should be vaccinated against classical swine fever and two unvaccinated. Pigs should be examined daily for increase of rectal temperatures and onset of clinical signs for up to 21 days, during which time blood samples should be collected daily for inoculation of primary pig leucocyte cultures (see (i) below) and for the "autorosette" technique (see (ii) below). When the pigs develop a temperature of 40°C or
higher or clear clinical signs due to ASF virus infection, all tissues will contain high titres of virus and both vaccinated and unvaccinated pigs should show signs of infection at approximately the same time after inoculation. At this time, or at the end of the 21-day observation period in the absence of clinical signs, the pigs should be killed and several tissues collected for virus isolation in primary pig leucocyte cultures and antigen detection by direct immunofluorescence. At the same time, the sera should be tested for antibodies. In doubtful cases a second passage in susceptible animals may be carried out. If a response is observed in the unvaccinated pigs, but not in the vaccinated pigs, the procedures for the diagnosis of classical swine fever should be carried out.

b) Haemadsorption test

The haemadsorption (HAD) test (3) can be carried out by inoculating the prepared suspensions of tissue from suspect pigs into primary pig leucocyte cultures or by preparing leucocyte cultures from the blood of pigs inoculated at the laboratory or blood collected in the field from suspect pigs.

i) HAD test in primary pig leucocyte cultures

A small number of cultures can be prepared from 10-20 ml of blood collected in heparin (100 IU per ml blood) which is centrifuged and the buffy coat cells washed in medium and resuspended in tissue culture medium containing 10-30% homologous pig serum and antibiotics at a concentration of $10^7$ cells per ml. The cell suspension is dispensed in aliquots of 1.5 ml in 160 x 16 mm tubes and incubated in a sloping position (5-10° from the horizontal) at 37°C. A larger number of cultures can be prepared from defibrinated or heparinised blood which should provide 300 tubes from each 100 ml of blood collected. The following points should be noted: all procedures must be carried out in such a way as to prevent contamination of the cultures; to prevent nonspecific haemadsorption, the medium should contain the plasma or serum from the same pig from which the leucocytes were obtained.

For routine diagnosis, only 2- to 4-day-old cultures are sufficiently sensitive, and three tubes are inoculated by adding 0.2 ml of prepared samples of tissue per tube. If the field material submitted is in poor condition, ten-fold and hundred-fold dilutions should also be inoculated into cultures. Positive and, whenever possible, negative controls must be included. Cultures are examined daily for 7-10 days for haemadsorption and cytopathic effect under a microscope; after 3 days, 0.2 ml of a fresh preparation of 1% pig erythrocytes in buffered saline are added. Haemadsorption, which consists of the attachment of large numbers of pig erythrocytes to the surface of infected cells, is diagnostic for ASF. A cytopathic effect consisting of a reduction in the number of adherent cells in the absence of haemadsorption may be due to the cytotoxicity of the inoculum, Aujeszky's disease virus or non-haemadsorbing ASF virus which can be detected by the direct immunofluorescence test on the cell
sediment (see (c) below). If no change is observed or if the results of the immunofluorescence test are negative, the supernatant should be sub-inoculated into fresh leucocyte cultures.

ii) HAD "autorosette" test with peripheral blood leucocytes from infected pigs

Blood from suspect pigs in the field or those inoculated in the laboratory is collected in heparin and leucocyte cultures prepared for direct examination for haemadsorption. This procedure is quicker than the preparation and inoculation of primary pig leucocyte cultures (i) and should give more rapid results in positive cases. It can be performed in laboratories which are not equipped for routine virological examinations; the minimum requirements are slides and cover slips, a microscope and sterile medium, tubes or bottles and pipettes. Twenty ml of whole blood is collected in a syringe containing 2,000 IU heparin in 2 ml of saline, mixed and transferred to a glass tube or bottle which is placed vertically in an incubator or water bath at 37°C to allow the cells to settle. Sedimentation is improved by the addition of 2 ml of a plasma volume expander such as "Dextravan 150" which is a solution of Dextran 150 in 0.9% NaCl for injection (Fisons UK). After 6 to 8 hours' incubation, the cultures are examined every 2 to 3 hours by transferring small aliquots of the white-cell-rich supernatant, together with some erythrocytes, onto a glass slide and identifying haemadsorbing cells under a microscope.

c) Antigen detection by direct immunofluorescence

The fluorescent antibody test (FAT) (1) can be used to detect antigen in tissues of suspect pigs in the field or those inoculated at the laboratory. In addition, it can be used to detect ASF virus antigen in leucocyte cultures in which no HAD is observed and can thus identify non-haemadsorbing strains of virus. It also distinguishes between the cytopathic effect produced by ASF virus and that produced by other viruses, such as Aujeszky's disease virus or a cytotoxic inoculum.

Cryostat sections or smears of test tissues, or spreads of cell sediment from inoculated leucocyte cultures on slides are air dried, fixed with acetone for 10 minutes at room temperature and stained with fluorescein isothiocyanate (FITC)-conjugated anti-ASF virus immunoglobulin for one hour at 37°C in a moist chamber. Control preparations are treated similarly. After washing in phosphate buffered saline (PBS), stained tissues are mounted in PBS/glycerol and examined under an ultraviolet light microscope with suitable barrier and exciter filters. Tissues are positive if specific granular cytoplasmic fluorescence is observed.

2. Serological tests

Antibodies persist in recovered pigs for long periods after infection, sometimes for life, and a number of tests are available for detecting these antibodies, although only a few of them have been developed for routine use in diagnostic laboratories
The most commonly used is the ELISA (9, 11), which is suitable for examining either serum or fluid from the tissues. Confirmatory testing of ELISA-positive samples should be carried out in critical cases using an alternative test such as the indirect fluorescent antibody test (IFA) or immunoblotting (6). Neutralising antibody to ASF virus is not demonstrable in pigs recovering from the disease.

Where ASF is endemic, confirmation of suspected cases of disease is best done by using a standard serological test (ELISA), combined with an alternative serological test (IFA) or an antigen detection test (FAT). In some countries, over 95% of positive cases have been identified by using a combination of indirect and direct immunofluorescence tests (8).

Both the CIEP and ELISA tests can be used for the large-scale screening of sera, although the ELISA test is more sensitive in detecting individual positive sera and has been used extensively as part of eradication programmes.

The method used depends on the staff and facilities available.

ELISA

The ELISA (9, 11) is a direct test which can detect antibodies to ASF virus in pigs which have been infected by the virus.

The ELISA antigen is prepared from infected cells grown in the presence of pig serum (2). Cells are harvested at 24 hours post infection, washed in PBS, sedimented at 650 g for 5 minutes, washed again in 0.34 M sucrose/5 mM tris-HCl (pH 8.0) and centrifuged. The following steps are performed on ice: after resuspension in 67 mM sucrose/5 mM tris-HCl (pH 8.0), the cells are incubated for 10 minutes, then lysed by adding nonidet P-40 detergent to a final concentration of 1% (w/v) for 10 minutes. Sucrose is added to a final concentration of 10% (w/w) and nuclei are pelleted at 1,000 g for 10 minutes. The supernatant is mixed with EDTA (2 mM final concentration), β-mercaptoethanol (50 mM final concentration) and NaCl (0.5 M final concentration). After 15 minutes' incubation at 25°C, the mixture is centrifuged at 100,000 g for 1 hour at 4°C over a 20% (w/w) sucrose layer. The fraction over the sucrose layer is used as ELISA antigen.

ELISA microtitre plates are coated with antigen by adding 100 μl of the recommended dilution of antigen in 0.05 M carbonate/bicarbonate buffer (pH 9.6) to each well. Plates are incubated at 4°C for 16 hours (overnight) and then washed 5 times in PBS (pH 7.2).

Test sera and control positive and negative sera are diluted 1:30 in 0.05% Tween 20 in PBS (pH 7.2) and 100 μl of each diluted serum added to the duplicate wells of the antigen-coated plate. Forty sera can be tested on one plate if 4 pairs of each positive and negative control serum are added to wells in different parts of the plate. Plates are incubated at 37°C for one hour on a plate shaker. After 5 washes in PBS, 100 μl of protein A-horseradish
peroxidase conjugate at the predetermined or pre-titrated dilution in 0.05% Tween 20 in PBS, are added to each well, the plates incubated at 37°C for one hour on a plate shaker and washed 5 times in PBS. Hydrogen peroxide is added to the substrate solution (0.04% orthophenylenediamine in phosphate-citrate buffer pH 5.0) at the rate of 10 μl per 25 ml and 100 μl of substrate added to each well. The reaction is stopped after incubation at room temperature for approximately 10 minutes by the addition of 100 μl of 1 M sulphuric acid to each well.

Clearly positive sera can be read by eye, but it is better to read the optical density (OD) in each well spectrophotometrically, at 492 nm in an ELISA reader. Any serum is considered positive if it has an OD value of more than twice the mean OD value of the control negative sera on that plate.

b) Indirect fluorescent antibody test

A suspension of ASF-virus-infected pig kidney or monkey cells at a concentration of 5 x 10^5 cells per ml is prepared and small drops spread on glass slides, air dried and fixed with acetone at room temperature for 10 minutes. Fixed slides can be stored at -20°C until ready for use.

To carry out the test (7), heat-inactivated (56°C for 30 minutes) test sera and positive and negative control sera at appropriate dilutions in buffered saline are added to slides of both infected and control uninfected cells and incubated for one hour at 37°C in a moist chamber. After washing in PBS and distilled water, suitable dilutions of anti-pig immunoglobulin-FITC or protein A-FITC conjugate are added to all slides, which are incubated for one hour at 37°C in a moist chamber, washed with PBS and distilled water, mounted in PBS/glycerol and examined with a UV-light microscope (as in section 1c). The control positive serum on infected cells must be positive and all other controls negative before the test can be read. Sera are positive if infected cultures show specific fluorescence.

C) Counter immunoelectrophoresis (immuno-electro-osmophoresis) test

This test (4) can be carried out rapidly and specific antibody can be detected in some sera 30 minutes after the test is set up. It requires the use of electrophoresis equipment (electrophoresis chamber, slide frames, gel cutter) and a 500-volt constant-current power supply.

The required number of 2.5 x 10 cm glass slides is placed in the slide frame on a level table and covered with the recommended volume of 0.6% agarose in veronal-acetate buffer (pH 8.6, ionic strength 0.025) containing 0.1% sodium azide and allowed to set. Four pairs of wells, 3 mm in diameter, are cut 10 mm apart in the gel on each slide as shown below.

The wells are filled with the appropriate reagents, including control positive and negative antigens and antisera, the frames placed in the electrophoresis chamber and run for 30 minutes with a constant voltage of 19 volts per cm.
After electrophoresis, slides are examined over an indirect light source for specific lines of precipitation. Slides are washed in 2% NaCl solution overnight and for 2 hours in several changes of distilled water before drying. Dried slides are stained with 0.075% amido black in equal volumes of methanol, 12% acetic acid and 1.6% sodium acetate, containing 0.007% glycerol, for 5 to 10 minutes and destained with three 10-minute washes in an aqueous solution of 45% methanol and 10% glacial acetic acid. Any line of precipitation observed on the stained slides is positive.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

At present there is no vaccine for ASF.

REFERENCES


SUMMARY

The highly variable clinical picture of hog cholera (HC) often precludes a diagnosis on clinical and pathological grounds alone. Laboratory methods are therefore essential for an unambiguous diagnosis of the disease.

Identification of the agent: The direct immunofluorescence (IF) test on cryostat sections of organs from affected pigs is used for the detection of HC antigen. The character of the fluorescence is identified as either HC or bovine virus diarrhoea (BVD) antigens by a panel of monoclonal antibodies. In negative IFT cases, the isolation of HC virus in the PK-15 cell line should be attempted. The cultures are examined for virus growth by direct immunofluorescence; positive results are further characterised by the use of monoclonal antibodies.

Serological tests: Detection of virus-specific antibodies is particularly useful in herds suspected of being infected with HC strains of low virulence. Serological methods are also valuable when there is a low incidence of disease, and are essential if a country wishes to be internationally recognised as being free from the disease.

Since antibodies against BVD virus are frequently observed in breeding pigs, only tests that differentiate between BVD and HC antibodies are relevant. For this purpose, the neutralisation peroxidase-linked assay (NPLA), the fluorescent antibody virus neutralisation (FAVN) test and the complex-trapping-blocking ELISA are very suitable. All three tests are specific and sensitive. The NPLA and ELISA can be read macroscopically and can be automated.

Requirements for biological products: Effective inactivated vaccines are not available.

Vaccines against HC are based on live virus that has been attenuated by passage through cell cultures or through a suitable host species that is not of the family Suidae.

Vaccine production is based on a seed lot system that has been validated with respect to virus identity, sterility, purity, safety, non-transmissibility, stability and immunogenicity.

A. DIAGNOSTIC TECHNIQUES

The viruses that cause hog cholera (HC), bovine virus diarrhoea (BVD) and Border disease (BD) are members of the family Togaviridae, genus Pestivirus, and
are closely related, both antigenically and structurally. Since BD strains isolated from sheep cannot be distinguished in vitro or in vivo from BVD virus strains (both have the potential to cross-infect the bovine and ovine species and to induce comparable congenital defects), the agents can be considered as being identical and will be referred to as BVD virus in the present paper.

Clinical signs and lesions seen post-mortem in pigs affected with HC are highly variable due to the occurrence of virus strains that vary in virulence. Furthermore, congenital infections with BVD virus strains in pigs give rise to clinical disease that is indistinguishable from HC (7, 8, 11).

Conspicuous signs are a rapid spread of disease in all age groups, accompanied by leucopaenia, pyrexia, huddling, conjunctivitis, anorexia, vomiting, constipation followed by diarrhoea, and a staggering gait. Several days after the onset of clinical signs, the ears, abdomen and inner thighs may show a purple discoulouration. Peracute and acute clinical cases die within 1-2 weeks. Sudden death in the absence of clinical illness is not symptomatic of HC.

Strains of HC virus of low virulence can cause subacute or chronic clinical illness which can be protracted for 2-4 weeks or even months. Chronic illness leads to a stunting of growth, whereas congenital, persistent infections may go undetected for months. Persistent infections may be confined to only a few pigs in a herd, showing typical signs, such as intermittent diarrhoea, anorexia and retarded growth. Mortality rates may be slightly above the expected level.

In peracute cases, gross pathological lesions are often inconspicuous or absent. In the acute form, the lymph nodes are marbled red, and haemorrhages occur on the serosae of the heart, kidneys as well as urinary bladder, in the skin and subcutis. In subacute and chronic cases, necrotic or "button" ulcers may be observed in the mucosae of the gastrointestinal tract, epiglottis and larynx, in addition to the above lesions.

Histopathological findings are not pathognomonic. Lesions may include parenchymatous degeneration of lymphatic tissue, cellular proliferation of vascular interstitial tissue, and a non-suppurative meningo-encephalomyelitis, with or without vascular cuffing.

The variability of the clinical signs and post-mortem lesions do not provide firm evidence for unequivocal diagnosis. Other diseases, such as African swine fever and salmonellosis, may be confused with acute HC. A tentative diagnosis based on clinical signs and post-mortem lesions must therefore be confirmed by laboratory investigations. This is all the more necessary in view of the serious consequences of an outbreak for trade in pigs and pig products.

Laboratory methods for HC are aimed at detection of the virus or viral antigens, or detection of specific antibodies.
1. Identification of the agent

a) Detection of antigen

The direct immunofluorescence (IF) test is both rapid and reliable, and can be used to detect HC antigen in cryostat sections of tonsils, spleen, kidney, or distal portions of the ileum. Tissues should be collected from several animals and transported without preservatives under cool conditions, but not frozen. Cryostat sections are stained directly with anti-HC conjugated antiserum for examination by fluorescence microscopy. Tonsillar tissue is the most suitable, as this is the first to become affected by the virus, irrespective of the route of infection (3). In subacute and chronic cases, the ileum is frequently positive and occasionally may be the only tissue to display fluorescence. A negative IF test result is not significant, and further samples should be obtained or attempts made at virus isolation in PK-15 cells.

The IF test involves the use of a fluorescein conjugate prepared from a polyclonal antibody to HC which will not distinguish between the antigens of different pestiviruses.

Strains of modified live virus vaccine multiply mainly in the regional lymph nodes and in the crypt epithelium of the tonsils. Pigs vaccinated with modified live virus strains may yield a positive IF test for 2 weeks after vaccination (2, 4). Rabbit inoculation is used to differentiate between lapinised and field strains of HC virus. In contrast to field strains, lapinised strains given intravenously cause a febrile reaction and induce an immune response in rabbits.

Pigs infected with BVD virus can give false positive IF test reactions. Congenital infections with BVD virus can cause clinical signs and pathological lesions indistinguishable from those in chronic HC (7, 8, 11). Infections by HC or BVD virus can be differentiated by testing sera from the dam and littermates, or from other contacts of an IF positive piglet, for antibodies to either virus. Another method of differentiating these viruses is by the inoculation of sero-negative piglets with a suspension of suspect material, followed 5 weeks later by neutralisation (VN) tests on their sera for the respective antibodies. However, VN tests and animal inoculation methods may take several days or even weeks. The use of a set of conjugated monoclonal antibodies of different specificities will allow an unambiguous differentiation between field and vaccine strains of HC virus on the one hand, and between HC virus and other pestiviruses on the other (10).

b) Isolation of virus

Isolation of virus in cell cultures is a more sensitive but slower method for diagnosis of HC than immunofluorescence on frozen sections. Isolation is best performed in rapidly dividing PK-15 cells seeded onto coverslips simultaneously with a 2% suspension of the tonsil. The cultures are examined for fluorescing foci by direct IF test after 24-72 hours.
The tonsil is the organ of choice for virus isolation. If this is not available, the spleen can be used.

A detailed procedure for virus isolation is as follows:

i) A 100-fold strength of a glutamine-antibiotic stock solution is prepared. Glutamine (2.92 g) is dissolved in 50 ml distilled water (solution A) and sterilised by filtration. The following antibiotics are each dissolved in 5-10 ml sterile distilled water: penicillin $10^6$ IU, streptomycin 1 g, mycostatin $5 \times 10^5$ U, polymixin B $15 \times 10^4$ U, and kanamycin 1 g. These solutions are pooled (solution B). Solutions A and B are mixed aseptically, made up to 100 ml with sterile distilled water, and stored in aliquots at -20°C. For use, thaw 1 bottle stock solution and add 1 ml to 10 ml of tissue suspension or 0.2 ml to 10 ml of cell suspension.

ii) Cut 1-2 g of tonsillar tissue into small pieces and, using a mortar and pestle, grind in a small amount of cell culture medium with sterile sand into a homogeneous paste.

iii) Make a 20% (w/v) suspension by adding Hanks’ balanced salts solution (BSS) or Hanks’ minimum essential medium (MEM); 1 ml of the glutamine-antibiotic stock is added for each 10 ml of suspension. This mixture is held at room temperature for 1 hour.

iv) Centrifuge at 3,000 rpm for 15 minutes.

v) A PK-15 monolayer is trypsinised, the cell suspension centrifuged at 800 rpm for 10 minutes, and resuspended to contain $2 \times 10^6$ cells/ml in growth medium (Earle’s MEM, 5% foetal bovine serum free from antibodies to BVD, and 0.2 ml of the glutamine-antibiotic stock solution per 10 ml cell suspension).

vi) Mix 9 parts cell suspension (v) and 1 part of supernatant fluid (iv) and inoculate 6-8 Leighton tubes with coverslips. Three tubes are inoculated with cell suspension alone as controls. (Instead of Leighton tubes, flat-bottomed microtitre plates can be used for virus isolation. Plates are fixed and stained as described for the neutralising peroxidase-linked assay.)

vii) At 1, 2 and 3 days after inoculation, 2 cultures together with a control culture, are washed (2 x 5 minutes) in Hanks’ BSS or Hanks’ MEM, fixed with cold acetone (Analar) for 10 minutes, and stained with a direct anti-HC virus conjugate at its appropriate working dilution.

viii) After washing in phosphate buffered saline (PBS) 3 times for 5 minutes each, the coverslip cultures are mounted in 90% carbonate-bicarbonate buffered glycerol, pH > 8.0, and examined for fluorescing foci.
2. **Serological tests**

Detection of virus-specific antibodies is particularly useful on premises suspected of having infections with low virulent strains. Due to the immunosuppressive effect of HC virus, antibodies cannot be detected with certainty until 4 weeks after infection. Serological investigations may also be useful in a terminal phase of HC eradication aimed at detecting residual foci of infection, especially in breeding herds.

As the incidence of BVD infection may be high in breeding stock, only tests that will discriminate between HC and BVD antibodies are useful. For this purpose, VN and the ELISA using monoclonal antibodies satisfy the requirements for specificity and sensitivity.

Neutralisation tests are performed in cell cultures, using a constant virus-varying serum method. As HC virus is non-cytopathic, any non-neutralised virus must be detected, after multiplication, by an indicator system. The fluorescent antibody virus neutralisation (FAVN) test (1) and the neutralising peroxidase-linked assay (NPLA) (5) are the most commonly used techniques. Both tests can be carried out in microtitre plates. The peroxidase system has the advantage that the results can be read with the naked eye.

### a) **To perform the FAVN test**

i) A suspension of PK-15 cells at a concentration of $2 \times 10^5$ cells/ml is seeded into 5 cm petri dishes with coverslips spread over the bottom, or in Leighton tubes with a coverslip.

ii) Incubate the cultures for 1-2 days at $37^\circ C$ in a CO$_2$ cabinet until they reach 70-80% confluency. An ordinary incubator may be used for stoppered Leighton tubes.

iii) For screening, equal volumes of a 1:5 dilution of heat-inactivated sera and a virus suspension containing 200 TCID$_{50}$/ml are incubated for 1-2 hours at $37^\circ C$.

iv) Remove coverslips from petri dishes or Leighton tubes, wash briefly in serum-free medium, overlay the cell sheet with the serum-virus mixture (iii) and incubate for 1 hour at $37^\circ C$ in a humidified atmosphere.

v) Place the coverslip in a clean Leighton tube and incubate the cultures in maintenance medium for 2 more days.

vi) Remove the coverslips from the Leighton tubes, wash the monolayers for 2 x 5 minutes in PBS pH 7.2, fix in pure acetone for 10 minutes and stain with the working dilution of the conjugate for 30 minutes at $37^\circ C$ before washing.

vii) Mount the coverslips on grease-free microscope slides with 90%
carbonate-bicarbonate buffered glycerol, pH>8.0, and examine for fluorescence.

When the FAVN test is performed in microtitre plates, the procedure for the NPLA (see below) can be followed up to point (viii). The plates are then stained with the working dilution of the conjugate for 30 minutes at 37°C and examined for fluorescence.

b) To perform the NPLA test

The NPLA test is carried out in flat-bottomed microtitre plates. Sera are first inactivated for 30 minutes at 56°C. Large numbers of sera to be screened are tested at a single dilution of 1:25. Appropriate controls to ensure specificity and sensitivity of reactions are incorporated into each test.

Occasionally, sera from pigs infected with BVD virus react in the NPLA at low dilution as if they were infected with HC virus. The extent of cross-reactivity depends on the strain of BVD virus involved, the interval between infection and time of sampling, as well as on the strain of HC virus used for neutralisation. The high antibody levels reached after exposure to HC infection, including strains of low virulence, allow the use of comparatively high initial dilutions in NPLA tests for HC antibody, thus avoiding most cross-reactions (5, 6). In case of continued doubt, comparative NPLA tests using the Oregon CV24 or 1138 strains of BVD virus have proved useful.

i) Dilutions of serum in growth medium (Eagle's MEM, 5% foetal bovine serum and antibiotics) are dispensed in 50 µl volumes into duplicate wells of a microtitre plate. The foetal bovine serum should be free from both BVD virus and antibodies to it.

ii) Add 50 µl of virus suspension to the wells, diluted in growth medium to contain approximately 100 TCID₅₀/50 µl and mix the contents on a microplate shaker for 20 seconds.

iii) Incubate the plates in a CO₂ incubator for 1 hour at 37°C.

iv) Add to all wells 50 µl of growth medium containing 2 x 10⁵ cells/ml.

v) Allow the cells to grow in 5% CO₂ to become confluent, usually within 4 days.

vi) Discard the growth medium and rinse the plates once in 0.15 M NaCl.

vii) Drain the plates by blotting on a towel.

viii) The cell monolayers may be fixed in one of two ways:

- The plates are incubated for 45 minutes at 37°C, and then for at least a further 45 minutes at -20°C. The plates are removed from the freezer,
the wells filled with 100 μl 4% paraformaldehyde in PBS and re-incubated for 5-10 minutes at room temperature. The paraformaldehyde is discarded and the plate rinsed with 0.15 M NaCl; or,

- The plates are incubated at 70-80°C for 1-2 hours.

ix) Add to each well 50 μl of a hyperimmune porcine HC antiserum, diluted in 0.5 M NaCl containing 1% Tween 80 and 0.1% sodium azide, pH 7.6. Incubate at 37°C for 15 minutes. The working dilution of the antiserum should be determined by prior titration: i.e. a serum with an NPLA titre of 1:30,000 could be used at 1:100.

x) Wash the plates 5 times in 0.15 M NaCl containing 1% Tween 80, pH 7.6.

xi) Add to each well 50 μl of an anti-porcine IgG-HRPO (horseradish peroxidase) conjugate, diluted to its working concentration in 0.5 M NaCl with 1% Tween 80, pH 7.6, and then incubate for 10 minutes at 37°C.

xii) Repeat the washing procedure (x).

xiii) Add 50 μl of chromogen-substrate solution to each well and stain for 15-30 minutes at room temperature. This solution consists of 3-amino-9-ethyl carbazole (4 mg) dissolved in N,N-dimethyl formamide (1 ml) as stock solution A; 0.05 M aqueous sodium acetate, pH 5.0 (19 ml), as solution B; and 30% hydrogen peroxide as solution C. Both A and C are kept in the dark at 4°C. Stock solution A can be kept at 4°C for at least 6 months, and solution C for 1 year. Immediately before use, add 1 ml of solution A slowly to 19 ml of solution B and mix gently. Then add 10 μl of solution C, mix well and stain the plates.

xix) The test is read visually. Infected cell sheets are completely or partially stained reddish brown. In doubtful cases, the monolayer should be examined by low-power microscopy. The cytoplasm of infected cells is stained dark red.

c) ELISA

Competitive, blocking and indirect techniques may be used on any suitable support. The tests used should minimise cross-reactions with BVD virus and other pestiviruses. However, the test system must ensure identification of all HC infections, and at all stages of the immune response to infection.

Antigen: The antigen should be derived from or correspond to viral proteins of one of the recommended HC virus strains. Cells used to prepare antigen should be free of any other pestivirus infection.

Antisera: Polyclonal antisera for competitive or blocking assays should be raised in pigs or rabbits by infection with one of the recommended HC virus strains or with the lapinised C strain. Monoclonal antibodies should be directed
against or correspond to an immunodominant viral protein of HC virus. Indirect assays should use an anti-porcine immunoglobulin reagent that detects both IgG and IgM.

The sensitivity of the ELISA should be high enough to score positive any serum that reacts in the neutralisation test. The ELISA may only be used with serum or plasma samples derived from individual pigs. If the ELISA procedure used is not HC-specific, then positive samples should be further examined by differential tests to distinguish between HC and other pestiviruses.

An example of a specific ELISA is the complex-trapping-blocking (CTB) ELISA (9), which is designed to screen a large number of pig sera for antibodies against HC virus. Its major components are two monoclonal antibodies (MAbs), each of which recognises a different epitope on the envelope protein E1 of the HC virus. The wells of an ELISA plate are coated with one MAb, which is used as capture antibody. After the wells are washed, a second MAb conjugated to horseradish peroxidase (HRPO) is added to the wells. HC virus antigen is pre-incubated with the test serum, and the serum-antigen mixture is then added to the conjugate in the wells of the coated ELISA plate. After incubation, the wells are washed again and chromogen-substrate solution is added. If both MAbs have bound to the antigen, the HRPO induces a chromogenic reaction, indicating that the test serum is negative for HC virus antibodies. If one or both of the epitopes are blocked by antibodies from the test serum, the HRPO-conjugate will be washed away and the wells will remain clear, indicating that the test serum contains antibodies against HC virus.

The CTB-ELISA is a single dilution test. The test sera are diluted 2.5 times in a dummy microtitre plate and incubated with the HC virus antigen. The test is fast and easy to perform, and detects antibodies against low virulent strains of HC virus at an early stage after infection. Since the MAbs are specific for HC virus, the CTB-ELISA will not detect antibodies against BVD virus.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Effective inactivated vaccines against HC are not available.

Live vaccines are produced from HC virus strains that have been attenuated by passage either in cell cultures or in a suitable host species not belonging to the family Suidae. Production is carried out in cell cultures, or in non-Suidae animals, based on a seed lot system. This must be validated with respect to identity, sterility, purity, safety, non-transmissibility, stability and immunogenicity.

To produce a seed lot and a final vaccine of high quality, the optimal conditions for virus yield must be determined. For vaccines produced in cell cultures, growth curve experiments must be done to study the effect of composition of the medium, regulation of pH and atmospheric CO₂ content, starting concentration of seeded cells, ratio between cell sheet surface and medium volume, phase of the cell growth
Explanation of terms used in this chapter

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>Seed lot</td>
<td>Seed virus used for pilot experiments</td>
</tr>
<tr>
<td>Vaccine</td>
<td>Registered product</td>
</tr>
<tr>
<td>&quot;Vaccine&quot;</td>
<td>Non-registered product</td>
</tr>
<tr>
<td>Vaccination</td>
<td>Application of a registered product according to instructions of manufacturer</td>
</tr>
<tr>
<td>&quot;Vaccination&quot;</td>
<td>Application of a non-registered product, whether or not according to instructions of manufacturer; or, application of a registered product NOT according to instructions</td>
</tr>
<tr>
<td>Vaccine dose</td>
<td>Amount of virus in final product and ready for use for one pig</td>
</tr>
<tr>
<td>&quot;Vaccine dose&quot;</td>
<td>Amount of test sample representing one vaccine dose.</td>
</tr>
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at the time of viral infection, stationary or rolling conditions during viral replication, etc. For vaccines produced in animals, their age, breed, weight, the size of inoculum (number of animal ID50), pathogenesis of the infection, and the clinical signs are factors to be investigated to determine the peak of virus growth and the tissues to be harvested.

Regardless of the production method, the substrate should be harvested under aseptic conditions and be subjected to a cycle of freezing and thawing to release cell-associated virus. Coarse cell or tissue elements are removed by filtration or low-speed centrifugation. A stabiliser, such as lactose at a final concentration of 5%, is added. The vaccine is homogenised before lyophilisation to ensure a uniform batch.

The vaccine virus in the final product should not differ by more than 5 passages from the material used for validating the seed lot. The commercial vaccine should be produced in batches in lyophilised form as a homogeneous product.

1. **Seed Management**

   a) Characteristics

   To validate a seed lot for a modified live virus HC vaccine, samples of the seed lot must first pass pilot experiments. Except for tests to confirm identity, sterility, purity and stability of attenuation, pilot experiments may also be performed with representative samples of the final commercial product. These samples must originate from the same seed lot as tested above.

   Except where otherwise specified, all pigs used in pilot tests are 6-8 weeks of
age, healthy, free of antibodies against HC and BVD viruses, of the same breed and origin, grouped at random if necessary and kept under the same conditions. Pregnant sows must be of equal parity.

The seed virus must be sterile and induce specific neutralising antibodies against a virulent strain of HC virus in pigs.

b) Culture

Production is performed in cell cultures or in a suitable host of species not of the family Suidae.

c) Validation as a vaccine

i) Purity

It must be virologically pure.

Each of 3 seronegative pigs is inoculated intramuscularly with an amount of virus equivalent to 10-fold the amount of virus contained in 1 dose of vaccine. This is repeated 3 weeks later using the same dose and route of administration. Serum samples are collected 2 weeks after the last inoculation, and tested by the most sensitive method for freedom from antibodies to the viruses of African swine fever, Aujeszky's disease, BVD, foot and mouth disease (all types), transmissible gastroenteritis, swine vesicular disease, and porcine influenza (types H1N1 and H3N2); and for porcine adenoviruses, porcine enteroviruses (types 1 and 2), and porcine parvovirus.

ii) Safety

For tests of safety, each of 10 seronegative pigs is inoculated intramuscularly with 10 vaccine doses. Ten other pigs serve as controls. All pigs are observed for 3 weeks thereafter. Body temperatures are recorded and blood samples are collected daily, with an anticoagulant, for the first week. Body weights are recorded at inoculation and 2 weeks later. No animal should die or show signs of illness caused by the vaccine (seed lot) virus. A daily group average body temperature should not reach 40.5°C or more throughout. The average weight gain should not fall significantly (P < 0.05) below that of the controls. Leucopaenia (WBC count < 7 x 10^6 cells/ml) may be permitted only in 1 pig for 1 day.

Each of 10 pigs is immunosuppressed by daily injections, each of 2 mg prednisolone/kg body weight for 5 consecutive days. On the third day, each animal is inoculated with 1 "vaccine" dose, and kept under observation for 3 weeks thereafter. No animal should die or become ill due to the vaccine virus.
Each of 10 sows, 25-35 days pregnant, is inoculated intramuscularly with 1 dose of "vaccine". A further 10 animals of the same parity and gestation serve as controls. The "vaccine" should not interfere with normal gestation to term, and the number of live piglets born from the test group should not be significantly less (p < 0.05) than that for the control pigs.

For field trials, a minimum of 200 pigs is used, farrowed and reared by at least 20 dams, and seronegative for HC and BVD. The litters are equally distributed over at least 2 farms. Half of the piglets in each litter are inoculated intramuscularly at 7-14 days of age with one "vaccine" dose. The uninoculated littersmates are controls. All piglets are weighed at inoculation and 2 weeks later; they are kept under observation for 3 weeks. A mortality rate that exceeds 5% due to causes other than "vaccination" invalidates the trial. No animal should die or show signs of disease due to the vaccine virus. The average weight gain of the inoculated pigs in the litters should not be more than 20% below that of the controls during the 2 weeks after inoculation.

iii) Non-transmissibility

To confirm non-transmissibility, 24 seronegative pigs are divided into 4 equal groups. Five pigs in each group are inoculated intramuscularly with 1 dose of "vaccine". The remaining pigs represent in-contact controls. All pigs are challenged 6 weeks later with at least $10^5$ PID$_{50}$ (median pig infectious dose) of a virulent strain of HC virus. All in-contact animals should be serologically negative at the time of challenge, and then die within 3 weeks. All "vaccinated" pigs should remain healthy and survive.

iv) Stability of attenuation

To confirm stability of virus attenuation, 2 pigs are each inoculated intramuscularly with 100 doses of vaccine, and then killed 6-7 days later. The tonsils of both pigs are pooled and made into a 10% suspension in PBS, pH 7.2. This is used to inoculate 2 further pigs intramuscularly with 2 ml, and these are then killed 6-7 days later. This protocol is repeated 5 times. During these passages, the tonsillar tissue may be stored at 4°C, if for less than 24 hours, or at -70°C for longer periods. At the same time, the presence of HC antigen is confirmed at each passage by direct immunofluorescence in cryostat sections of the tonsils, or by titration in a suitable substrate. If HC virus or antigen cannot be demonstrated after a certain passage, a second series of passages is performed, commencing with the last 2 pigs of the previous series to show infection.

Five pigs are inoculated intramuscularly with the sixth pig passage of the seed lot virus equivalent to 1 vaccine dose, or, if this passage has not been reached, the highest passage of the 2 series where virus or viral antigen was detected. Five further pigs are similarly inoculated with 1 dose of the seed lot virus equivalent to 1 vaccine dose. All pigs are weighed at the time of
inoculation and again 2 weeks later. Blood is collected daily into anticoagulant during the first week, and all pigs kept under observation for 3 weeks. No animal should die or become ill from the vaccine virus. The average weight gain of the 2 groups during the first 2 weeks should not differ significantly (P<0.05). Leucopenia (WBC count of <7 x 10^6/ml) is permitted, at the most, in one pig of either group for one day.

v) Immunogenicity

To demonstrate adequate immunogenicity, 10 pigs are each inoculated with 1 "vaccine" dose, and 2 others are housed separately as uninoculated controls. All pigs are challenged 7 days later with 10^5 PID_{50} of a virulent strain of HC virus. Only the controls should die.

In a test for duration of immunity, 10 pigs are each inoculated with 1 dose, and 2 others maintained separately as controls. Six months later, the sera of the inoculated pigs are tested for HC antibodies; at least 8 pigs should be positive. All pigs are then challenged with at least 10^5 PID_{50} of a virulent strain of HC virus, and observed for 3 weeks. Only the controls should die.

Under the storage conditions prescribed by the manufacturer for the final product, the "vaccine" must maintain its immunogenicity at least until the end of the expiry period.

2. Manufacture

Each batch of modified live virus HC vaccine must be derived from the same seed lot that has been used for the pilot tests. Also, each batch must be prepared according to the production protocol and under the conditions laid down for the registration of the final product. The properties of each batch and those of the seed lot must be verified as uniform.

The protocol for production will depend upon the vaccine strain, the production system (animals or cell cultures), and available facilities. The norms for cell culture vaccines may vary according to the production system, namely, primary cultures, cell lines, monolayers or suspension cultures.

3. In-process and batch control

All pigs used in batch control tests are 6-8 weeks of age and free of antibodies to HC and BVD viruses. Again they must be uniform in origin, breed, husbandry, and randomly distributed into any groups where necessary.

a) Identity

The vaccine must induce specific neutralising antibodies against a virulent strain of HC virus.
b) Sterility

Tests for sterility and freedom from contamination of biological material may be found in the Chapter on General Information.

c) Safety

Each of 10 pigs is inoculated with 10 vaccine doses. The pigs are observed for 3 weeks thereafter and body temperatures taken daily for the first week. No pig should die or show signs of disease attributable to the vaccine, and the average daily body temperature must at no time reach 40.5°C or more.

d) Purity

The batch must be virologically pure. To test for this, 3 pigs are each inoculated intramuscularly with 10 vaccine doses. Serum samples are collected at the time of inoculation and again 5 weeks later. These are tested for antibodies to BVD (neutralisation for 1 hour at 37°C) and porcine parvovirus (haemagglutination inhibition using 4 haemagglutinating units). All 3 pigs must remain free. Tests for virological purity need not be carried out when using vaccines produced in rabbits.

e) Potency

Potency is expressed as the number of 50% protective doses (PD$_{50}$) contained in one vaccine dose. One vaccine dose is at least 100 PD$_{50}$.

Ten pigs are each inoculated intramuscularly with 1:100 dilution of one vaccine dose in PBS, pH 7.2. Two uninoculated pigs are housed separately as controls. All pigs are challenged intramuscularly with 10$^5$ PID$_{50}$ of a virulent strain of HC virus 2 weeks later. They are observed for 3 weeks thereafter, during which time the controls should die. At least 5 of the inoculated pigs must survive without showing signs of disease.

This potency test may be replaced by an infectivity assay, provided that the manufacturer can show that there is a distinct and reproducible relationship between the virus content in the vaccine and the protection it will confer on pigs against challenge.

f) Stability

The period of validity of a batch of lyophilised HC vaccine should not be less than one year.
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SUMMARY

The aetiological agents of avian influenza, the virulent form of which is also known as fowl plague, are influenza type A viruses, which are members of the Influenzavirus genus of the family Orthomyxoviridae. It is the only orthomyxovirus known to affect birds. Diagnosis is preferably made by isolation and characterisation of the virus. This is because infections in birds can give rise to a wide variety of clinical signs that may vary according to the host, the strain of virus, the presence of any secondary exacerbative organisms and the environmental conditions.

Identification of the agent: Suspensions in antibiotic solution of tracheal and cloacal swabs (or faeces) taken from live birds, or from pools of faeces and organs from dead birds, are inoculated into the allantoic cavity of 9- to 11-day-old embryonated chicken eggs. The eggs are incubated at 35-37°C for 4-7 days. The allantoic fluid of any eggs containing dead embryos, as well as all eggs at the end of the incubation period, are tested for the presence of haemagglutinating activity. The presence of influenza A virus can be confirmed by an immunodiffusion test between concentrated virus and an antiserum to the nucleocapsid or matrix antigens common to all influenza A viruses.

For subtyping the virus, the laboratory must have monospecific antisera prepared against the isolated antigens of each of the 14 haemagglutinin (H1-H14) and 9 neuraminidase (N1-N9) subtypes of the virus which can be used in immunodiffusion tests. Alternatively, the newly-isolated virus may be examined by haemagglutination and neuraminidase inhibition tests against a battery of polyclonal antisera to a wide range of strains covering all the subtypes.

As the term 'fowl plague' refers to infection with virulent strains of influenza A virus, it is necessary to assess the virulence of an isolate for domestic poultry. Whereas all virulent strains isolated to date have been either of the H5 or H7 subtype, most H5 or H7 isolates have been of low virulence. The determination of strain virulence for birds is based on a recommendation of the First International Symposium on Avian Influenza, Beltsville, Maryland, USA. This involves the inoculation of a minimum of 8 susceptible 4- to 8-week-old chickens with infectious virus, strains being considered highly pathogenic if they cause more than a 75% mortality rate within 8 days.

Serological tests: Since all type A influenza viruses have antigenically similar nucleocapsid and antigenically similar matrix antigens, agar gel immunodiffusion tests are used to detect antibodies to these type antigens.
Concentrated virus preparations containing either type antigen are used in such tests. Not all birds develop demonstrable precipitating antibodies, these being notably absent in waterfowl. Haemagglutination inhibition (HI) tests have also been employed in routine diagnostic serology; but it is possible that this technique may miss some particular infections because the haemagglutinin is subtype specific.

Requirements for biological products: There are commercially available vaccines whose use has been restricted to inactivated oil emulsion vaccines to combat strains of low virulence causing problems in turkeys.

A. DIAGNOSTIC TECHNIQUES

Avian influenza (fowl plague) is caused by infection with type A viruses of the *Influenzavirus* genus of the *Orthomyxoviridae*. Influenza A viruses are the only orthomyxoviruses known to affect birds; chickens and turkeys are the main birds of economic importance to be affected, although many species of birds may carry the virus. Influenza A viruses share antigenically related nucleocapsid or matrix polypeptides which are classified into subtypes on the basis of their haemagglutinin (H) and neuraminidase (N) antigens (8). At present 14 H subtypes (H1-H14) and 9 neuraminidase subtypes (N1-N9) are recognised. To date, the highly virulent influenza A viruses that produce acute clinical disease, also known as fowl plague, have been associated only with the H5 and H7 subtypes, although the reasons for this are not clear. Many viruses of H5 and H7 subtype isolated from birds have been of low virulence (1).

Depending on the age and type of bird and on environmental factors, the highly pathogenic disease may vary from one of sudden death with little or no overt signs to a more characteristic disease with respiratory signs, excessive lacrimation, sinusitis, oedema of the head, cyanosis of the unfeathered skin and diarrhoea. However, none of these signs can be considered pathognomonic. Diagnosis of the disease, therefore, depends on the isolation of the virus and a demonstration of its virulence for an appropriate host. Serological methods may supplement diagnosis but are not yet suitable for a detailed identification.

1. Identification of the agent

Samples taken from dead birds should include oro-nasal swabs. Samples from lungs, air sacs, intestine, spleen, brain, liver and heart may also be collected and processed either separately or as a pool.

Samples from live birds should include both tracheal and cloacal swabs, although swabs of the former site are preferred. Small delicate birds may be harmed by swabbing, but the collection of fresh faeces may serve as an adequate alternative.

The samples should be placed in phosphate-buffered isotonic saline (PBS) at pH 7.0-7.4 containing antibiotics. The antibiotics can be varied according to local
Avian influenza (Fowl plague) (A15)

conditions but could contain penicillin (2,000 units/ml), streptomycin (2 mg/ml), gentamycin (50 μg/ml) and mycostatin (1,000 units/ml) for tissues and tracheal swabs, but at 5-fold higher concentrations for faeces and cloacal swabs. It is important to re-adjust the pH of the solution to pH 7.0-7.4 following the addition of the antibiotics. Faeces and finely minced tissues should be prepared as 10-20% (w/v) suspensions in the antibiotic solution. Samples should be processed as soon as possible, first leaving them for 1-2 hours at room temperature. Where this is not possible, samples may be stored for several days at 4°C.

The preferred method of growing avian influenza A viruses is by the inoculation of embryonated specific pathogen-free (SPF) chicken eggs. The supernatant fluids of faeces or tissue suspensions obtained through clarification by centrifugation at 1,000 g are inoculated into the allantoic sac of at least 5 embryonated SPF chicken eggs of 9-11 days incubation. These are incubated at 35-37°C for 4-7 days. Eggs containing dead or dying embryos, together with all eggs remaining at the end of the incubation period, should first be chilled at 4°C and the allantoic fluids tested for haemagglutination (HA) activity. Fluids that yield a negative reaction should be passaged into at least 1 further batch of eggs.

Immunodiffusion is the accepted method of detecting influenza viruses by demonstrating the presence of the nucleocapsid or matrix antigens that are common to all members of the influenza A genus. The antigens consist of concentrated virus or extracts of infected chorio-allantoic membranes; these are tested against known positive antisera. Virus may be concentrated from infective allantoic fluid by ultracentrifugation, or by precipitation under acid conditions. The latter method consists of the addition of 1.0 M HCl to infective allantoic fluid until it is approximately pH 4.0. The mixture is placed in an ice-bath for 1 hour and then clarified by centrifugation at 1,000 g at 4°C. The supernatant fluid is discarded. The virus concentrates are resuspended in glycine-sarcosyl buffer: this consists of 1% sodium lauroyl sarcosinate buffered to pH 9.0 with 0.5 M glycine. These concentrates contain both nucleocapsid and matrix polypeptides. Preparations of nucleocapsid-rich antigen can also be obtained from chorio-allantoic membranes for use in agar gel immunodiffusion tests (4).

Negative contrast electron microscopy may also be used to distinguish between ortho- and paramyxoviruses, since in preparations of the latter the distinctive herringbone nucleocapsid is invariably seen whilst it is rare to see the smaller and less distinct influenza nucleocapsid. This technique is not used routinely.

Any HA activity of sterile fluids harvested from the inoculated eggs is most likely to be due to an influenza virus or to an avian paramyxovirus. There are currently 9 recognised serotypes of avian paramyxoviruses. Most laboratories will have antiserum specific for Newcastle disease virus (avian paramyxovirus type 1), and in view of its widespread occurrence and universal use as a live vaccine in poultry, it is best to evaluate its presence by haemagglutination inhibition (HI) tests.

The method recommended for definitive subtyping of influenza A viruses by the WHO Expert Committee (1981) involves the use of highly specific antisera,
prepared in an animal giving minimum non-specific reactions (e.g. goat), directed against the H or N subtypes (7). An alternative technique is the use of polyclonal antisera raised against a battery of intact influenza virions. Subtype identification by this technique is beyond the scope of most diagnostic laboratories not specialising in influenza viruses. Assistance is available from the OIE Reference Laboratory, Central Veterinary Laboratory, Weybridge, Surrey, UK.

The term 'fowl plague' implies the involvement of virulent strains of virus. It is used to describe a disease of chickens with clinical signs such as excessive lacrimation, respiratory distress, sinusitis, oedema of the head and face, cyanosis of the unfeathered skin and diarrhoea. Sudden death may be the only sign. These signs may vary enormously depending on the host, age of bird, presence of other organisms and environmental conditions. In addition, viruses that normally cause only a mild or no clinical disease may mimic 'fowl plague' if exacerbating conditions exist.

It was therefore resolved at the First International Symposium on Avian Influenza held in 1981 (2) to abandon the term 'fowl plague' and to define any highly pathogenic strains responsible for the virulent disease as follows: 'Influenza viruses highly pathogenic for avian species be considered any influenza virus that results in not less than 75% mortality within 8 days in at least 8 susceptible chickens, 4-8 weeks old, inoculated by the intramuscular, intravenous or caudal air sac route with bacteria-free infectious allantoic or cell culture fluid. This assumes the use of standard operating procedures.'

The OIE subsequently adopted the following criteria for classifying an avian influenza virus as highly pathogenic:

i) Any influenza virus that is lethal for 6, 7 or 8 of eight 4- to 8-week-old susceptible chickens within 10 days following intravenous inoculation with 0.2 ml of a 1:10 dilution of a bacteria-free, infectious allantoic fluid.

ii) The following additional test is required if the isolate kills 1 to 5 chickens but is not of the H5 or H7 subtype: growth of the virus in cell culture with cytopathogenic effect or plaque formation in the absence of trypsin. If no growth is observed the isolate is considered not to be a highly pathogenic avian influenza (HPAI) isolate.

iii) For all H5 and H7 viruses of low pathogenicity and for other viruses, if growth is observed in cell culture without trypsin, the amino acid sequence of the connecting peptide of the haemagglutinin must be determined. If the sequence is similar to that observed for other HPAI isolates, the isolate being tested will be considered to be highly pathogenic.

It should be noted that while there have been numerous isolations of strains of H5 and H7 subtypes of low pathogenicity, all the highly pathogenic influenza strains isolated to date have possessed either the H5 or H7 haemagglutinin. Further
information concerning the pathogenicity or potential pathogenicity of H5 and H7 subtypes may be obtained by sequencing the genome, as pathogenicity is associated with basic amino acids at the cleavage site of the haemagglutinin. Amino acid sequencing of the cleavage sites of H5 and H7 subtype influenza isolates of low virulence in vivo would identify viruses which have the capacity to become highly pathogenic for poultry by simple mutation.

2. Serological tests

a) Agar gel immunodiffusion (AGID) tests

All type A influenza viruses have antigenically similar nucleocapsid and antigenically similar matrix antigens. This fact enables the presence or absence of antibodies to any influenza A virus to be detected by AGID. Concentrated virus preparations, as described above, contain both matrix and nucleocapsid antigens; the matrix antigen diffuses more rapidly than the nucleocapsid antigen. AGID tests have been widely used routinely to detect specific antibodies in chicken and turkey flocks as an indication of infection. These have generally employed nucleocapsid-enriched preparations made from the chorio-allantoic membranes of embryonated chicken eggs (4) which have been infected at 10 days old, homogenised, freeze-thawed 3 times, and centrifuged at 700-1,000 g. The supernatant fluids are inactivated by the addition of 0.1% formalin, recentrifuged and used as antigen. Not all avian species may produce precipitating antibodies following infection with influenza viruses. Antibodies have been most notably absent in waterfowl.

To conduct the test, antigen is prepared as described in ref. 4. Using a template and cutter, wells of approximately 5 mm diameter, and 5 mm apart, are cut in the agar. A pattern of wells must place each suspect serum adjacent to a known positive serum and antigen. This will make a continuous line of identity between the known positive, the suspect serum and the ribonucleoprotein antigen. Approximately 50 μl of each reagent must be added to each well.

Precipitin lines can be detected after approximately 48 hours, but this can be dependent on the concentrations of the antibody and the antigen. These lines are best observed against a dark background while being illuminated from behind. A specific, positive result is recorded when the precipitin line between the known positive control wells is continuous with the line between the antigen and the test well. Crossed lines are interpreted as the test serum lacking identity with the antibodies in the positive control well.

b) Haemagglutination and haemagglutination inhibition tests

Variations of the procedures for HA and HI tests are practised in different laboratories. The following examples apply in the use of V-bottomed microwell plastic plates in which the final volume for both types of test is 0.075 ml.
HA test: Two-fold dilutions of 0.025 ml amounts of infectious allantoic fluid are made in PBS pH 7.2. For accuracy this should be done from a close range of an initial series of dilutions. Add 0.025 ml of PBS to each well followed by 0.025 ml of 1% v/v chicken red blood cells. Mix gently and allow to settle for 45 minutes. Agglutinated red blood cells will not settle to a button. The titration should be read to the highest dilution giving complete haemagglutination; this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

HI test: Two-fold dilutions of 0.025 ml amounts of the same serum to be tested are made in PBS and 4 HAU of antigen in 0.025 ml added to each well. After 15-30 minutes, 0.025 ml of 1% v/v chicken red blood cells is added with gentle mixing and left for 45 minutes. 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 red cells 'stream' at the same rate as the control cells that do not contain virus should be considered as showing inhibition.

Some sera from avian species other than chickens may possess non-specific haemagglutinins for chicken red cells. These agglutinins can be removed by absorbing the test serum with chicken red cells or, alternatively, by using the red cells of the species under investigation.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

No vaccines specifically designed to contain or prevent highly pathogenic influenza are currently available. However, in the USA there has been widespread use of experimental inactivated vaccines produced under special licence on a commercial basis (6). These vaccines have been used against viruses that are not highly pathogenic but which may cause serious problems in turkeys, especially in exacerbating circumstances. Significant quantities of vaccine have been used (3).

The existence of a large number of virus subtypes together with the known variation of different strains within a subtype pose serious problems in selecting strains to produce influenza vaccines. In addition some isolates do not grow to a sufficiently high titre to produce adequately potent vaccines without costly prior concentration. The vaccines produced have either been autogenous, i.e. prepared from isolates specifically involved in an epizootic, or else prepared from viruses possessing the same haemagglutinin subtype that yield high concentrations of antigen. In the USA some standardisation of the latter has been carried out in that the National Veterinary Services Laboratories have propagated and hold influenza viruses of each subtype for use as seed virus in the preparation of inactivated vaccines (3). These vaccines and those for use in Italy (5) have been prepared from infective allantoic fluid inactivated by betapropiolactone and emulsified with mineral oil.
REFERENCES


Newcastle disease is caused by a virus designated paramyxovirus type 1 (PMV-1) of the genus Paramyxovirus belonging to the family Paramyxoviridae. The avian paramyxoviruses consist of 9 subtypes designated PMV 1-9 respectively.

Strains of Newcastle disease virus (NDV) vary widely in the severity of the disease they may produce in birds. The less pathogenic strains may also induce severe disease when exacerbated by the occurrence of other organisms or by adverse environmental conditions. The preferred method of diagnosis is virus isolation and subsequent characterisation.

Identification of the agent: Suspensions in an antibiotic solution prepared from tracheal and cloacal swabs (or faeces) obtained from live birds, or of pooled organs taken from dead birds, are inoculated into the allantoic cavity of 9- to 11-day-old embryonated chicken eggs. The eggs are incubated at 37°C for 4-7 days. The allantoic fluids of those containing dead embryos as they arise, together with those of all eggs at the end of the incubation period, are tested for haemagglutinating activity.

Any haemagglutinating agents should be tested for specific inhibition against an NDV monospecific antiserum. The virus shows some antigenic cross-relationship with other avian PMV subtypes.

The pathogenicity of any newly-isolated virus can be assessed by determining the mean death time (MDT) in eggs, the intracerebral pathogenicity index in day-old chicks or by the intravenous pathogenicity index in 6-week-old chickens. In some countries variations of these standard techniques are used.

Sero logical tests: The haemagglutination inhibition test is most widely used in NDV serology. Its use in diagnosis depends on the vaccinal immune status of the birds to be tested and on prevailing disease conditions.

Requirements for biological products: Live viruses of low virulence (lentogenic) or of moderate virulence (mesogenic) are used for the vaccination of poultry depending on the disease situation. Inactivated vaccines are also used.

Live vaccines may be administered to poultry by various routes. They are usually produced by harvesting the infective allantoic/amniotic fluid from infected embryonated chicken eggs; some are prepared from infected cell cultures. The final product should derive from the expansion of master and working seeds.
Inactivated vaccines are usually given intramuscularly or subcutaneously. They are usually produced by the addition of formaldehyde to infectious virus, or by treatment with betapropiolactone. Most inactivated vaccines are prepared for use by emulsification with a mineral or vegetable oil.

International standards for live vaccines, inactivated vaccines and antisera are available.

A. DIAGNOSTIC TECHNIQUES

Newcastle disease (ND) is caused by a virus, Newcastle disease virus (NDV), which is a member of the genus Parainfluenza virus of the family Paramyxoviridae. The paramyxoviruses isolated from avian species have been classified by serological testing into 9 distinct serotypes, designated PMV-1 to PMV-9; NDV has been designated PMV-1 (1).

Since its recognition in 1926, ND has been regarded as being endemic in many countries throughout the world. Prophylactic vaccination is practised in all but a few of the countries producing poultry on a commercial scale.

One of the most characteristic properties of different strains of NDV has been their great variation in pathogenicity for chickens. Strains of NDV have been placed into 5 groups or pathotypes on the basis of the clinical signs seen in infected chickens (7). These are:

- Viscerotrophic velogenic – a highly pathogenic form in which haemorrhagic intestinal lesions are frequently seen
- Neurotrophic velogenic – with a high mortality usually following respiratory and nervous signs
- Mesogenic – with respiratory signs, occasional nervous signs, but with a low mortality
- Lentogenic or respiratory – with mild or subclinical respiratory infection
- Asymptomatic enteric – consisting usually of a subclinical enteric infection.

Pathotype groupings are rarely clear-cut (2), and even in infections of specific pathogen free (SPF) birds considerable overlapping may be seen. In addition, exacerbation of the clinical signs induced by the milder strains may occur in superimposed infections by other organisms or when adverse environmental conditions are present.

Since signs of clinical disease in chickens vary widely and diagnosis may be complicated further by the different responses to infection by different hosts, clinical signs alone may not present a reliable basis for diagnosis of ND. However, the characteristic signs and lesions associated with the virulent pathotypes will give
rise to strong suspicions of the disease.

1. **Identification of the agent**

Samples from dead birds should consist of oro-nasal swabs, as well as samples collected from lung, air sac, intestine, spleen, brain, liver and heart tissues. These may be collected separately or as a pool.

Samples from live birds should include both tracheal and cloacal swabs. Where opportunities of obtaining samples may be limited it is important that cloacal swabs (or faeces) and tracheal swabs (or tracheal tissue) are examined as well as organs or tissues that are grossly affected or associated with clinical disease.

The samples should be placed in phosphate buffered isotonic saline (PBS) at pH 7.0-7.4 containing antibiotics. The antibiotics can be varied according to local conditions but could contain penicillin (2,000 units/ml), streptomycin (2 mg/ml), gentamycin (50 μg/ml) and mycostatin (1,000 units/ml) for tissues and tracheal swabs, but at 5-fold higher concentrations for faeces and cloacal swabs. It is important to readjust the pH of the solution to pH 7.0-7.4 following the addition of the antibiotics. Faeces and finely minced tissues should be prepared as 10-20% (w/v) suspensions in the antibiotic solution. Samples should be processed as soon as possible, first leaving them for 1-2 hours at room temperature. Where this is not possible, samples may be stored for several days at 4°C.

The supernatant fluids of faeces or tissue suspensions obtained through clarification by centrifugation at 1,000 g are inoculated into the allantoic sac of at least 5 embryonated SPF chicken eggs of 9-11 days incubation. These are incubated at 35-37°C for 4-7 days. Eggs containing dead or dying embryos, together with all eggs remaining at the end of the incubation period, should first be chilled at 4°C and the allantoic fluids tested for haemagglutination (HA) activity. Fluids that yield a negative reaction should be passaged into at least one further batch of eggs.

Haemagglutination activity detected in bacteriologically sterile fluids harvested from inoculated eggs may be due to the presence of any of the 13 subtypes of influenza or of the 8 other paramyxovirus serotypes. NDV can be confirmed by the use of specific antiserum. Usually chicken antiserum is used, prepared against one of the strains of NDV.

a) **Haemagglutination test**

Two-fold dilutions of 0.025 ml amounts of infective allantoic fluid are made in PBS pH 7.2. For accuracy this should be done from a close range of an initial series of dilutions. Add 0.025 ml of PBS to each well followed by 0.025 ml of 1% v/v chicken red blood cells. Mix gently and allow to settle for 45 minutes. Agglutinated red blood cells will not settle to a button. The titration should be read to the highest dilution giving complete haemagglutination; this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.
b) **Haemagglutination inhibition test**

Two-fold dilutions of 0.025 ml amounts of the same serum to be tested are made in PBS and 4 HAU of antigen in 0.025 ml added to each well. After 15-30 minutes, 0.025 ml of 1% v/v chicken red blood cells is added with gentle mixing and left for 45 minutes. The haemagglutination inhibition (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 red cells 'stream' at the same rate as the control cells that do not contain virus should be considered as showing HI.

Cross-reactions in HI tests between NDV and PMV-3 may cause some problems (1) which may be resolved by the use of suitable antigen and antiserum controls.

Some sera from avian species other than chickens may possess non-specific haemagglutinins for chicken red cells. These agglutinins can be removed by absorbing the test serum with chicken red cells or, alternatively, by using the red cells of the species under investigation.

**Pathogenicity indices**

The extreme variation in virulence of different NDV isolates and the widespread use of live vaccines means that the identification of an isolate of NDV from birds showing clinical signs does not confirm a diagnosis of ND, so that an assessment of the virulence of the isolate is also required. No *in vitro* marker of pathogenicity has so far been determined for NDV isolates, so that an assessment of pathogenicity is usually based on one or more of the following tests:

a) **Mean death time in eggs (MDT)**

Dilute fresh infective allantoic fluid in sterile saline to give a 10-fold dilution series between $10^{-6}$-$10^{-9}$. For each dilution inoculate 0.1 ml into five 9- to 10-day-old embryonated SPF chicken eggs and place at 37°C. Retain the remaining virus dilutions at 4°C and inoculate another 5 eggs with 0.1 ml of each dilution 8 hours later and place at 37°C. Each egg is examined twice daily for 7 days and the times of any embryo deaths recorded. The minimum lethal dose is the highest virus dilution which causes all the embryos inoculated with that dilution to die. The MDT is the mean time in hours for the minimum lethal dose to kill embryos.

The MDT has been used to classify NDV strains into velogenic, taking less than 60 hours to kill; mesogenic, taking between 60-90 hours; and lentogenic, taking more than 90 hours.

b) **Intracerebral pathogenicity index (ICPI)**

Fresh infective allantoic fluid with an HA titre of greater than $2^4$ is diluted 1:10 in sterile isotonic saline, with no additives, of which 0.05 ml is injected
intracerebrally into each of 10 one-day-old chicks hatched from an SPF flock. The birds are examined every 24 hours for 8 days. At each observation the birds are scored; normal birds are assigned a value of 0, sick birds 1, and dead birds, 2. The ICPI is the mean score per bird per observation over the 8-day period. The most virulent viruses will give indices which approach the maximum score of 2.0, whereas lentogenic strains will give values close to 0.0.

c) **Intravenous pathogenicity index (IVPI)**

Fresh infective allantoic fluid with an HA titre greater than $2^4$ is diluted 1:10 in sterile isotonic saline and 0.1 ml of the diluted virus is injected intravenously into 10 six-week-old SPF chickens.

Birds are examined daily for 10 days and scored at each observation: normal birds are given 0, sick birds 1, paralysed birds 2, and any dead birds, 3. The IVPI is the mean score per bird per observation over the 10 day period. Lentogenic strains and some mesogenic strains will have IVPI values of 0, whereas the indices for virulent strains will approach 3.0.

Some minor variations have been recommended in these tests. Swabbing of the cloaca and conjunctiva of 8-week-old chickens with undiluted allantoic fluid has been substituted for the IVPI test (11). The intention is to distinguish between viscerotropic velogenic and other velogenic viruses.

d) **Interpretation of pathogenicity indices**

Interpretation of the pathogenicity indices obtained with a view to imposing trade or movement restrictions, or other policies, is not necessarily straightforward. Since viruses capable of producing quite severe disease may have IVPI values of 0, the ICPI test is most frequently used for such assessments. However, as different strains can show a complete range of values from 0.00 to 2.00 in this test, it is clear that any value used for definition must be decided arbitrarily. In countries in which the only live vaccines used are lentogenic, an ICPI value of 0.7 or above is usually given as the value for viruses requiring government action.

2. **Serological tests**

ND virus may be employed in a wide variety of serological tests as an antigen, enabling neutralisation or enzyme-linked immunosorbent assays (ELISA) to be used for diagnosis. At present the HI test is most widely used. Chicken sera rarely give non-specific positive reactions in this test and any pre-treatment of sera is unnecessary. Sera from species other than chickens may sometimes cause agglutination of chicken red cells, so this property should first be determined and then removed by absorption of the serum with chicken red cells.

An international ND reference antiserum is available from the WHO/FAO International Laboratory for Biological Standards, Central Veterinary Laboratory,
New Haw, Weybridge, Surrey, UK. This is used in standardising laboratory reference antisera (6).

The value of serology in diagnosis is clearly related to the immune status of the affected birds. HI titres may be regarded positive if there is inhibition at serum dilution of 1:8 or more against an antigen dose of 4 HAU.

HI titres may be used to assess the immune status of a flock. In vaccinated flocks which are being monitored serologically, it may be possible to identify anamnestic responses as the result of a challenge infection with field virus (6), but great care should be exercised as variations may occur from other causes. For example, it has been demonstrated that PMV-3 virus infections of NDV-vaccinated turkeys will result in substantially increased titres to NDV (3).

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

A detailed account of all aspects of NDV vaccines, including their production and usage, has been published (6), and reference should be made thereto for details of the procedures outlined here.

NDV strains used in commercial live virus vaccines fall into 2 groups: lentogenic vaccines such as Hitchner-B₁, La Sota and F, and mesogenic vaccines such as Roakin, Mukteswar and Komarov. Strains from both these groups have been subjected to selection and cloning to fulfil different criteria in their production and application.

Most live virus vaccines are grown in the allantoic cavity of embryonated chicken eggs but some, notably some mesogenic strains, have been adapted to a variety of tissue culture systems.

Live virus vaccines may be administered to birds by incorporation in the drinking water, delivered as a coarse spray or by intranasal or conjunctival instillation. Some mesogenic strains are grown by wing-web intradermal inoculation. Vaccines have been constructed to give optimum results through application by specific routes. In general the more immunogenic live vaccines are more virulent, and are therefore more likely to cause adverse side effects. For example, vaccination with the La Sota strain will cause considerably greater problems in young susceptible birds than the Hitchner-B₁ strain, although La Sota induces a stronger immune response.

Inactivated vaccines are considerably more expensive than live vaccines, since their use entails handling and injecting individual birds. They are prepared from allantoic fluid that has had its infectivity inactivated by the addition of formaldehyde or beta-propiolactone. This is incorporated into an emulsion with mineral oil, and is administered intramuscularly or subcutaneously. Individual birds thus receive a standard dose. There is no subsequent spread of virus or adverse respiratory reaction. Both virulent and avirulent strains are used as seed virus although, from the aspect of safety control, the use of the latter appears more suitable. As no virus multiplication takes place after administration, a much larger amount of antigen is
required for immunisation than for live virus vaccination. A high yield of virus to produce a potent vaccine is important, and the Ulster 2C strain is very suitable for this.

1. **Seed management**

a) **Characteristics**

The first principle to consider in selecting a strain for a live NDV vaccine is whether it is to be used as a primary or secondary vaccine, the main consideration being its pathogenicity. The method of application and frequency of use are valid considerations. The use of monoclonal antibodies has demonstrated considerable variation in the antigenicity of different strains (4, 14). This may indicate a need to tailor vaccines more carefully to relate to any prevalent field virus.

The most important consideration in selecting a seed for the preparation of inactivated vaccine is the amount of antigen produced when grown in embryonated eggs; it is rarely cost effective to concentrate virus. Both virulent and lentogenic strains have been used as inactivated vaccines but the former offer an unnecessary risk, since the manipulation of large quantities of virulent virus is involved, as well as the dangers of inadequate inactivation and possible subsequent contamination. Some lentogenic strains grow to very high titres in eggs. Exceptionally high titres can be obtained by the Ulster 2C strain which has been recommended as a seed for inactivated vaccine (10). However, successful commercial inactivated vaccines are produced when the Hitchner-B₁, La Sota or F strains are used as seeds.

b) **Culture**

A master seed is established, and from this a working seed. If the strain has been cloned by limiting dilution or plaque selection the establishment of a master culture may only involve producing a large volume of infective allantoic fluid (minimum 100 ml) which can be stored as lyophilised aliquots (0.5 ml).

c) **Validation as a vaccine**

Seed viruses of unknown pedigree should be passaged through SPF eggs and cloned before producing the master seed. Some passage through SPF chickens may also be desirable (6). In either case the master seed should be checked for sterility, safety, potency and extraneous agents after preparation.

2. **Manufacture**

For vaccine production a working seed is first established. Batches of vaccine are produced from this by expansion of an aliquot of master seed to a sufficient volume to allow vaccine production for 12-18 months. It is best to store the working seed in liquid form at -60°C since lyophilised virus does not always multiply to high titre on subsequent first passage (6).
The majority of ND vaccines are produced in embryonated chicken eggs, and live virus vaccines should be produced in SPF eggs. The method of production is basically that of propagating the virus aseptically; all procedures are performed under sterile conditions.

It is usual to dilute the working seed in sterile PBS, pH 7.2, so that about $10^3$-$10^4$ EID$_{50}$/0.1 ml are inoculated into the allantoic cavity of 9- or 10-day-old embryonated SPF chicken eggs. These are then incubated at 37°C. Eggs containing embryos dying within 24 hours should be discarded. The incubation time will depend on the virus strain being used and will be pre-determined to ensure maximum yield with the minimum number of embryo deaths.

The infected eggs should be chilled at 4°C before being harvested. The tops of the eggs are removed and the allantoic fluids aspirated after depression of the embryo. The inclusion of any yolk material and albumin should be avoided. All fluids should be stored immediately at 4°C and tested for bacterial contamination before large pools are made for lyophilisation or inactivation. Live vaccines are usually lyophilised. The methodology depends on the machinery used and the expertise of the manufacturers, but this is a very important step as inadequate lyophilisation results in both loss of titre and a reduced shelf-life.

In the manufacture of inactivated vaccines the harvested allantoic fluid is treated with either formaldehyde (a typical final concentration is 1:1,000) or beta-propiolactone (a typical final concentration is 1:2,000-1:4,000). The time required must be sufficient to ensure freedom from live virus. Most inactivated vaccines are not concentrated; the inactivated allantoic fluid is usually emulsified with mineral or vegetable oil. The exact formulations are generally commercial secrets.

### 3. In-process control

Each batch of live virus vaccine should be tested for viability and potency. For those produced in eggs the most important process control is testing for bacterial and fungal contamination. This is necessary because of the occasional occurrence of putrefying eggs which may remain undetected at the time of harvest.

For inactivated vaccines the efficacy of the process of inactivation should be tested in embryonated eggs, taking 25 0.2 ml aliquots from each batch and passaging each 3 times through SPF embryos.

### 4. Batch control

Most countries have published specifications for the control of production and testing of NDV vaccines (e.g. ref. 13) which include the definition of the necessary testing of vaccines during and after manufacture.

It is necessary to test the infectivity of live virus vaccines to enable adequate levels of virus to be administered. This is usually titrated in embryonated chicken eggs to...
give the EID$_{50}$ (egg-infectious dose). This involves making 10-fold dilutions of virus; 0.1 ml of each dilution is inoculated into between 5 or 7 9- to 10-day-old embryonated chicken eggs. After 5-7 days incubation at 37°C the eggs are chilled and tested for the presence of haemagglutinin activity which is an indication of the presence of live virus. The EID$_{50}$ end point is calculated using a standard formula such as Spearman-Karber (5).

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

b) Safety

The use of chickens for the testing of vaccines involves the inoculation of 10 or more birds of stated age that originate from an SPF flock. Ten doses of live vaccine are administered supraconjunctivally to each bird and the birds are then observed for 21 days. No chicken should show serious clinical signs and none should die from causes attributable to the vaccine (9).

For inactivated vaccines a double dose is administered by the recommended route to 10 3-week-old birds, and these are observed for 2 weeks for absence of clinical signs of disease.

c) Potency

Various methods for the testing of NDV vaccines for potency have been put forward. The importance of using a suitable challenge strain for assessment has been stressed (6). A suitable strain is the Herts 33 strain. The method recommended (9) involves the vaccination of 20 birds at the minimum recommended age by the suggested route using the minimum recommended dose. After 14-21 days each vaccinated bird and 10 control birds are challenged intramuscularly with $10^5$ LD$_{50}$ of NDV challenge virus. The vaccine passes the test if at the end of 10 days 90% of the vaccinated chickens survive with no signs of disease but all controls die within 6 days.

It is not necessary to repeat the potency test on each batch if it has been shown that a representative batch of the final product from the master seed has passed the test.

d) Stability

When stored under the recommended conditions the final vaccine product should maintain its potency for at least one year. Accelerated stability tests such as reduction of infectivity following incubation at 37°C for 7 days (14) may be used as a guide to the storage capabilities of a batch of live virus vaccine. Live virus vaccines must be used immediately after reconstitution. Inactivated vaccines must not be frozen.
5. Vaccination programmes

The duration of immunity depends on the vaccination programme chosen. One of the most important considerations affecting vaccination programmes is the level of maternal immunity in young chicks which may vary considerably from farm to farm, batch to batch and among individual chicks. For this reason one of several strategies is employed. Either the birds are not vaccinated until 2-4 weeks of age when most of them will be susceptible, or birds are vaccinated at 1-day-old by conjunctival instillation or by the application of a coarse spray. This will establish active infection in some birds that will persist until maternal immunity has waned. Revaccination is then carried out 3-4 weeks later. It has been demonstrated that inactivated vaccines may also be usefully employed to vaccinate chicks which have a degree of maternal immunity at 1-day-old (8). Vaccination of fully susceptible birds at 1-day-old even with the most mild of live vaccines may result in respiratory disease, especially if common pathogenic bacteria are present in any number.

Vaccination after 3 weeks of age is normally only practised in breeding hens and should be done at sufficiently frequent intervals to maintain an adequate immunity. Vaccination programmes often employ slightly more pathogenic live virus vaccines to boost immunity than those used initially or when oil emulsion inactivated vaccines have been used.

When devising a vaccination programme, consideration should be given to the type of vaccine used, the immune and disease status of the birds to be vaccinated and the level of protection required in relation to any possibility of infection with field virus under local conditions (6). Two examples of vaccination programmes which may be used in different disease circumstances are listed here. For the first example, when the disease is mild and sporadic it is suggested that the following order of vaccination be adopted: live Hitchner-B₁ by conjunctival or spray administration at 1-day-old; live Hitchner-B₁ or La Sota at 18-21 days of age in the drinking water; live La Sota in the drinking water at 10 weeks of age, and an inactivated oil emulsion vaccine at point of lay. For the second example, when the disease is severe and more widespread, the same protocol as above is adopted up to 21 days of age followed by revaccination at 35-42 days with live La Sota in the drinking water or as an aerosol; this revaccination is repeated at 10 weeks of age with an inactivated vaccine (or a mesogenic live vaccine) and again repeated at point of lay (6).

6. International standard preparations

Several international standard preparations to assist in the manufacture of ND vaccines are available from the WHO/FAO International Laboratory for Biological Standards, Ministry of Agriculture Fisheries and Food, Central Veterinary Laboratory, New Haw, Weybridge, Surrey KT15 3NB, UK (15).

An international reference Newcastle disease antiserum is available as a lyophilised chicken antiserum. It is intended to be used for the standardisation of laboratory reference sera for use as a serum control in HI tests.
An international standard Newcastle disease live virus vaccine consists of lyophilised infective allantoic fluid from embryonated eggs infected with Hitchner-B₁. Each ampoule contains 109.5 mg dry weight. It is intended for use as a control preparation in titrating a national or laboratory standard.

An international standard Newcastle disease inactivated vaccine consists of lyophilised allantoic fluid from eggs infected with NDV which has been inactivated with formaldehyde and adsorbed onto aluminium hydroxide. Each vial contains 525 mg and an International Unit is defined as the amount of activity produced by 1 mg of the international standard. It is intended for the calibration of the potency of laboratory or national standards.

REFERENCES


Summary

Bacillus anthracis can readily be isolated from tissues, blood, exudates or clarified serum samples.

Samples of blood can be collected by aspiration from the jugular vein of cadavers by syringes. All samples should be as fresh as possible and the animal should not have been treated previously with antibiotics. Putrefaction will diminish the chances of isolating vegetative anthrax bacilli. Investigation of wool, hides, bone-meal or other material suspected of containing anthrax spores requires preparation of these materials before culture or before animal inoculation. Spores are never found in the animal body during life. Care must be taken to differentiate B. anthracis from other biologically related aerobic Gram-positive sporing bacilli.

Identification of the agent: B. anthracis is a Gram-positive non-acid fast rod, 3-8 μm by 1-2 μm, straight or slightly curved with truncated ends. In films made from body exudates or blood the organism stains blue with a distinctive pink-purple capsule with McFadyean's methylene blue. The capsule can be stained specifically with Wright-Giemsa, toluidine blue or Old's method using saffranin solution. The spores are ellipsoidal in shape and non-motile.

Growth on agar is arranged characteristically in long segmented chains of bacilli. Growth on tryptose agar enriched with 5% bovine blood at 37°C occurs in 8-12 hours in relatively pure culture. After incubation for 24-36 hours, the colonies appear rough and granular, with wisps of "medusa hair" extensions, possessing a butyrous or ground glass appearance. B. anthracis is largely non-haemolytic on blood agar, although a very weak incomplete haemolysis may develop after a few days. This distinguishes B. anthracis from the strongly haemolytic B. cereus.

A rapid presumptive diagnosis on field specimens (blood or spleen) can be made by inoculating a Mueller-Hinton agar plate with suspect material, placing a 10-unit penicillin-sensitivity disc onto it and then placing a glass coverslip adjacent to the disc. Within 3 hours chains of B. anthracis can be observed under the coverslip at a magnification of x 1,000, their typical form making them appear like strings of pearls.

The identity of B. anthracis is confirmed by its penicillin sensitivity and susceptibility to lysis by specific B. anthracis gamma bacteriophage. Pathogenicity tests can be performed in guinea pigs, rabbits or mice by inoculating material subcutaneously or intraperitoneally.

Specific staining with fluorescein-labelled antibody can be employed. A
Anthrax (Bl)

thermoprecipitation reaction (Ascoli test) is used in some parts of the world for anthrax diagnosis on decomposed cadavers. Indirect immunoradiometric assays may also be used for the detection of spores. Other tests that are available include indirect haemagglutination, agglutination and ELISA.

Serological tests: Antibody detection tests are not often used for diagnostic purposes. Indirect haemagglutination test and ELISA are occasionally employed for detection of specific antibodies.

Requirements for biological products: Anthrax spore vaccines are used to immunise horses, cattle, sheep and goats. Immunity develops within 10-14 days, but takes longer in horses. Annual revaccination is recommended, or every 6 months in more heavily infected areas.

A. DIAGNOSTIC TECHNIQUES

The organism Bacillus anthracis can be recovered and identified from tissues, blood, or exudates of animals that have succumbed to the disease. Its identification can be delayed by the normal bacterial flora found in the body orifices, or in soil, which may inhibit its development or overgrow it in culture.

All samples should be as fresh as possible. Exposure to the effects of putrefactive bacteria in an unopened carcass at temperatures greater than 25-30°C for as little as 48 hours will render the recovery of vegetative anthrax bacilli difficult. This is because the organism can neither sporulate nor continue to survive in the absence of oxygen or in the presence of an increased CO₂ concentration (18).

Due to failure of the blood to clot, the safest way to avoid contamination is to aspirate a small quantity of blood with a syringe from a peripheral blood vessel, such as the caudal or jugular veins, which should then be emptied into a small test tube and sealed with a rubber stopper soaked in a disinfectant. It is safer practice to make air dried blood films immediately, and take a swab of blood or exudate for subsequent culture.

Another method (7) of sample collection is by the use of sterile tape and tubes. Three 5 x 1 inch strips of sterile tape are put in a tube. A sample of blood is collected from a suspected case by syringe, and the tape inoculated with 3 to 5 drops. A short length of uninoculated tape is extended beyond the stopper before closing the tube. On receipt of the specimen, the stopper is removed for 2 to 3 hours to allow the blood to dry. Unlike other specimens, the inoculated tape does not require refrigeration during transit. Any prior antibiotic therapy given to the animal concerned will interfere with diagnosis.

The opening of carcasses for post-mortem examinations should not be carried out on suspected anthrax cases until the examination of blood smears has proved them negative, because of the risk of spore dissemination. Pigs and horses may not have demonstrable anthrax bacilli in the blood (5), and pigs often show less acute clinical signs before death than cattle. In these species, the bacilli may be demonstrated in
oedema fluid from the abdominal cavity (21), or in smears from the submandibular lymph nodes. In the rare cases where these examinations are negative and anthrax is still suspected, samples of spleen should be taken as described for smears and culture.

Formalinised and freshly refrigerated tissues, such as spleen, should be sent for identification safely and securely packed. The samples should include portions of all internal organs, and fluids (urine, ocular, cerebrospinal fluid, other body fluids, blood).

1. **Identification of the agent**

The newly isolated anthrax bacillus is rarely difficult to identify, but several of its important characteristics, such as capsule formation, the inverted fir-tree growth in slab gelatin, and its pathogenicity for laboratory animals may be lost on prolonged subculture.

a) **Morphology**

*B. anthracis* forms relatively large rods that may be slightly curved, tending to be rectangular, with truncated ends. On agar the organism grows characteristically in long segmented chains. Unjointed filaments are not infrequent in culture, whereas *in vivo* it occurs mostly in pairs or chains of 3 or 4 organisms. It is non-motile. The capsule which surrounds the entire bacillary chain contains D-glutamic acid and develops *in vivo* and when grown on serum media, and on bicarbonate media in the presence of excess CO₂. This capacity for capsule formation is lost when grown on agar. Plasmid PX02 is involved in the formation of capsules and is associated with the production of a toxin (9). Spores are not formed *in vivo*; they are equatorial, ellipsoidal, and of the same size as the bacillus. After the spore is fully formed the residual protoplasm of the bacillus disintegrates. Germination occurs by absorption of the spore coat.

b) **Staining**

*B. anthracis* is strongly Gram-positive and non-acid fast. McFadyean's methylene blue, Wright-Giemsa and toluidine blue stains are commonly employed to demonstrate anthrax bacilli in blood films. Such films are made on slides, dried by passing rapidly through a Bunsen flame, and then stained with McFadyean's polychrome methylene blue for a few seconds, washed and dried. An amorphous purplish material can be seen between the bacteria, representing the disintegrated capsules: this is characteristic of the anthrax bacillus.

To stain spores, a drop of Giemsa in 1 ml of water is used as the staining solution for 10 minutes; the bacilli stain a dark brown. Old's stain consists of a 3% saffranin solution which is briefly brought to the boil on the slide: the bacillus stains a reddish brown and the capsule yellow. Toluidine blue staining reveals a central or paracentral spore in organisms which have been incubated for at least 24 hours.
B. anthracis is an aerobic facultative anaerobe. Its optimum growth temperature is 35-37°C within the limits of 12-44°C. Some strains haemolyse sheep red cells. Growth is good on ordinary media, but this is not improved by including serum, and only slightly so by glucose. A reddish pigment is formed when grown on bicarbonate media (23). On agar, irregular colonies 2-3 mm in diameter appear after 8-12 hours but later assume a more typical appearance, with a tessellated or reticular structure. On examination by low-power microscopy, they have a wavy margin, often likened to locks of hair, presenting a "medusa head" appearance. The colony is one continuous convoluted chain of bacilli, has a membranous consistency and is difficult to emulsify. On bicarbonate media in the presence of excess CO₂, virulent strains form smooth mucoid colonies. On an agar slope, growth is thick, spreading and greyish yellow, with an uneven surface. It has the appearance of innumerable tiny air bubbles beneath the surface. After about a week irregular round scales appear on the surface of the growth.

In a gelatin slab culture, growth occurs along the slab and is characterised by outgrowths of delicate lateral extensions giving an inverted fir-tree effect. Liquefaction occurs very slowly, starting at the top of the growth. In broth culture, there may be a very fine floccular turbidity with a moderate floccular deposit, consisting of interwoven threads which may partly disintegrate on shaking. There is usually no surface growth although pellicle formation can occur.

On blood serum medium, there is an abundant, creamy-yellow confluent growth with an uneven surface. Coagulated serum is partially liquefied. On sheep blood agar the anthrax bacillus is non-haemolytic or slightly haemolytic as compared with the saprophytic members of the genus, which are themselves markedly lytic.

During growth, B. anthracis forms acid but no gas in glucose, maltose, or sucrose. It produces ammonia but not hydrogen sulphide; methylene blue is partially reduced. It is catalase positive. Litmus milk is coagulated and decolorised, and later, peptonised. It gives a weak lecithinase reaction (23).

When B. anthracis is grown on bicarbonate media in an atmosphere with a high partial pressure of CO₂, the smooth mucoid encapsulated form is produced and this can be demonstrated by the appropriate stains. Capsules are formed only in infected tissues. They are subject to variation and when the capsule is absent or imperfectly developed the colonies tend to be moist and slimy and may be devoid of the characteristic wreathed margins. This is well seen in cultures which have been attenuated in virulence by growth at temperatures above the optimum, for example at 42-43°C. The typical colony, as described above, is the "rough" form; the variant is small, "smooth" and without the characteristic wreathed appearance. The bacilli in this type of colony are arranged in bundles, not in a convoluted chain. Virulence is associated with the "rough" form, the "smooth" variant being relatively avirulent.

The vegetative form is as susceptible as other non-spore-forming bacteria. The
thermal death-point occurs on heating at about 60°C for 30 minutes. Spores are very resistant to chemical and physical changes but are killed in 10 minutes by boiling.

Media containing haematin and lysozyme, inhibiting many aerobic spore-bearing contaminants, can be used to isolate *B. anthracis* from contaminated soil. Another selective medium incorporates propamidine and polymixin B as inhibitors of saprophytic bacteria (11). PLET medium contains polymixin, lysozyme, disodium ethylenediamine tetra-acetate, and thallous acetate (5, 12).

Susceptibility to lysis by specific gamma bacteriophage or to penicillin helps to confirm that an isolate is a strain of *B. anthracis*. However, some strains resist infection by bacteriophages. Both these characteristics can be observed on a blood agar plate or a slide. The suspect colony is picked off and one half of the blood agar plate is streaked. A slide is then prepared for Gram staining with what remains on the loop. To the other half of the plate, a known culture of *B. anthracis* f.ex., a reconstituted Sterne vaccine strain, is added to control bacteriophage lysis and penicillin sensitivity. A drop of the appropriate dilution of bacteriophage and a 10-unit penicillin disc are then placed on each side of the plate. Results can usually be interpreted after 5-6 hours incubation at 37°C. After 12 hours there is a clear zone where the bacteriophage was inoculated, and a wide zone of inhibition surrounds the penicillin disc (5).

Media impregnated with 0.5 units of penicillin per ml can induce a typical "string of pearls" appearance to *B. anthracis* (15). The typical long chains are converted to rounded, bead-shaped structures resembling a string of pearls. A modification of the "string of pearls" test (13) is that instead of incorporating penicillin into the medium, a 10-unit penicillin sensitivity disc is placed onto an inoculated Mueller-Hinton agar plate. A glass coverslip is placed adjacent to the disc. Within 3 hours chains of *B. anthracis* occur beneath the coverslip. As the penicillin diffuses into the agar, the optimal concentration of antibiotic moves further from the disc so that the exact location of the string of pearls continues to grow outwards for several hours. This test is useful in making presumptive diagnoses of anthrax from field specimens (i.e. blood or spleen samples) in a matter of hours. Instead of Mueller-Hinton agar, tryptose agar without blood can be used (5).

Caution must be exercised when doing laboratory and animal pathogenicity studies (5).

For direct isolation by animal inoculation, 50 ml or more of heat-treated fluid are centrifuged at 1,500 r.p.m. for 15 minutes. The supernatant fluid is discarded and the residue inoculated intramuscularly into a guinea pig which has been passively immunised 24 hours previously with a mixture containing *Clostridium perfringens* antitoxin 1,000 and *Cl. septicum* antitoxin 500 units; *Cl. novyi* antitoxin 100 and tetanus antitoxin 500 units; or a polyvalent gas-gangrene serum with added tetanus antitoxin. Death due to anthrax occurs in 2-3 days. Death from gas-gangrene, usually due to *Cl. septicum* or *Cl. sordelli*, occurs
earlier. In the latter cases aerobic cultures from the local lesion and spleen should be made as they may yield *B. anthracis*. The minimum infecting dose is 30-50 spores (6).

For putrid specimens, a scratch technique has been described (18) in which the tail or skin of a mouse is scratched with a needle dipped in the suspect material. Clostridial antitoxins may be administered to passively immunise the animals against those contaminants (5).

Pathogenicity tests are usually conducted in guinea pigs or mice by injecting 0.25 ml of a broth suspension either subcutaneously or intraperitoneally, or 0.5-1.0 ml aliquots of heated material subcutaneously, intramuscularly or intraperitoneally. Death in laboratory animals usually ensues within 24-48 hours in mice and as late as 72 hours in guinea pigs. Animals succumb to respiratory failure, and show evidence of general toxicity and multiple organ haemorrhages. Animals inoculated subcutaneously have subcutaneous gelatinous exudates and haemorrhagic oedema at the point of injection. Impression smears of the enlarged spleen or unclotted blood show an enormous number of Gram-positive encapsulated rods. The organism is easily recovered from infected tissues and blood. Deaths that occur before 24 hours, or later than 4 to 5 days, are considered non-specific. All animals that die should be autopsied. There is a relationship between dose, number of organisms and susceptibility to toxin challenge (Table I). The criteria for identification should consist of typical colony morphology, absence of haemolysis, penicillin sensitivity, and susceptibility to specific bacteriophages.

The detection of the organism in contaminated specimens presents difficulties. Anthrax bacilli are rapidly destroyed in unopened carcasses at temperatures of 25-30°C, and by 72-80 hours after death few, if any, bacilli are viable. By 48 hours after death, at 25-30°C they may be isolated only with difficulty. At 5-10°C, *B. anthracis* has been isolated for as long as 4 weeks after death. Higher temperatures promote proliferation of putrefactive bacteria, mainly anaerobes, that rapidly remove oxygen needed by aerobic *B. anthracis*. Specimens from animals that have been dead for several hours and animal by-products, such as wool, hides, bones and bone-meal, are usually heavily contaminated with other spore formers and rapidly growing contaminants, such as *Pseudomonas* and *Proteus*, that may be antagonistic or overgrow *B. anthracis*.

To isolate the bacterium from hair or wool (21), soak hair or wool in distilled water or weak potassium hydroxide solution for 4 hours, or prepare a 10-20% suspension in distilled water or saline and filter through gauze or allow to settle at room temperature. Or, treat a saline suspension with 1 to 2% phenol for 1 hour. The supernatant fluids are heated at 60°C for 20-30 minutes to destroy vegetative cells and activate spores. Following heat treatments, some workers recommend centrifugation at 1,000-2,000 g for 20-30 minutes and examination of the sediment obtained by both cultural and animal inoculation tests. For direct culture, samples of the heated material are streaked on nutrient agar plates enriched with 5% blood (sheep, rabbit, or bovine) and these are observed for typical colonies (5). With heavily contaminated materials, a
Table I

Relation between infective dose, number of organisms per ml of blood at death, and susceptibility to toxin challenge*

<table>
<thead>
<tr>
<th>Species</th>
<th>Relative resistance to parenteral challenge of spores</th>
<th>Parenteral spore dose to establish anthrax (spores/ml)</th>
<th>Toxin dose i/v to cause death (units/kg)</th>
<th>Quantitation of blood at death (bacilli per ml)</th>
<th>toxin units per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>V. susceptible</td>
<td>5</td>
<td>1,000</td>
<td>10^6.9</td>
<td></td>
</tr>
<tr>
<td>Guinea pig</td>
<td>Susceptible</td>
<td>50</td>
<td>1,125</td>
<td>10^8.3</td>
<td>50</td>
</tr>
<tr>
<td>Rhesus monkey</td>
<td>Susceptible</td>
<td>3,000</td>
<td>2,500</td>
<td>10^6.8</td>
<td>35</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>Susceptible</td>
<td>4,000</td>
<td>10^8.9</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Resistant</td>
<td>10^6</td>
<td>15</td>
<td>10^4-10^6</td>
<td>15</td>
</tr>
<tr>
<td>Dog</td>
<td>V. resistant</td>
<td>10^7.3</td>
<td>60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


portion is shaken with water and allowed to stand for 3-4 hours with occasional shaking. The material is teased out and the supernatant fluid heated to 70°C for 10 minutes. Different volumes (0.2 ml - 2.0 ml) of this fluid are added to melted agar, plates poured and incubated at 37°C for 12-15 hours. It is essential to examine the plates early for deep colonies which have a typical filamentous appearance sometimes likened to knotted string. A rich culture medium is essential and plates should not be too crowded with colonies. Confirmation is obtained by inoculating them subcutaneously into a mouse or guinea pig (1).

Many ubiquitous saprophytic species of aerobic spore-forming bacilli are hard to classify or to distinguish from *B. anthracis*, except on the basis of pathogenicity. Those most commonly encountered are *B. cereus, B. subtilis, and B. licheniformis*. The DNA base composition indicates that *B. cereus* is most closely related to *B. anthracis*. Bacilli more or less closely resembling the anthrax bacillus have been isolated from soil, water, meat-, fish-, and bone-meal, wool, dust, oil-cake, and less often from animals and man. These organisms have been termed "*B. pseudoanthracis*" or "*B. anthracoides*". Other *Bacillus* species have occasionally been responsible for disseminated infections.
in immunologically compromised hosts. B. cereus infection in man causes two forms of food poisoning. In cattle it gives rise to mastitis which may occasionally be fatal.

Criteria to differentiate B. anthracis from pseudoanthrax bacilli are given in Table II. An inverted fir-tree growth in gelatin occurs with some strains of pseudoanthrax bacilli, but the branches are thick and interlaced, quite different from the regular, delicate lateral outgrowths of B. anthracis. The most important distinguishing character of anthrax bacillus is its lack of motility and its peculiar pathogenicity; no other member of the Bacillus group will kill a guinea pig within 48 hours when injected subcutaneously as 0.5 ml of a 24 hour broth culture.

c) Morphology and staining

Certain bacteria tend to resemble the anthrax bacillus. Others, such as B. cereus, B. mucoides and B. megaterium are shorter with rounded ends, and several motile species with peritrichous flagellae occur, such as B. subtilis. The spore is central or eccentric as in B. subtilis, or subterminal or terminal, as in B. mucoides. "B. mesentericus" is relatively small, not exceeding 0.8 μm, and B. megaterium is large, up to 1.8 μm (6).

The optimum temperature for the growth of anthracoid organisms is usually low, about 20°C, although certain types grow best between 30-37°C. Some are thermophilic, with an optimum temperature of 55°C. They are characteristically aerobic, but usually also facultative anaerobes. Abundant growth occurs on all the ordinary culture media. B. subtilis produces a white, glistening, adherent, somewhat membranous growth which tends to spread. Similar growths occur with other species. Certain types produce colonies that are practically identical to B. anthracis with the same "medusa head" appearance. The colonies of B. mucoides are initially similar to those of the anthrax bacillus, but are easily differentiated by their feathery appearance due to long projecting and branching threads radiating out from the central growth. The growths may be dry or moist, and white, greyish-white, yellowish or brown. Certain species produce a black pigment. On potato, characteristic cultural appearances may be noted, for example B. mesentericus develops a thick wrinkled or folded layer of growth which assumes a brownish colour. Pellicle formation in broth is a frequent character. B. cereus is commonly found in heat-treated biological materials and it may be identified by culture on egg-yolk agar (5).

d) Ascoli test

A thermoprecipitin reaction, the Ascoli test, has been used to detect anthrax bacilli in infected hides, organs and tissues from suspected carcasses. It can be applied to putrefied material. Anthrax immune serum contains antibodies capable of forming a precipitate with an antigenic extract of B. anthracis. Capsular antigens are glutamic acid peptides, while those in the cytoplasm are polysaccharide compounds. The former is not specific for B. anthracis as it also occurs in members of the subtilis-mesentericus group. The polysaccharide
### Table II

**Differentiation between *B. anthracis* and other sporulating aerobes**

<table>
<thead>
<tr>
<th>B. anthracis</th>
<th>Anthracoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-motile</td>
<td>Generally motile</td>
</tr>
<tr>
<td>Capsulated</td>
<td>Non-capsulated</td>
</tr>
<tr>
<td>Produces long chains</td>
<td>Produce short chains</td>
</tr>
<tr>
<td>Forms colonies on blood agar with many on 0.7% bicarbonated media (CO₂)</td>
<td>Few or none (<em>B. cereus</em>)</td>
</tr>
<tr>
<td>Reddish, raised, mucoid</td>
<td>Flat, dull</td>
</tr>
<tr>
<td>No turbidity or pellicle in broth</td>
<td>Often turbidity &amp; pellicle in broth</td>
</tr>
<tr>
<td>No growth on penicillin agar (10 μg/ml)</td>
<td>Usually good growth on penicillin agar</td>
</tr>
<tr>
<td>Inverted fir-tree growth in gelatin</td>
<td>Fir-tree growth absent or atypical</td>
</tr>
<tr>
<td>Methylene blue reduced weakly</td>
<td>Methylene blue usually reduced strongly</td>
</tr>
<tr>
<td>Haemolysis of sheep RBCs weak or absent</td>
<td>Often strongly β-haemolytic</td>
</tr>
<tr>
<td>Liquefaction of gelatin absent or slow</td>
<td>Liquefaction of gelatin rapid</td>
</tr>
<tr>
<td>Ferments salicin slowly</td>
<td>Often ferment salicin rapidly</td>
</tr>
<tr>
<td>Lecithinase reaction weakly positive</td>
<td>Lecithinase reaction strongly positive with <em>B. cereus</em> and <em>B. mucoides</em> (not <em>B. subtilis</em> group)</td>
</tr>
<tr>
<td>Polysaccharide precipitin reaction strongly positive</td>
<td>Polysaccharide precipitin reaction weakly positive</td>
</tr>
<tr>
<td>Produces toxin neutralised by <em>B. anthracis</em> antitoxin</td>
<td>Any toxic substances not neutralised by <em>B. anthracis</em> antitoxin</td>
</tr>
<tr>
<td>Pathogenicity for animals (mouse, guinea pig)</td>
<td>Mostly non-pathogenic: if pathogenic, produce disease unlike anthrax</td>
</tr>
<tr>
<td>Culture filtrates non-toxic to tissue culture cells</td>
<td>Culture filtrates (<em>B. cereus</em>) toxic to tissue culture cells</td>
</tr>
<tr>
<td>Fluorescent antibody to <em>B. anthracis</em> positive</td>
<td><em>(B. anthracis)</em> antibody negative</td>
</tr>
<tr>
<td>Lysis by gamma phage</td>
<td>Not susceptible to gamma phage</td>
</tr>
</tbody>
</table>

(Modified from Wilson G. et al. [23])
antigen is specific however. It is present in organs of infected animals following any lysis of the bacilli.

The test is specific, with the limitation that occasionally it gives positive reactions when large numbers of bacilli of the subtilis-mesentericus group are present. The test is therefore unsuitable for use on foodstuffs. Otherwise positive results are significant, but negative results are not since the amount of anthrax antigen present may be too low, particularly in cases of localised infections.

The antigen is prepared by preliminary culture of *B. anthracis* for 24 hours at 37°C on brain-heart infusion agar. These are washed and again re-cultured for 24 hours at 37°C on the same agar. The bacteria are re-washed, centrifuged and the deposit resuspended in 0.5% carbol saline. This is extracted by heating at 100°C for 30 minutes and re-centrifuged. The supernatant fluid is the antigen.

A high-titred antiserum to *B. anthracis* with a high IgM content is required. Immune sera vary in their precipitin content, and a test serum with known precipitating properties must be selected. Towards the end of immunisation of an animal, most suitably a horse, relatively large doses of antigen must be administered intravenously. The animal is bled within a week of the final inoculation. Rabbits may also be used. Suitable antisera will yield reactions when diluted 1:50.

About 2 mg of tissue are minced, diluted with 5-10 ml of carbol saline containing acetic acid 1:1,000 and boiled for 5 minutes. The fluid is cooled and filtered. If identifying a suspect culture, it is washed off the agar into 10 ml saline, heated at 100°C for 30 minutes and filtered. 0.5 ml of antiserum is put in a small tube and the filtrate carefully superimposed. The development of a ring of turbid precipitate at the interface within 15 minutes indicates a positive reaction (5). Alternatively, a chloroform extract of suspect tissue can be used. The controls consist of parallel tests on normal serum and an extract from a known normal tissue. Reports of non-specific reactions have caused the Ascoli test to be discounted (21) although it is still used in many countries.

e) Agglutination test

This uses a corpuscular antigen from unencapsulated, vegetative bacteria (2). As suspensions of *B. anthracis* tend to agglutinate spontaneously, the bacilli must be disaggregated by ultrasonic treatment. The antigen consists of the supernatant fluid to which is added 0.1% thiomersal and the density adjusted to 80% for macroscopic tests, or to 60% for microtitre systems (2).

f) Agar gel immunodiffusion test

This is used for the detection and quantitation of *B. anthracis* antibodies in sera of previously exposed or vaccinated animals and man (3).
g) **Immunofluorescence**

A fluorescent antibody technique may be employed on smears obtained from lesions of animals after initiation of antibiotic therapy, or for the examination of isolates grown for capsule production in the appropriate medium. Direct and indirect versions of an immunofluorescence assay have been described in which bacterial spores are dried and heat-fixed onto the walls of plastic coated multispot microscope slides (15). Indirect tests are more valuable.

h) **Immunoradiometric assay**

Radioactive labelled antibodies have been introduced for the rapid detection of *B. anthracis*. Indirect immunoradiometric assays (IRMA) have the advantage that they are also suitable for measuring antibacterial antibody in clinical samples. Due to the costs of labelling antibodies the use of such methods has not yet been widely adopted.

2. **Serological tests**

Antibody detection tests are not often used for diagnostic purposes.

a) **Indirect haemagglutination test**

A 4-fold rise in titre in paired sera obtained at least 2 weeks apart indicates infection or vaccination (23). A single titre of 1:8 or higher may represent past infection or immunity as a result of vaccination.

b) **Enzyme-linked immunosorbent assay (ELISA)**

This can be used to detect antibodies in man with acute anthrax or in convalescents. Vaccinated individuals give high extinction values. The use of this test for evaluating vaccination responses in animals is under investigation (10).

B. **REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

Anthrax vaccines are prepared as spore vaccines and are used to protect animals such as horses, cattle, pigs, sheep and goats.

For the preparation of suitable vaccines it is necessary to take account of the properties of the organism and of its virulence.

1. **Seed management**

a) **Characteristics**

The formation of R and S forms is determined mainly by culture conditions, either on a medium containing bicarbonate under an atmosphere of 5-45%
Anthrax (B1)

CO₂, or on an ordinary medium in air. Three strain types can be distinguished on the following criteria:

**Type I (S) (virulent):**
- capsule formation in CO₂ + bicarbonate agar: positive
- capsule formation in air + nutrient agar: negative
- colony formation in CO₂ + bicarbonate agar: mucoid
- colony formation in air + nutrient agar: rough

**Type II (avirulent):**
- capsule formation in CO₂ + bicarbonate agar: positive
- capsule formation in air + nutrient agar: positive
- colony formation in CO₂ + bicarbonate agar: mucoid
- colony formation in air + nutrient agar: mucoid

**Type III (R) (avirulent):**
- capsule formation in CO₂ + bicarbonate agar: negative
- capsule formation in air + nutrient agar: negative
- colony formation in CO₂ + bicarbonate agar: rough
- colony formation in air + nutrient agar: rough

Thus only strains which form a capsule on bicarbonate agar in the presence of CO₂ can be virulent. Intermediate avirulent forms (type II) also occur. Every population of anthrax bacilli contains R forms, since these can arise spontaneously from S forms. *In vivo* there is selection for S forms having a capsule which provides protection against the defense mechanisms of the host, and R forms are obtainable from an infected animal only just before death. For isolation and identification of R types the nutrient agar should contain 0.5% sodium bicarbonate, and long-term culture should be set up under air containing 10-75% CO₂ at 37°C (20).

The pathogenicity of *B. anthracis* results from two closely linked factors: ability to multiply within the body, and ability to form toxin. Multiplication is promoted by the CO₂ and O₂ present in the blood and tissues, while capsule formation hinders phagocytosis and the effect of the humoral immune response. The toxin contains 3 components and has various physio-pathological effects on the infected animal, together with a strong proteolytic action *in vivo* and *in vitro* (17, 14).

The virulence of a population of *B. anthracis* is correlated to the ratio of S to R forms and the degree of toxin formation. Ability to form spores is not inevitably linked to virulence, although strains which have lost the ability to form spores are also avirulent (4).

**b) Culture**

The optimum temperature for growth of *B. anthracis* is between 30-37°C. The lower limit is 15°C and the upper 45°C. The pH of the medium should lie between 7.5-7.8.
In liquid media *B. anthracis* forms a flocular deposit without forming a mould-like crust. After 24 hours the uppermost two-thirds of the fluid is usually still clear. When a high density of vegetative forms is required, it is necessary to culture for 12 hours with continuous shaking.

On account of the risk of contamination during lyophilisation, and for the sake of simplicity in manipulation, the following procedure is used for spore-forming strains: 1% extra agar is incorporated into the trypticase agar and the mixture is poured into screw-topped tubes as agar slopes. The surface of the medium is thoroughly coated with inoculum and incubated for 48 hours. For the next 8-14 days the screw-cap is loosened to provide sufficient air for good spore formation. Then the cap is screwed tight to prevent drying out, and the culture is kept at 4-10°C. Every other year the culture should be tested for viability and drying out.

c) Validation as a vaccine

Strains are maintained by the usual microbiological techniques. It is necessary to subculture frequently to prevent their dissociation into R forms; alternatively a permanent culture can be established immediately.

There are 3 antigenic components to *B. anthracis* infection, namely the capsule, the vegetative cell and the spore. Components of the toxin also possess serological activity. There is a wide variety of structural relationships within the genus *Bacillus* between these immunologically active structures. The immunologically active component of the capsule is a polypeptide, and that of the cell wall a polysaccharide. In purified intact spores, the exosporium is the antigenically active portion.

Following infection with *B. anthracis*, the animal responds by forming specific antibodies. The extent of the immune response depends on the mode of infection. Pulmonary and intestinal infections involve the mediastinal or intestinal lymph nodes, and the immune response is stronger than after cutaneous or peracute forms of infection.

In general there is little information on immunological events in a naturally acquired infection. Animals that survive are usually immune for a long time. It is usually impossible to detect humoral antibodies to capsule, cell wall or toxin, so that a negative result in serological tests is not necessarily significant (16).

The storage life of a freeze-dried vaccine is at least 2 years when stored at 4°C.

2. Manufacture

A considerable advance in the preparation of spore vaccines was the production of variants, practically avirulent *in vivo* and *in vitro*, by incubating *B. anthracis* at 42°C in different concentrations of CO₂ (18). Such variants produce good immunity in sheep and guinea pigs. Immunity is enhanced by incorporating adjuvants like glycerol, aluminium hydroxide or saponin (18). For
vaccine preparation Sterne's attenuated strains "B" or "34F2" are used. They are stored lyophilised and propagated on solid or fluid nutrient media. A suspension of these bacteria is inoculated onto production cultures which are incubated for 5-8 days at 37°C. Providing the proportions of sporulated cells is at least 70%, the spore culture is washed off in normal saline and the vegetative cells are killed by heating on a water bath at 65°C. The density of spores in the vaccine is standardised.

A fermenter is used for preparing vaccines in fluid medium, with an incubation time of 30-48 hours.

3. **In-process control**

For the supply of live spore vaccines against anthrax, outside the provisions of the European Pharmacopoeia, the minimum requirements laid down by the WHO Expert Committee for Biological Standardisation are adhered to. The vaccine must be produced by the seeding system and the exact spore content declared.

4. **Batch control**

   a) **Sterility**

   Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

   b) **Safety**

   The usual tests for safety of any vaccine product are carried out.

   c) **Potency**

   Anthrax vaccines are tested for efficacy in guinea pigs, rabbits or sheep, according to the lethality of the production strain for each species. For example, the minimum dose is injected subcutaneously into 10 sheep. During the following 21 days, no more than 2 sheep may die from causes unrelated to the vaccine. Then a strain of *B. anthracis* pathogenic for the particular species used is injected subcutaneously, using a dose of 100 MLD50 for each vaccinated animal and 10 MLD50 for each of 3 left unvaccinated as controls. During the following 18 days all vaccinated animals must survive and all the controls must die from anthrax. If a vaccinated animal dies, the test must be repeated, and if deaths recur the vaccine is rejected.

   In the United States, anthrax spore vaccine is deemed potent if it protects 27 out of 30 guinea pigs from a challenge that kills at least 10 out of 12 controls. Each vaccine dose must contain at least twice the spore count of that used in the immunogenicity test but not less than $2 \times 10^6$ spores. No untoward reactions should be observed in 2 lambs each inoculated with 2 vaccine doses.
REFERENCES


SUMMARY

Aujeszky's disease, also known as pseudorabies, is caused by a virus that infects the central nervous system and other organs such as the respiratory tract in virtually all mammals except man and the tailless apes. It is associated primarily with swine, which remain latently infected following clinical recovery. The disease is caused by an alpha-herpesvirus and is controlled by containment of infected herds. The disease is also controlled by the use of vaccines and removal of latently infected animals.

A diagnosis of Aujeszky's disease is established by isolating the virus post-mortem as well as by detecting a serological response in the live animal.

Identification of the agent: Isolation of Aujeszky's disease virus can be made by inoculating a tissue homogenate, for example of brain and tonsil or material collected from the nose/throat, into a sensitive cell line such as porcine kidney (PK15) or SK6 or primary or secondary kidney cells. The specificity of the cytopathic effect is verified by immunofluorescence, immunoperoxidase or neutralization with specific antiserum.

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

Until recently, it was not possible to distinguish between antibodies resulting from natural infection and those from vaccination. Such a distinction can now be made for animals vaccinated with gene-deleted live vaccines.

Requirements for biological products: The spread of Aujeszky's disease may be diminished by the use of vaccines containing either modified live or inactivated virus antigens. More recently, these conventional vaccines have been supplemented by r-DNA derived gene-deleted or naturally deleted live pseudorabies virus (PRV) vaccines. The virus used in these new vaccines, sometimes referred to as marker vaccines, lacks a specific glycoprotein (GX, GI, or GIII).

A. DIAGNOSTIC TECHNIQUES

Aujeszky's disease, also known as pseudorabies, is caused by an alpha-herpesvirus, a member of the family Herpesviridae. It principally affects pigs, also herbivores and carnivores, but can infect all mammalian species other than man and most species of primates.
Whereas isolation of the virus will assist in a provisional diagnosis in the case of lethal forms of Aujeszky's disease or clinical disease in pigs, other techniques and serological tests are required for diagnosis of latent infections. Many affected animals, however, except pigs, do not live long enough to produce any marked serological response.

1. Identification of the agent

The diagnosis of Aujeszky's disease can be confirmed by isolating the virus from oro-pharyngeal fluid of living pigs or post-mortem following fatalities in unweaned piglets, and in the face of such clinical signs as abortion, or encephalitis in herbivores or carnivores. For the examination, preferably samples of brain, tonsil and lung tissue should be obtained. In cattle, infection is characterised by a pruritus, in which case a sample of the corresponding section of the spinal cord may be required in order to isolate the virus. In latently infected pigs, the trigeminal ganglion is the most consistent site for virus isolation.

The samples are homogenised in normal saline and the resulting suspension clarified by low-speed centrifugation at 900 g for 10 minutes. The supernatant fluid is used to inoculate any sensitive cell culture system. Numerous types of cell line or primary cell cultures are sensitive to Aujeszky's disease virus, but a porcine kidney cell line (PK15) is generally employed. The overlay medium for the cultures should contain antibiotics (penicillin 200 IU/ml, streptomycin 100 μg/ml, polymixin 100 μg/ml, fungizone 3 μg/ml).

The virus induces a cytopathic effect (CPE) which usually appears within 24-72 hours. The monolayer develops accumulations of birefringent cells, which is then followed by complete detachment of the cell sheet. Syncytia also develop, the appearance and size of which are variable. The virus is identified by immunofluorescence, immunoperoxidase or neutralisation using specific antiserum. In the absence of any obvious CPE it is advisable to make one blind passage into further cultures, which might make virus detection possible. Confirmation may be obtained by staining infected coverslip cultures with haematoxylin and eosin to demonstrate the characteristic herpesviral acidophilic intranuclear inclusions with margination of the chromatin. Laboratories that are not equipped for cell culture still resort to the intramuscular inoculation of suspect material into rabbits. Samples containing the virus induce the pathognomonic signs of pruritus at the inoculation site which is followed by death after 2-5 days.

The isolation of the virus makes it possible to confirm Aujeszky's disease, but failure to isolate it does not necessarily signify that the animal was free from infection.

2. Serological tests

Whichever serological technique is used should give the expected positive result with the International Reference serum. This serum (obtainable from the OIE Reference Laboratory for Aujeszky’s Disease, Ecole Nationale Vétérinaire, 94704 Maisons-Alfort, France) is diluted with 1.4 ml distilled water before use. For
international trade purposes, the recommended sensitivity of the test used should at least equal that of the reference serum diluted 1:2 (i.e. diluted with 2.8 ml distilled water).

The standard serological method has been virus neutralisation (VN), because of its specificity (3, 12). This has been replaced by the enzyme-linked immunosorbent assay (ELISA) because of its sensitivity and suitability for large-scale testing (1, 6, 7, 9).

A latex agglutination test has recently been developed.

a) Virus neutralisation

Virus neutralisation in cell culture can be performed in various ways which vary according to the length of incubation of the virus-serum mixtures (1 hour at 37°C or 24 hours at 4°C), and the presence or absence of complement. Most laboratories use a reaction period of 1 hour at 37°C in the absence of complement, because this is easy and rapid. However, an incubation period of 24 hours at 4°C facilitates the detection of antibody levels 10-15 times lower than those demonstrable after incubation for only 1 hour.

The VN technique may be conducted in 96-well microtitre plates using an incubation period of 24 hours at 4°C for virus-serum interaction. Other methods can be used, such as tests done in tubes.

Test sera can be inactivated by heating at 56°C for 30 minutes, but this is not essential as unheated sera will yield somewhat higher titres. Cell cultures sensitive to Aujeszky's disease virus are used, such as PK15 or SK6 or primary or secondary kidney cells. The culture medium depends on the cell line; for PK15 cells this is Eagle's minimum essential medium (MEM) plus Earle's salt (2 g sodium bicarbonate/litre), glutamine (2 mM) and non-essential amino acids. Various antibiotics are included (penicillin 100 IU/ml, streptomycin 100 µg/ml, if necessary kanamycin 150 µg/ml and amphotericin B 2.5 µg/ml). Serum (5-10%) from new-born calves is added to the maintenance medium, and is also included for the performance of the VN tests. This serum should be tested for its suitability prior to use. A strain of Aujeszky's disease virus is maintained at -70°C until required.

The VN tests may be qualitative or quantitative. For the qualitative test, 50 µl of undiluted test serum are added to each well; however, false positive reactions may occur. For the quantitative test, 2-fold dilutions are made in culture medium (without calf serum) directly in the plate, using automatic diluting equipment or 50 µl diluters. Each serum sample is tested in duplicate. For each sample there should be at least 1 well containing serum together with 50 µl of diluent instead of viral suspension to ensure that the serum has no toxic effect on the cells.

To prepare a stock of virus, the cell monolayer sheet of a 75 ml flask is drained of medium, and inoculated with 7 ml of viral suspension with a titre of
10^7 TCID_{50} doses/ml. This is incubated at 37°C for 1 hour whilst agitating the flask. The cells are overlaid with 30 ml of medium without serum and re-incubated. At the onset of CPE, which will be within about 18-20 hours, the cultures are subjected to a cycle of freezing and thawing. The resulting suspension is centrifuged at 1,500 g for 10 minutes and the supernatant fluid divided into aliquots after the addition of 1% lactose solution. These aliquots are stored at -70°C in vials until required.

To calculate the titre of the stock virus, a sample vial is thawed rapidly at 37°C, and titrated by making a series of 10-fold dilutions, using at least 5 wells for each dilution (optimum 8 wells). The results are expressed as the percentage of wells in which the cells show a CPE at each dilution. The virus titre is calculated by Spearman-Karber or Reed and Muench methods.

For VN tests, the virus is first diluted with culture medium (with or without 1% serum from new-born calves) to provide 2 x 10^3 TCID_{50} (median tissue culture infective doses) of virus per ml (or 100 TCID_{50} doses/50 μl). Aliquots of 50 μl of viral suspension are added to the test serum in each well, except those containing control serum. The plates are incubated in a moist chamber at 37°C for 1 hour or else at 4°C overnight. Aliquots of 150 μl of cell suspension containing about 150,000 cells/ml are added to each well. The plates are sealed with sterile film, incubated at 37°C for at least 72 hours, and the cell sheets examined.

Positive and negative sera are included as controls in each test. The titre of the virus used in each test is also controlled by back-titration. Ten-fold dilutions of the viral suspension are added in aliquots of 50 μl to at least 5 wells for each virus dilution (10^1, 10^2, 10^3 TCID_{50}), and the titre calculated from the results. Results of VN tests are read after two and three days of incubation at 37°C starting with the positive and negative controls, and the virus titration. The results of VN tests are valid only when the titre of virus used is 10^{1.5}-10^{2.5} TCID_{50} doses/50 μl and the control sera give their expected titres to the nearest 2-fold dilution. Any evidence of cytotoxicity or contamination of the sera under test renders the result of VN tests invalid.

For the qualitative test, a serum is judged positive when there is no trace of CPE in the respective wells. If the CPE is indefinite or less than that seen in the wells with the negative control serum, or else present in only some wells, the result should be regarded as doubtful. This has to be taken into account when interpreting the overall results obtained with other samples from the same group of animals. Sera which yield doubtful results should be retested, or further samples requested. A serum is considered to be negative when a CPE is present in the wells.

For the quantitative test, the serum titre is expressed as the denominator of the highest initial dilution of serum which effects complete neutralisation of viral CPE.
b) Enzyme-linked immunosorbent assay (ELISA)

ELISA kits available commercially use direct or competitive techniques for measuring antibody levels (Table Ia). They differ in their mode of preparation of antigen, conjugate, or substrate, in the period of incubation and in interpreting results. The general advantage is that they enable the rapid processing of large numbers of samples. This can also be automated and the results analysed by computer. Some of these kits (Table Ib) make it possible to differentiate between vaccinated and naturally infected animals (4, 10, 11).

The sensitivity of ELISA is superior to that of the VN test which employs one hour of virus-serum interaction without the presence of complement. Some weakly positive sera are more readily detected by VN tests with long-term incubation of virus-serum mixtures than by ELISA. Some other weakly positive sera are more readily detectable by ELISA. It is now possible to use as a supportive technique filter paper disks which are moistened with a few drops of blood obtained by puncturing some superficial vein. This technique makes it convenient to collect blood samples from pigs (2, 8). It is possible to distinguish between antibodies resulting from natural infection and those from immunisation with certain vaccines, using filter paper discs.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Aujeszky's disease may be controlled by the use of vaccines containing either modified live or inactivated virus antigens. More recently, these conventional vaccines have been supplemented by r-DNA derived gene-deleted or naturally deleted virus pseudorabies virus (PRV) vaccines. The virus used in these new vaccines, sometimes referred to as marker vaccines, lacks a specific glycoprotein (GX, GI, or GIII). These gene-deleted marker vaccines have the advantage over conventional whole virus vaccines that it is possible to distinguish vaccinated animals from infected ones by checking the antibodies. Therefore, in countries where the eradication of Aujeszky's disease is planned, these marker vaccines are the vaccines of choice. Standards applicable to the manufacture of live virus vaccines will be described.

1. Seed management

a) Characteristics

Vaccines are made using a seed-lot system, in which a master seed virus (MSV) is prepared from a suitable strain of Aujeszky's disease virus. A number of strains are used for vaccine manufacture. The antigen in an inactivated vaccine can be one of a number of wild-type strains, such as Funkhouser, Iowa, or Missouri, or the naturally deleted Bucharest virus or r-DNA-derived gene-deleted virus. Modified live conventional vaccines and r-DNA engineered vaccines use the Bartha, Bucharest, or Iowa strains, or are derived from Aujeszky's original isolate or from other field isolates such as the NIA-3 strain.
TABLE I. Principal characteristics of some commercially available ELISA kits (this table gives examples only and is not comprehensive)

a) Conventional kits

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>IDEXX (screening)</th>
<th>IDEXX (verification)</th>
<th>BOMMELI</th>
<th>SVANOVABYTECH</th>
</tr>
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<td>anti-pig Ig + peroxidase</td>
<td>anti-pig Ig + peroxidase</td>
<td>monoclonal antiGII + peroxidase</td>
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<tr>
<td>Substrate</td>
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<td>$H_2O_2 + ABTS$</td>
<td>$H_2O_2 + ABTS$</td>
<td>$H_2O_2 + TMB$</td>
</tr>
<tr>
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<td>100 Fore St, Portland, ME 04101, USA</td>
<td>Langgas Str. 7, CH 3001, Bern, Switzerland</td>
<td>S75183 Uppsala, Sweden</td>
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b) Kits for GI antibodies

<table>
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<td>S75183, Uppsala, Sweden</td>
</tr>
</tbody>
</table>

ABTS: 2,2'-azino-di-3-ethylbenzthiazoline sulfonate TMB: tetramethylbenzidine
A virus identity test (using either a fluorescent antibody test, neutralisation test, constant serum-decreasing virus method, or any other suitable identity test) must be conducted on the MSV.

b) Culture

Most of the cell lines used to propagate Aujeszky's disease virus are continuous lines such as the porcine kidney (PK-15). A master cell stock (MCS) is established at a specified passage level. The MCS and the highest passage level (MCS + n) intended for use in the preparation of a biological product is specified in an Outline of Production. Both MCS and MCS + n are monitored by a variety of procedures to characterise the cell line and to ensure freedom from adventitious agents. The MCS must be monitored for species of origin. A minimum of 50 mitotic cells should be examined at both the MCS and MCS + n passage levels. The modal number in the MCS + n must not exceed 15 percent of the modal number of the MCS. Any marker chromosomes in the MCS must also be present in the highest cell passage.

If there is evidence that the cell line may induce malignancies in the species for which the product is intended, the cell line is tested for tumorigenicity and oncogenicity.

c) Validation as a vaccine

i) Purity

The cell line and MSV must be shown to be free of mycoplasma, bacteria, fungi, cytopathogenic or haemadsorbing viruses, haemagglutinating parvoviruses, porcine circoviruses, cytopathic and non-cytopathic ovine and bovine pestiviruses, and other extraneous agents, as determined by culturing and by fluorescent antibody procedures. Porcine cells are tested for transmissible gastroenteritis virus, porcine adenovirus, bovine virus diarrhoea/classical swine fever virus, and rabies virus. Some workers also believe that tests should be carried out for rotavirus.

ii) Safety test

Two swine of the minimum age for which the product is recommended are each injected with the equivalent of 10 doses of the viral vaccine. The vaccine is administered in accordance with the label recommendations. The animals are observed for absence of adverse reactions.

iii) Efficacy

Immunogenicity is determined by vaccination challenge of susceptible pigs (20 vaccinates and 5 controls) of the youngest age for which the vaccine is recommended. For a successful test, at least 4 of the 5 controls must develop central nervous symptoms or die, and at least 19 of the 20
Aujeszky's disease (B2)

vaccinated pigs must remain free of signs of pseudorabies. A geometric mean titre of the vaccine used in the efficacy test is determined and used for subsequent potency tests.

2. Manufacture

Only MSV that has been established as pure, safe and immunogenic may be used as seed for a vaccine product. All serials of vaccine must be from the first through the fifth passage of MSV.

3. In-process control

Cells from the MCS are propagated in a variety of growth media, such as minimum essential medium supplemented with 5 to 10 percent fetal calf or bovine serum, in stationary vessels, roller flasks, or suspension cultures. When cells become confluent, the growth medium is discarded and the cultures are refed with maintenance medium containing 2 percent fetal calf or bovine serum containing MSV. At a predetermined virus-cell ratio, infected cell cultures are propagated at 36° ± 1°C for 2 to 5 days and are harvested when the cells become round, degenerate, and/or start to slough off the culture walls. Virus harvests are usually stored at 3°C or frozen at -40°C to -70°C, while in-process tests, such as sterility and virus titres, are carried out.

Virus lots are thawed and pooled, and both stabiliser and diluent are added prior to filling and lyophilisation. After desiccation, a cap is set on each vial while under vacuum. Prior to release for sale and distribution, each batch of the product is subjected to the tests described below.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

b) Safety test

Two swine of the minimum age for which the product is recommended are each injected with the equivalent of 10 doses of the viral vaccine. The vaccine is administered in accordance with the label recommendations. The animals are observed for absence of adverse reactions. With products derived from r-DNA technology, other appropriate tests may be required on each serial of the product. For example, all engineered pseudorabies vaccines are thymidine kinase (TK) negative (5), and each MSV as well as each serial of product is tested to ensure freedom from this enzyme.

c) Potency test

Each serial or subserial must have a virus titre that exceeds the titre of the
vaccine used in the immunogenicity test by a specified value. For example, if the efficacy trial conducted with a virus titre of $10^{3.0}$ TCID$_{50}$ per dose was satisfactory, the value of $10^{0.7}$ is added for test variation between laboratories and $10^{0.5}$ is added for dating. Thus each serial of the product would be released with a titre of $10^{4.2}$ TCID$_{50}$/dose. A virus identity test (using either a fluorescent antibody test, neutralisation test, constant serum-decreasing virus method, or any other suitable identity test) must be conducted on each serial of product.

d) Duration of immunity

The instructions for use accompanying most live pseudorabies virus vaccines recommend that healthy swine, 3 days or 3 weeks of age or older, be vaccinated with 2 ml either by the intramuscular or subcutaneous route or by intranasal installation of 1 ml/nostril. Sows and gilts are normally vaccinated with 2 ml before breeding and some manufacturers advise semiannual vaccination for animals retained for breeding. Piglets nursing immune sows should be revaccinated 30 to 90 days later, when maternal antibody levels have declined.

REFERENCES


ECHINOCOCCOSIS/HYDATIDOSIS
(B3)

SUMMARY

Diagnosis of echinococcosis in dogs or other carnivores depends on the demonstration of cestodes of the Echinococcus genus in their faeces or small intestine. In intermediate hosts this depends upon detection of the larval cyst form that can infect almost any organ, particularly the liver and lungs.

Identification of the agent: At present four species of the genus Echinococcus are regarded as taxonomically valid. These are Echinococcus granulosus, Echinococcus multilocularis, Echinococcus oligarthrus and Echinococcus vogeli. These 4 species are morphologically distinct in both adult and larval stages. Also, a number of inter- and intra-specific variants have been described.

It should be remembered that there is a risk of contamination of human operators in handling these organisms, and suitable precautions must therefore be observed.

Larval forms of Echinococcus can often be visually detected in organs. Special care has to be taken for a specific diagnosis of echinococcosis in instances where Taenia hydatigena is also a problem. Histological examination may confirm the diagnosis after formalin fixed material is processed by conventional staining methods. The presence of a PAS-positive acellular laminated layer with or without an internal cellular, nucleated germinal membrane can be regarded as a specific characteristic of metacestodes of Echinococcus.

Only the small intestine is required for the collection of adult Echinococcus spp. The technique of carrying out surveys with the use of arecoline has been generally adopted for determining the prevalence of Echinococcus spp. in dogs.

Serological tests: There are no satisfactory serological tests for the diagnosis of adult echinococcosis.

Requirements for biological products: The principal product used for diagnosis of the adult Echinococcus is arecoline hydrobromide.

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A. DIAGNOSTIC TECHNIQUES

The diagnosis of echinococcosis in dogs or other carnivores requires the demonstration of the cestodes of *Echinococcus* spp. in their faeces or the small intestine. In the intermediate host, diagnosis depends on the detection of the larval cyst form.

1. **Identification of the agent**

Four species of the genus *Echinococcus* are accepted taxonomically at present, namely *Echinococcus granulosus*, *Echinococcus multilocularis*, *Echinococcus oligarthrus*, and *Echinococcus vogeli*. These are morphologically distinct both in their adult and larval stages.

**Echinococcus granulosus**

The adult varies between 2-7 mm in length and usually possesses 3-4 segments, rarely up to 6. The penultimate segment is mature, and the genital pore normally opens posterior to the middle in both mature and gravid segments. There are rostellar hooks on the protoscolex in 2 rows. The size of the hooks varies between 22-45 μm in the first row, and 18-38 μm in the second row.

The gravid uterus has well developed sacculations. The larval stage is a fluid-filled bladder that is unilocular, although communicating chambers also occur. Growth is expansive and endogenous daughter cysts may be produced. Individual bladders may reach up to 30 cm in diameter.

**Echinococcus multilocularis**

The adult varies between 1.2-3.7 mm in length and usually possesses 4-5 segments. The ante-penultimate segment is characteristically mature, and the genital pore is anterior to the middle in both mature and gravid segments. The gravid uterus is sac-like. On the rostellum, there are hooks which vary in size between 27.6-34.3 μm (large) and 22.7-31.0 μm (small).

The metacestode is a multivesicular structure consisting of conglomerates of small vesicles usually not exceeding a few millimetres in diameter. Unlike *E. granulosus*, the larval mass often contains a semi-solid rather than a fluid matrix. It proliferates by budding and this results in infiltration of tissues. It is commonly referred to as the alveolar hydatid.

**Echinococcus oligarthrus**

The adult varies between 1.9-2.9 mm in length, and normally possesses 3 segments, the penultimate of which is mature. The genital pore is anterior to the middle in mature segments and approximately at the middle in gravid segments. The gravid uterus is sac-like.
The metacestode is polycystic and fluid-filled with a tendency to become septate and multi-chambered. The rostellar hooks of the protoscolex vary in length between 25.9-27.9 μm. The single cyst may reach a diameter of about 5 cm. Its preferred sites appear to be internal organs and muscles.

**Echinococcus vogeli**

The adult varies between 3.9-5.6 mm in length, and usually has 3 segments, the penultimate of which is mature. The genital pore is situated posterior to the middle in both the mature and gravid segments. The gravid uterus has no lateral sacculations and is characterised by being relatively long and tubular in form, as opposed to the other segments, which are sac-like.

The metacestode is similar to that of *E. oligarthrus*. It has been reported that the two species can be distinguished by comparing differences in the dimensions and proportions of the rostellar hooks on the protoscolex. The hooks on *E. oligarthrus* vary in length between 25.9-37.9 μm (average 33.4 μm) and 22.6-29.5 μm (average 25.45 μm) for large and small hooks respectively. Those of *E. vogeli* vary between 19.1-43.9 μm (average 41.64 μm) and 30.4-36.5 μm (average 33.6 μm) for the large and small hooks respectively.

Large numbers of inter-and intraspecific variants have been described, including 15 species and numerous subspecies. Only the 4 species described above are accepted taxonomically (1-3, 5).

Several subspecies of *E. granulosis* have been suggested, such as *E. g. granulosus* which infects dogs and ungulates, and *E. g. canadensis* which is indigenous to wolves and wild ungulates in the arctic regions of North America. Two subspecies of *E. multilocularis* (*E. m. multilocularis* and *E. m. sibiricensis*) have been proposed, but none for *E. oligarthrus* or *E. vogeli*. To avoid future taxonomic confusion, minor morphological variants within a genus should be designated as strains until their significance has been clarified. Morphology may not be the sole criterion for discrimination between strains, particularly when such variations may reflect phenotypic adaptations of the adult parasite to different environments rather than genotypic differences. *In vitro* cultivation, biochemistry, development in definitive hosts and intermediate host specificity should all be considered when differentiating between strains.

a) **Diagnosis of larval echinococcosis**

Whereas diagnosis in domestic animals may take place in licensed slaughter houses, that in wildlife has to be done by field surveys. There are no satisfactory techniques of immunodiagnosis, so that autopsies have to be carried out. Specimens should be preserved by fixation in 4-10% formol saline, or kept cool and deep frozen for subsequent examination.

Echinococcal larvae can be observed in organs, but in large animals, such as sheep and cattle, palpation or incision should be done. Pigs, sheep and goats
may be infected with *T. hydatigena* and it is nearly impossible to differentiate between these two parasites when they occur in the liver. *Ascaris suum* has also been incriminated as a cause of "white spot" in sheep livers. In wild animals such as ruminants and rodents, several other larval cestodes should be considered for differential diagnosis.

Formalin fixed material can be stained by conventional histological techniques. The presence of a periodic acid-Schiff (PAS)-positive acellular laminated layer with or without an internal cellular, nucleated germinal membrane can be regarded as a specific characteristic of metacestodes of *Echinococcus*. Isoenzyme electrophoresis and electrofocusing may assist in differentiating larval cestodes. Radiographic methods of diagnosis have also been used (4, 6).

**b) Diagnosis of adult parasites in carnivores**

Autopsy surveys and transmission experiments are employed in the study of wildlife echinococcosis. It should be emphasised that it is necessary to isolate and identify the adult *Echinococcus* since under normal conditions of faecal examination the eggs of *Echinococcus* cannot be differentiated from those of *Taenia* spp. *Echinococcus* adults must be examined to confirm the diagnosis.

The small intestine is removed as soon as possible after death, and tied at both ends. If the material is not formalin fixed, it should be examined quickly since the parasite can be digested within 24 hours. Concentrations of 4-10% formalin do not kill eggs.

The intestine is divided into several sections and immersed in saline at 37°C for examination. Worms adhering to the intestinal wall may be observed and counted by means of a hand lens. For accurate counts, the unfixed intestine is best divided into 4 or 6 sections, opened up and immersed in saline at 37°C for 30 minutes to release the parasites. The contents are washed into another container for detailed examination, and the intestinal wall scraped with a spatula. All material is boiled and washed by sieving to eliminate most of the particulate material. The washed intestinal contents and scrapings are placed on a black tray and the worms counted with the aid of a hand lens or stereoscopic microscope.

The worms are fragile and are best handled in normal saline with a Pasteur pipette. They are washed free of other material and left for about 30 minutes for all movement to cease. After removal of the fluid, cold 5-10% formalin (5°C) or FAA fixative (80 ml 95% ethanol, 10 ml 37-40% formaldehyde, 5 ml glacial acetic acid) is added and left for a further 12 hours.

For staining, the worms are washed in water for 15 minutes and transferred to Mayer's paracarmine (1.0 g carminic acid, 0.5 g aluminium chloride, 4.0 g calcium chloride, 100 ml 70% ethanol) for 12-24 hours. Excess stain is removed by immersion in 0.5-1.0% hydrochloric acid solution for a few seconds. Dehydration is accomplished by serial passage in ascending concentrations of
alcohol (35, 50, 70, 85, 95, 100%) for at least 15 minutes in each, with two changes in 100%. The alcohol is removed by xylol (10 minutes) and cleared with methyl salicylate or creosote.

Prior to mounting in any suitable medium such as balsam, picolyte, etc., the specimens should be returned to the xylol for few minutes.

e) Arecoline surveys

These are generally adopted as a means of determining the prevalence of *Echinococcus* within dog populations. Under laboratory conditions, the drug arecoline will eliminate large numbers of worms from some dogs but none from others. It is a safe procedure but nine or more treatments may be required to free all dogs of *E. granulosus* on a group basis. The recommended dose is 1.75-3.50 mg/kg, but doubling or halving the rate does not alter its efficacy.

The treatment of healthy dogs is a safe procedure, but any vomiting which occurs after administration of the drug will reduce the effectiveness of the treatment. In some dogs, arecoline may cause abnormally severe and prolonged stimulation of mucous secretions, characterised by excess salivation and anal discharge of watery mucus. This may lead to dehydration which should be countered by access to water at all times. In animals that actively resist treatment, a significant amount of the drug may be absorbed through the mucosa of the mouth or throat during dosing; this can lead to a fall in blood pressure, incoordination, loss of consciousness, cyanosis, and laboured respiration. These effects can be reversed by artificial respiration and the injection of between 0.3-4.0 mg, according to size of the animal, of the antagonist atropine sulphate. One third of the dose is given intravenously, and the remainder subcutaneously or intramuscularly. Pregnant bitches and animals with cardiac abnormalities should not be treated with arecoline.

To obviate any possibility of bowel puncture by bone fragments, dogs should not be fed bone for three days before treatment. Dosing should not be carried out on a full stomach, although preliminary starvation is not necessary. Older dogs that are habitually constipated may require a dose of liquid paraffin 1-2 days before treatment. As some dogs resist purgation, the additional use of arecoline hydrobromide as an enema has been recommended, but the worms may not be reached by this procedure and it may thus lead to false negative interpretations. Such dogs should be dosed again after a rest of 3-4 days.

Dogs that purge successfully produce at least two motions; the first will be formed faeces and can be ignored, but the mucus that follows may be productive. This can be divided into several samples and each examined separately. This method is not recommended as the worms will be difficult to detect. Preferably, the mucus sample (about 4 ml) is diluted with 100 ml tap water, covered with a thin layer of 1 ml of kerosene (paraffin) and boiled for 5 minutes. The kerosene prevents foaming and reduces the smell.
d) Flotation technique

This technique is independent of a piped water supply and relies on the fact that *Echinococcus* floats in a medium with specific gravity of about 1.2, whereas most other faecal debris will sink. The faecal sample is placed directly into a fine meshed (80-100 meshes/linear inch) wire basket (no. 1 in diagram) and boiled for 5 minutes (7). If a faecal mass contains large particles it may be placed in a coarse mesh (10 meshes/linear inch) basket (no. 2 in diagram) which fits inside the fine mesh basket and will retain the larger particles.

After boiling, a stainless-steel collecting pan with a cone in the centre and having a 5 cm opening at the apex and a wall 15 cm deep and 10 cm wide (no. 3 in diagram) is attached to the top of the fine-meshed basket. Both basket and pan are slowly immersed in a saturated salt (NaCl) solution. The contents of the pan are then poured into a black tray for examination. If no worms are detected initially, the flotation should be repeated at least twice.
e) Sieving technique

This involves the washing of a faecal sample under pressure through a sieve, and examining the retained material for the presence of worms. Another method employs a constant stream of water to wash boiled mucous faeces through a sieve. Material retained by the sieve is washed into a black tray for examination. For field surveys, a mesh of 80-90 meshes per linear inch can be used; and for laboratory studies, a mesh of 120 meshes per linear inch, in order to obtain a cleaner sample.

f) Sedimentation technique

For exact quantitation, a sedimentation technique is required. A boiled faecal sample is suspended in 0.85% saline and the worms allowed to sediment for at least 30 minutes. The supernatant fluid is removed and the worms resuspended in saline. This should be repeated several times, until the majority of the light flocculent material has been removed in the supernatant fluid. The sediment is examined for worms by pouring a small amount at a time into a black tray containing saline. This is considered to give more accurate results and is less damaging to the worms than other methods. It is however too time-consuming for large numbers of samples.

2. Serological tests

There are no satisfactory serological methods of diagnosis for adult echinococcosis.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Characteristics of arecoline

Arecoline is used to perform surveys of worm infestation, particularly in dog populations. It is the chief alkaloid of the areca nut, the seed of *Areca catechu*. Arecoline hydrobromide is a white crystalline powder, odourless, with a bitter taste. It should be stored in well-closed containers, protected from light and moisture. Arecoline-acetarsol is the arecoline salt of 3-acetamido-4-hydroxyphenyl-arsonic acid and is a white or very pale yellow, somewhat glistening powder, almost odourless.

Arecoline is a parasympathomimetic agent. Its action results in sweating, and stimulation of salivary, lachrymal, gastric, pancreatic, and intestinal glands. It increases intestinal tonus and mobility of smooth muscle, and this effect is responsible for purgation. The liver is the principal site of detoxication. Arecoline also has a direct action on the worm itself, by causing its paralysis but not death, making it relax its hold on the intestinal wall. Thus, it must be administered by the oral route. The accompanying purgation carries the worms out with the faeces.
Administration of the drug

The use of the drug in tablet form and administered by means of a tablet gun is strongly recommended. Standard tablets of arecoline hydrobromide are preferably coated with sugar to disguise its taste and to avoid the development of allergic reactions in handlers with skin sensitivity.

The accepted dose rates for tablets each containing 25 mg arecoline hydrobromide are:

Minimum: 1 tablet per 14 kg body weight
Maximum: 1 tablet per 7 kg body weight
Optimum: 1 tablet per 10 kg body weight

Arecoline-acetarsol tablets may also be used, and are available under a number of trade names. It has been suggested that this preparation has fewer side effects than the hydrobromide salt. A standard liquid solution may be recommended as follows:

Arecoline hydrobromide: 15 mg/ml
Saccharine (or sugar): 1.5 mg/ml
Dose: 1 ml/5 kg body weight

In a Tasmanian programme, the dose rate was reduced from 3.25 mg/kg to 1.62 mg/kg. The solution used was 0.82% arecoline hydrobromide given at a rate of 1.6 mg/kg up to a maximum of 32 mg. Dogs which failed to be purged within one hour were re-dosed at the same level.

A schedule of a divided dose treatment frequently gives improved diagnostic results without increasing the side effects of the drug. This schedule, in which the minimum dose is given and followed 15-30 minutes later with a similar dose, is recommended provided that the total dose is not greater than the maximum recommended dose rate.

REFERENCES


Heartwater is a rickettsial disease of ruminants caused by Cowdria ruminantium. A provisional diagnosis of heartwater is based on an assessment of the history of the disease outbreak, the spectrum of clinical signs and the character of the lesions observed post-mortem. A history of recent movement of animals into or through an endemic area is highly relevant, particularly when disease outbreaks occur 2-4 weeks later.

Clinical signs range from the subclinical to the peracute. Peracute cases die suddenly. Acute cases last for 3-6 days with pyrexia throughout, and subsequently with central nervous system (CNS) impairment which progresses to an incoordinated gait, blinking and twitching of the eyelids, opisthotonus and death. Newborn calves have a high innate resistance to the disease.

Identification of the agent: Diagnosis of heartwater depends on the demonstration of rickettsias in vascular endothelial cells. Squash preparations are made of several areas of cerebellum, cerebrum, and hippocampus which are the best tissue sources. Smears of the intima of the jugular vein or larger blood vessels are also acceptable. These preparations are dried, fixed with absolute alcohol, dried and stained by Giemsa. Fixed tissue smears can be transported unrefrigerated. The rickettsias are visible in clusters in the cytoplasm of infected cells.

An entire brain may be submitted provided it is kept refrigerated. Biopsies of brain can be obtained from live animals under strict aseptic conditions. Blood (10-20 ml) in an anticoagulant, such as heparin or EDTA, can be collected during a period of pyrexia. The spleen should be removed immediately after death. Blood and spleen samples should be kept in insulated containers for examination as soon as possible. Specimens of ticks can be transported frozen in dry ice, or preferably in liquid nitrogen.

Fresh or uncoagulated whole blood from suspect animals taken during the febrile period may be subinoculated into susceptible bluetongue-immune sheep to confirm the disease in areas where bluetongue is endemic. The results are not available for 2-3 weeks. Ferrets and some strains of mice are also susceptible.

Serological tests: Serological tests include complement fixation, indirect fluorescent antibody tests, flocculation, and enzyme linked immunoassay (ELISA). The antigen may be derived from infected cell cultures. Peritoneal macrophages from mice infected with the Kumm isolate have been employed in immunofluorescence tests, and primary neutrophil cultures provide a rapid and simple method of obtaining large amounts of C. ruminantium antigen.
Requirements for biological products: No biological products are available.

A. DIAGNOSTIC TECHNIQUES

Heartwater (cowdriosis) is caused by the rickettsia *Cowdria ruminantium* and affects domestic and wild ruminants (7, 8, 9). It is also known by the synonyms veld poisoning, péricardite exudative infectieuse (French), Herzwasser (German), and hidrocarditis infectiosa (Spanish). It exists as an enzootic in the regions of Africa and Madagascar (36, 37) that are infested with ticks of the *Amblyomma* species. The disease has also been reported in the Caribbean (5, 30, 39, 40). Many wild ruminants, including antelopes, are susceptible and can act as reservoir hosts (3, 29). Indigenous breeds of cattle, goats and sheep may be more resistant than imported breeds.

A provisional diagnosis of heartwater is based on an assessment of the history of the disease outbreak, the spectrum of clinical signs and the character of the lesions observed post-mortem. A history of recent movement of animals into or through an endemic area is highly relevant, particularly when disease outbreaks occur 2-4 weeks later (1).

Clinical signs range from the subclinical to the peracute. Peracute cases die suddenly. Acute cases last for 3-6 days with pyrexia throughout, and subsequently with central nervous system (CNS) impairment which progresses to an inco-ordinated gait, blinking and twitching of the eyelids, opisthotonus and death. Newborn calves have a high innate resistance to the disease (17).

1. Identification of the agent

In the mammalian host, *C. ruminantium* is found in colonies or clusters (7-9, 10, 15, 31). The size varies from 200-500 nm. In stained smears under light microscopy, the small coccoid forms appear uniform, whereas the larger ones are pleomorphic and include rings, horseshoe shapes and bacillary forms (31). With the exception of the semicarbazones (gloxazone), the rifamycins, the sulphonamides and the tetracyclines, the organism is not susceptible to antibiotics.

Electron microscopy reveals that the rickettsias occur as clusters within a vacuole in the cytoplasm of infected endothelial cells (31). Each rickettsia is surrounded by 2 unit membranes. It multiplies mainly by binary fission, sometimes by multiple budding (31). There is a divergence of opinion regarding the localisation of *C. ruminantium* and the infectivity of the different components of blood. Infectivity has been associated with the leucocytes (18) as well as the red cells (14). The only certainty is that *C. ruminantium* multiplies in the vascular endothelial cells of ruminants (15) and appears in the circulation. It has been found in neutrophil cells (23). There is also evidence that *Cowdria* multiplies in reticulo-endothelial cells (10).

The known vectors of cowdriosis are three host ticks of the genus *Amblyomma* in which infection is transstadial and not transovarial. Transovarial infection apparently occurs only very rarely (4). In *Amblyomma* ticks, *C. ruminantium* has
been observed in the epithelial cells and lumen of the gut, where they occur as densely packed colonies (21). They also occur in the tick salivary gland (20).

Heartwater is not a natural disease of laboratory mice, although they can be infected by strains of Cowdria that vary in virulence. The Kumm strain is highly virulent for mice (13). Most strains are not strongly infective for mice, and some will cause only scattered deaths in mice or eventually fail to infect them on repeated passage. Some strains will passage in mice, inducing clinical signs (26), and will retain their virulence for sheep. Some strains can be adapted to mice through alternating passages in mice and sheep.

Fresh whole blood collected from febrile goats and maintained at 4°C for 72 hours is still infectious for mice (26). Blood collected from febrile goats in an anticoagulant and preserved with 10% dimethyl sulphoxide at -70°C or at -196°C remains infective for goats (23). Neutrophils derived from an infected goat and maintained in modified RPMJ 1640 medium at 37°C for 68 hours are also still infective for goats. Neutrophils similarly treated and maintained at 37°C are infective for mice. C. ruminantium will persist for 90 days in white mice when infected intraperitoneally (16); this is a convenient means for transportation of the organism over a prolonged period.

Following natural infection by ticks the incubation period for the disease is 1-3 weeks (occasionally but rarely 5 weeks), with an average of 9-14 days. In acute cases, the animals develop a pyrexia of above 40°C for several days. Clinical signs may vary from anorexia to depression accompanied by varying degrees of nervous involvement. Animals with nervous signs rarely recover. The mortality rate is about 90%, depending on species and breeds.

The most common lesions are pulmonary oedema and lung congestion with marked interlobular septal oedema. Frothy exudate occurs in the trachea, bronchi and nasal passages. There may be hydrothorax, and a hydropericardium is frequently found. Subendocardial and brain haemorrhages occur, but these are not common in sheep, and splenomegaly and lymphadenopathy can be found in experimentally infected small ruminants. Splenomegaly and lymphadenopathy are commonly seen in natural cases.

In infected mice there is no pyrexia, but tachypnoea, lethargy, anorexia, incoordination, clinical hypothermia and anorexia may occur. The body temperature falls 10°C before death. Tissues are invaded by the sixth day after infection, coinciding with the hypothermia. Post-mortem lesions resemble those found in large animals. Mice that die have a pulmonary oedema and acute interstitial pneumonia, with concentrations of the organism in endothelial cells of the lung capillaries. Their occurrence is rare in the brain, but they may be seen in the kidney glomeruli.

The organism can be grown in an endothelial cell line (2). These cells are of bovine fetal origin and are used at their seventieth passage level. Cultures can be irradiated by ultraviolet light and then inoculated with tick-derived stabilate prepared from
A. hebraeum nymphs. Cultures are used for the production of large quantities of C. ruminantium antigen for serology and potential vaccine purposes.

Difficulties in diagnosis can arise for a number of reasons, such as lack of adequate facilities in the field, the acuteness of disease onset, the collection of inappropriate samples, a lack of pathognomonic post-mortem lesions, a failure to obtain adequate squash preparations of brain, and the lack of simple serological tests. The disease can be confused with the acute forms of other conditions, such as anthrax, haemoparasitaemia, pasteurellosis, rabies, rinderpest, foot and mouth disease or poisoning. A tentative diagnosis of heartwater may be possible on clinical grounds and herd history. A preponderance of Amblyomma ticks in certain seasons may also lead to suspicion of the disease. Laboratory confirmation is made by demonstrating rickettsias microscopically.

The specimens required include:

i) Smears prepared from several areas of the cerebral cortex, cerebellum, and hippocampus. Brain tissue smears are made by squashing matched, bead-like pieces between 2 microscope slides, and then drawing them apart, so that 2 smears are obtained. The best brain crush smears are made by alternately crushing-smearing-lifting, crushing-smearing-lifting, etc., along the length of the slide. A modification (22) of the smear technique is to make a zig-zag smear of about 5 mm wavelength on a slide: the capillaries are deposited on each wave, and do not all accumulate at the end of the smear as with the other technique. Each smear is air-dried, fixed in absolute alcohol or methanol for 5 minutes, and stained by Giemsa.

ii) Smears similarly prepared from the intimal lining of the jugular vein. The vessel lumen is freed of blood, rinsed in phosphate buffered saline (PBS) and then scraped onto a microscope slide. If brain smears are available, intimal smears are unnecessary.

iii) Whole blood (5-10 ml) collected into an anticoagulant from animals during the febrile period.

iv) The spleen removed immediately after death.

All fixed smears are forwarded unrefrigerated: blood and spleen specimens should be packed in ice for immediate transport.

To detect the agent in the squash preparations of brain (22, 32), areas containing several unconvoluted capillaries are located first by low power microscopy. Individual capillaries can then be examined more precisely by high power microscopy. The organisms stain reddish-purple to blue, but numbers may be scanty, especially in peracute cases.

It has not been definitively established whether the cerebellum or the cerebrum contains the most rickettsias. Both tissues need to be sampled, making several smear preparations. Suitable smears may be made of brain that has been
refrigerated at 6°C for less than a week (38), but better slides are made from fresh material. Brain biopsies may be obtained aseptically from a live animal (35) showing clinical signs for up to 16-18 days after infection. The area of the biopsy is subjected to local anaesthesia, an incision made on a line between the inner canthus of the eye and the frontal crest, two thirds of the distance towards the crest. A small hole is drilled in the cranium and a needle inserted to penetrate the brain tissue. A small amount is sucked into a syringe, and a squash preparation made as before (22, 32). Also, C. ruminantium can be demonstrated by the inoculation of susceptible animals with whole blood collected from sick animals during the febrile period. The ensuing disease will enable the rickettsias to be isolated and identified.

2. **Serological tests**

Vascular endothelial cells were the first mammalian cells to be identified as being infected with C. ruminantium, and the neutrophils of infected goats were found to carry the organism in the blood (25). More recently C. ruminantium has been propagated in the calf umbilical endothelial cell line (E-5). The use of this cell line has allowed the production of large quantities of the agent for use with serological tests.

An indirect fluorescent antibody test is the serological test currently being used. C. ruminantium is propagated in the E-5 cell line. The cells are treated with trypsin when early cytopathic effects are evident; about 30% of the cells are infected at this time. The cells are centrifuged onto glass slides using a Cytospin (Shandon Southern Products Ltd., Astmoor, Runcom, England), fixed with a mixture of methanol 60% and acetone 40%, and stored frozen. A 1:40 dilution of serum to be tested and negative and positive control serum are added to separate slides and incubated at 37°C for 30 minutes. The slides are rinsed with PBS, blotted, and anti-species antibody conjugated to fluorescein isothiocyanate is added. The slides are incubated again at 37°C for 30 minutes, rinsed, and blotted. They are then examined for specific fluorescence with an ultraviolet microscope. A 1:40 titre is considered positive. The tests can be used for large-scale screening.

Direct and indirect immunofluorescence tests have been used to identify C. ruminantium in infected cell cultures and in tick vectors (3, 11, 23, 28, 33, 34). Complement fixation tests using brain tissue, and whole blood as antigen, have been described (12, 27). These are unreliable and of questionable value. The use of enzyme immunoassay (ELISA) has been reported to detect the organism or humoral antibodies to it (41). The antigen in the latter case was purified infected brain tissue and tick stabilate. Applied in the case of known positive herds, the correlation was 100%, but known negative herds have yielded a number of false positive results.

There are antigenic differences between strains of C. ruminantium that can infect both rodents and ruminants and those that infect only ruminants (23, 24, 38, 40). These differences are of importance when carrying out epidemiological serum surveys for heartwater. A capillary flocculation test (18) may be done using antigen prepared from infected brain tissues. This test can detect antibodies up to 4 weeks after infection.
B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

No products are available at present.

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LEPTOSPIROSIS
(B6)

SUMMARY

Laboratory diagnostic procedures for leptospirosis fall into two groups. The first group consists of tests for the demonstration of leptospires in animal material. The second contains the tests for antibody detection. The selection of tests to be carried out depends on the purpose for which a diagnosis is to be made and the resources available.

Identification of the agent: The isolation of leptospires from, or their demonstration in:

a) the internal organs (such as liver, lung, brain) and body fluids (blood, milk, cerebrospinal, thoracic and peritoneal fluids) of clinically affected animals gives a definitive diagnosis of acute clinical disease or, in the case of a fetus, chronic infection of its mother.

b) the kidney, urine and/or the genital tract of unaffected animals is diagnostic only of a chronic carrier state.

Isolation, especially from clinical material, is time consuming and is a task for specialised laboratories, as is identification of isolates. Isolation from renal carriers is very useful in epidemiological studies to determine which serovars are present in an animal species, a particular group of animals or geographical location.

The demonstration of leptospires by immunochemical tests (immunofluorescence, immunoperoxidase and immunogold) is more suited to most laboratory situations. However these tests are "number of organisms"-dependent and lack the sensitivity of culture. They provide no information as to the infecting serovar. They require high IgG titre anti-leptospire sera which are not available commercially.

Serological tests: Serological testing is the most widely used means for the diagnosis of leptospirosis, and the microscopic agglutination test (MAT) is the standard serological test. Minimum antigen requirements are that the test should employ representative strains of all the serogroups known to exist in the particular country plus those known to be maintained elsewhere by the animal species under test.

The MAT is used primarily as a herd test. To obtain useful information at least 10 animals or 10% of the herd, whichever is the greater, should be tested. As an individual animal test, the MAT is very useful in diagnosing acute infection: a 4-fold change in antibody titres in paired acute and convalescent serum samples is diagnostic.
The MAT has limitations in the diagnosis of chronic infection in individual animals: both in the diagnosis of abortion and in the identification of renal or genital carriers (of particular interest to those involved in the import/export of live animals). Infected animals may have MAT titres below the widely accepted minimum significant titre of 1:100.

Requirements for biological products: Vaccines for veterinary use are suspensions of one or more serovars of Leptospira interrogans inactivated in such a manner that immunogenic activity is retained. While a range of experimental vaccines based on cellular extracts has been tested, commercial vaccines are invariably whole cell products. The leptospires are grown in suitable culture media which may contain serum. If used, serum must be removed from the final products. Vaccines may contain suitable adjuvants.

A. DIAGNOSTIC TECHNIQUES

Leptospirosis is a contagious disease of animals and man due to infection with Leptospira interrogans. There are 198 distinct serovars recognised; these are arranged in 23 serogroups (25).

The use, interpretation and value of laboratory diagnostic procedures for leptospirosis varies with the clinical history of the animal or herd, the duration of infection and the infecting serovar. Acute leptospirosis should be suspected in cases of: sudden onset of agalactia (in adult milking cattle and sheep); icterus and haemoglobinuria, especially in young animals; meningitis; and nephritis and hepatitis in dogs. Chronic leptospirosis should be considered in cases of: abortion, stillbirth, birth of weak offspring (may be premature); infertility; and cases of periodic ophthalmia in horses. Two major chronic microbiological sequels of leptospiral infection present particular diagnostic problems: these are the localisation and persistence of leptospires in the kidney and the male and female genital tract. Chronically infected animals may remain carriers for life.

1. Identification of the agent

The demonstration of leptospires in blood and milk of animals showing clinical signs suggestive of acute leptospirosis is considered diagnostic. However, isolation from blood is not often made because bacteriaemia is not always accompanied by clinical symptoms. The demonstration of generalised leptospiral infection in a range of organs taken at autopsy from animals which have died is taken as diagnostic. However, the demonstration of leptospires in the genital tract, kidneys and/or urine merely indicates that the animal was a carrier.

Failure to demonstrate leptospires in the urine of an animal does not rule out the possibility of the animal being a chronic renal carrier; it merely indicates that the animal was not excreting detectable numbers of leptospires at the time of testing.

The demonstration of leptospires in the body fluids or internal organs (usually kidney, liver, lung, brain or adrenal gland) of aborted or stillborn fetuses is
considered diagnostic of chronic leptospirosis of the mother, and is evidence of active infection of the fetus.

The isolation of leptospires is the most sensitive method of demonstrating their presence, provided that antibiotic residues are absent, that tissue autolysis is not advanced and that tissues for culture have been stored at a suitable temperature (4°C) and – in the case of urine – suitable pH, since collection.

Culture should be carried out in a semi-solid (0.1 to 0.2 percent agar) bovine serum albumin medium containing either Tween 80 (22) or a combination of Tween 80 and Tween 40 (13) and, optionally, a small amount of fresh rabbit serum (0.4 to 2 percent). A dilution culture method should be used (13). Contamination may be controlled by a variety of selective agents, e.g. 5-fluorouracil (23), nalidixic acid (24), fosfomycin (27), and a mixture of rifamycin, polymixin, neomycin, 5-fluorouracil, bacitracin and actidione (1). However, the use of selective agents, especially those used by Adler et al. (1), will reduce the chance of isolation where there are only small numbers of viable leptospires, and many isolated strains of leptospires will not grow in selective media containing multiple antibiotics. Liquid culture media or 1% BSA solution containing 5-fluorouracil at 200 μg/ml should be used as transport media for the submission of samples.

Cultures should be incubated at 29-30°C for at least 12 weeks, and preferably for 26 weeks (13). They should be examined by dark-ground microscopy every 1-2 weeks. It is important that a 100 watt light source and a good quality microscope be used.

Leptospires may also be demonstrated by a variety of immunochemical staining techniques, e.g. immunofluorescence (14), immunoperoxidase (12) and immunogold staining (29). These are useful in diagnosing infection in pathological material which is unsuitable for culture or where a rapid diagnosis is required. Since they are "number of organisms"-dependent, they are not suitable for diagnosing the chronic carrier state, where the numbers of organisms present may be very low or very localised. Leptospires do not stain satisfactorily with the aniline dyes, and silver staining techniques lack sensitivity and specificity (2).

Initial reports suggest that DNA probes (5, 31) and time-resolved immunofluorescent assays (37) may prove very useful techniques for the detection of leptospires but further evaluation on field material is required.

The identification of leptospire isolates is a task for specialised laboratories. A pure leptospire culture is identified to species level by a variety of tests: the ability to infect animals; the relative resistance to 8-azaguanine; lipase activity; salt and temperature tolerance; and G+C content of DNA (21).

Strains belonging to Leptospira interrogans can be further differentiated to serogroup level by cross-agglutination reactions (11). Further differentiation to the serovar level is traditionally done by cross-agglutination-absorption, although for an increasing number of serogroups this can now be done by less time-consuming methods: factor analysis (11), monoclonal antibodies (30, 32), and restriction endonuclease analysis (33, 34).
2. **Serological tests**

Serological testing is the laboratory procedure most frequently used to confirm the clinical diagnosis, and is also the method most widely used in herd prevalence and epidemiological studies. Leptospiral antibodies appear within a few days of onset of illness and persist for weeks or months and, in some cases, years. Unfortunately, antibody titres frequently fall to undetectable levels while animals remain chronically infected. To overcome this problem, the polymerase chain reaction may prove useful in the future.

A wide variety of serological tests, which show varying degrees of serogroup and serovar specificity, have been described (19). Two tests have a role in veterinary diagnosis: the microscopic agglutination test (MAT) and the ELISA test.

a) **Microscopic agglutination test (MAT)**

The microscopic agglutination test, using live antigens, is the most widely used serological test. It is the reference test against which all other serological tests are evaluated and is the test specified for import/export testing. For optimum sensitivity it should employ serovars of all the serogroups known to exist in the country in which the animals are found and, preferably, strains representing all the known serogroups. The presence of a serogroup is usually indicated by frequent reaction in serological screening or isolation of a serovar from clinically affected animals. The sensitivity of the test can be improved by incorporating local isolates rather than reference strains. The two methods for carrying out the test have been detailed in references 20 and 26.

The strains selected should be grown in liquid polysorbate 80-bovine albumin medium at 29°C and the cultures should be at least four days old, but not more than eight days. The transmittance of the antigen should be 60-70% using a spectrophotometer with a 400 nm filter. The number of antigens to be used is determined and a screening test may be performed with 1:50 serum dilutions. The positive samples are titrated to an end-point using doubling dilutions of serum starting at 1:50 through to 1:51,200 before addition of antigen. A volume of each antigen equal to the diluted serum volume is added to each well. The microtitration plates are incubated at 28-30°C for 2 hours. The plates are examined with a dark-field microscope. The degree of reaction is interpreted by estimating the percentage of leptospires that are agglutinated. If 100% of the leptospires are agglutinated, the reaction is 4+; 3+ = approximately 75% agglutination, 2+ = 50%, and 1+ = less than 50%. The end-point is the highest dilution with a 2+ or greater reaction. If a screening test is performed, any serum that has a 2+ reaction at a 1:100 dilution is titrated to the end-point, starting at a dilution of 1:100.

As an individual animal test, the MAT is very useful in diagnosing acute infection; the demonstration of a 4-fold change in antibody titres in paired acute and convalescent serum samples is diagnostic. The test has limitations in the diagnosis of chronic infection in individual animals, both in the diagnosis of abortion (15) and in the identification of renal or genital carriers (13). This is
particularly true with the host adapted leptospiral infections, e.g. serovar hardjo infection in cattle: when a titre of 1:100 or greater is taken, the sensitivity of the test is only 0.41, and even when the minimum significant titre is reduced to 1:10 the sensitivity of the test is only 0.67 (13). The demonstration of antibodies in fetal blood is diagnostic.

Since leptospirosis is a herd problem, the MAT has much greater use as a herd test. To obtain useful information, Cole et al. (9) have suggested that samples be taken from at least 10 animals, or 10% of the herd, whichever is the greater. In a study of hardjo infection in cattle, Hathaway et al. (20) found that a ten-cow sample usually provided little useful information other than establishing the presence or absence of infection in a herd. Increasing the sample size markedly improved epidemiological information, investigations of clinical disease, and public health tracebacks.

In making a serological diagnosis of leptospirosis as the cause of disease, account must be taken of the infecting serovar and of the clinical condition involved. In the case of L. pomona abortion in cattle, a high titre is commonly found because the clinical incident occurs during the acute phase of infection. Abortion in cattle due to L. hardjo is a chronic event; in this case the serological response at the time of abortion is more variable, with some animals seronegative and others showing high titres. Milk drop in cattle may occur during the acute phase of L. hardjo infection and is associated with high titres. Vaccination history must also be considered in the interpretation of MAT results.

b) ELISA

The major identified uses for the ELISA have been an IgM-ELISA in identifying recently infected animals (10, 16); and a total Ig-ELISA in identifying fully susceptible animals suitable for experimental challenge work (16).

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Leptospiral vaccines for veterinary use are suspensions of one or more strains of Leptospira interrogans inactivated in such a manner that immunogenic activity is retained. While a range of experimental vaccines based on cellular extracts has been tested (4), commercial vaccines are invariably whole cell products. The leptospires are grown in suitable culture media which may contain serum. If used, serum must be removed from the final product. Vaccines may contain suitable adjuvants.

1. Seed management

a) Characteristics

Proper selection of vaccine production strains is of utmost importance.
Immunity induced by vaccination is largely serovar-specific (8). A vaccine’s formulation should be based on its being used in a particular animal species in a particular geographical region. It should contain only those serovars – and preferably those genotypes – which cause problems in that animal species, or which are transmitted by that animal species to other species, in that geographical location.

Suitable strains should be further selected by their ability to grow to high yields under batch culture conditions.

b) Culture

Each component strain to be included in the final vaccine should be grown separately in liquid medium, which should preferably be a protein-free medium (3, 28) or a low-protein one (3).

The volume of each master-seed culture should be amplified by growth for 2-10 days at 29±1°C in a series of subcultures until a volume sufficient for use as a production seed culture is achieved. These should be aerated and agitated as required.

c) Validation as a vaccine

Strains selected for use as master-seed culture should be cloned on solid medium to ensure the absence of saprophytic leptospire contaminants.

Each subculture of the master seed culture should be checked for purity and for satisfactory growth. Purity can be checked by inoculating a loopful of culture onto blood-agar plates or onto fluid thioglycolate for incubation at 37°C for 2-5 days and by examining a Gram-stained smear of spun culture. Growth can be checked by dark-field microscopy.

Each production seed culture should also be checked against its homologous antiserum to ensure purity and homology.

2. Manufacture

Manufacture is carried out by batch culture in appropriately sized fermentor vessels. These should be equipped with ports for the sterile addition of seed culture, air and additional medium. They should also have sampling ports so that the purity and growth of the production culture can be monitored.

Ideally, low-protein or protein-free media are used for production. However, some strains require the presence of animal protein to achieve suitable yields; this is usually supplied as bovine serum albumin. All media components which are not degraded by heat should be sterilised by that method. This reduces the risk of contamination by water-borne saprophytic leptospires which are not removed by filter sterilisation.
After addition of the seed culture, the growth of the production culture is monitored at frequent intervals for the start of log-phase growth. Once this is observed the vessel is then agitated and aerated. The final yield can often be improved by the addition of more Tween 80 to the culture when log-growth is first observed to be slowing down. Adequate growth may require up to 10 days of incubation at 29 ± 1°C.

Inactivation is usually by the addition of formalin, but phenol and heat inactivation have also been used.

After the appropriate inactivation period, the culture may be concentrated and any protein material removed by ultrafiltration. Suitable volumes of the various strains to be included in the final vaccine can then be blended, and adjuvant and preservative added, if appropriate.

3. **In-process control**

During production daily or twice daily sub-samples should be taken and monitored for growth of leptospires and absence of contaminants. Growth is monitored either by counting leptospires in a counting chamber under dark-field microscopy or by a nephelometer. The absence of contamination can be monitored by the microscopic examination of Gram-stained preparations of spun culture.

Immediately prior to inactivation, a sample should be taken for checking against its homologous antiserum in a MAT. The inactivated culture must be checked for freedom from viable leptospires. This is done by inoculating aliquots of inactivated culture into an appropriate growth medium such as the medium of Johnson and Harris (22), incubating at 29±1°C for at least 4 weeks and examining weekly by dark-field microscopy for the presence of viable leptospires.

After blending, the levels of free inactivating agents, minerals present in adjuvants (such as aluminium) and preservatives (such as thiomersal) must be within prescribed limits.

4. **Batch control**

a) **Sterility**

Selected final container samples of the completed vaccine should be tested for the absence of viable bacteria and fungi (7, 17, 18, 35). Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

b) **Safety**

Final container samples of completed product should be tested for safety. Methods for this have been described elsewhere (6, 17, 36).
c) Potency

Samples of completed vaccine should be tested for potency in hamsters. Potency is usually measured by the vaccine's ability to prevent the death of the animal when challenged with between 10 and 10,000 LD$_{50}$. With some serovars, e.g., serovar hardjo, which are not hamster or guinea-pig lethal, potency must be measured against prevention of renal infection when the animals are challenged with between 10 and 10,000 ID$_{50}$.

d) Duration of immunity

Duration of immunity should be checked in the animal species for which the vaccine is intended. Vaccinal immunity should persist for at least 6 months.

e) Stability

When stored under the prescribed conditions, the vaccines may be expected to retain their potency for at least 2 years. Stability should be assessed by determining potency after storage at 4°C, room temperature and 37°C.

REFERENCES


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36. United States Department of Agriculture Standard Requirements. § 113.38.

Q fever occurs in most parts of the world and is caused by the organism Coxiella burnetii. It is responsible for febrile disease in man and various species of animals. In man it can cause influenza-like symptoms, granulomatous hepatitis and endocarditis. In cattle, sheep and goats it can induce abortion. In a flock or herd which is already infected and where abortion has occurred, the recovery of the organism may not in itself be conclusive since it can be isolated from the placenta after a normal parturition. It is present in the milk of infected animals, and pulmonary infection without gross lesions has been reported. Ticks may play an important role in the spread of the disease.

Personnel who handle C. burnetii should remember that the organism is hazardous and strict precautions must be observed in any manipulations.

Identification of agent: Samples should be collected from the placenta and vaginal discharges, from the freshly aborted fetus, as well as from milk or colostrum where available. The organism can be demonstrated by high-power microscopy in smears of placenta or other material after they have been suitably stained with 2% basic fuchsin and counter-stained with methylene blue or malachite green. These findings, when coupled with serological investigations, are sufficient for practical diagnosis. In doubtful cases and for more extensive investigations, the inoculation of laboratory animals, such as guinea pigs, is necessary.

Serological tests: Various techniques can be employed, such as complement fixation (CF), the indirect fluorescent antibody test, micro-agglutination, capillary agglutination, allergic tests and enzyme-linked immunosorbent assay (ELISA). CF antibody titres of 1:160-1:1,280 are indicative of a recent infection.

Requirements for biological products: Q fever vaccines are inactivated vaccines produced from the infected yolk sacs of embryonated chicken eggs. They may or may not incorporate an adjuvant such as an oil emulsion. An annual booster inoculation is recommended; the vaccine may be combined with a Chlamydia vaccine and so be suitable for use in sheep, goats and cattle.

A. DIAGNOSTIC TECHNIQUES

Q fever is an infectious disease which occurs in most parts of the world and is caused by the organism Coxiella burnetii. Different strains may show differences in pathogenicity. C. burnetii is responsible for febrile disease in man and various
species of animals. In man it can cause influenza-like symptoms, granulomatous hepatitis and endocarditis (6, 9). In cattle, sheep and goats it can induce abortion.

Serological evidence of the presence of *C. burnetii* has been found in most countries in Europe, Asia, Oceania and the Americas. Although this may indicate the presence of Q fever in a given species in a given country, it does not necessarily mean that the organism is responsible for the disease. Nor is the isolation of *C. burnetii* or the demonstration of specific antibodies always a definitive indication of the disease itself, since in an infected herd or flock in which rickettsial abortion may or may not have occurred recently, it can be recovered – albeit rarely – from the placenta after normal parturition. It is present in the milk of infected animals, and pulmonary infection without gross lesions has been reported. Ticks may play an important role in the spread of the disease.

It is most important to remember that *C. burnetii* is hazardous to man and must therefore be handled with appropriate precautions.

1. **Identification of the agent**

Samples should be collected from the placenta and vaginal discharges soon after abortion. Milk or colostrum samples can also be taken. *C. burnetii* can be demonstrated in various ways, depending on the type of sample and the purpose of diagnosis.

Smears are prepared on microscope slides of placental tissue from a case of abortion suspected of being due to a rickettsial infection. These are stained by a rapid method (Stamp) using 2% basic fuchsin solution, followed by rapid decolouration with 3% acetic acid solution, and counter-staining with 1% methylene blue or malachite green solution. The smears are examined microscopically (x 500). A very large number of thin, pink-staining bacillary bodies will be seen against a blue or green background. They may sometimes be difficult to detect because of their small size (0.3-1.5 μm long x 0.25 μm wide). Their small size is compensated for by their large numbers, which appear as red masses against the blue or green background. Microscopically, *Coxiella* can be confused with *Chlamydia* and *Brucella*. However, by the same staining procedure *Chlamydia* have sharper outlines, are round, small and may resemble globules. *Brucella* organisms are larger (0.6-1.5 μm long and 0.5-0.7 μm wide), more clearly defined and stain more intensely. Diagnosis made on the basis of microscopy, coupled with positive serological results, is usually adequate for routine purposes. Known positive slides of *Coxiella*, *Chlamydia* and *Brucella* should be available for comparison.

For specific laboratory investigations it may be necessary to isolate the agent in culture (5). Where microscopical examination has revealed large numbers of *Coxiella* and there appears to be only a low contamination rate with other bacteria, direct isolation by inoculation of embryonated chicken eggs may be attempted. For this a portion of placenta is homogenised in phosphate-buffered saline (PBS) containing streptomycin and penicillin. After low-speed centrifugation, dilutions of the supernatant fluid are inoculated into 5-day-old embryonated chicken eggs via the yolk sac. Any embryos which die during the first 5 days after inoculation are
discarded. The yolk sacs are harvested after 10-15 days of incubation. Stained smears of the yolk sac wall are examined to ensure the absence of bacterial contamination but to determine the presence of *Coxiella*. Further passages may be required to obtain an isolate in pure culture. Microscopical observation of rickettsial bodies is adequate for confirmation of diagnosis.

In the case of a more heavily contaminated sample, such as a placenta, or where there is only a small number of *Coxiella* in a body fluid, such as in milk, the inoculation of laboratory animals may be necessary. Mice and guinea pigs are best for this purpose. Guinea pigs are used in preference since they are about ten-fold more susceptible than mice. Following intraperitoneal inoculation, body temperature and antibody status are monitored. If pyrexia develops, the animal is killed and the spleen removed for recovery of the agent by inoculation into embryonated chicken eggs. This method should always be performed in conjunction with serological tests on other guinea pigs which have been inoculated with the same samples. These sera are collected 21 days after inoculation. Any positive results will confirm a diagnosis of *C. burnetii*.

2. **Serological tests**

Various techniques can be employed. The complement fixation test is used most often, generally employing the cold technique of Kolmer. The antigen should contain two strains, namely, the bovine Nine Mile strain and the human/ovine Henzerling strain. Anti-sheep red-cell serum diluted 1:1,000 is mixed with a 1.15% suspension of erythrocytes. Consideration should be given to the testing of acute phase and convalescent phase sera for diagnosis (6).

Serum titres between 1:10 and 1:40 indicate a past infection but titres of 1:160-1,280 indicate recent infection; such titres are obtained especially at the time of abortion.

The sandwich technique of indirect fluorescent antibody testing is also used, particularly in human medicine. A very pure antigen prepared by differential centrifugation is required (5, 7, 9).

The micro-agglutination technique of Giroud (9) is now being superseded by other tests. A drop of a suspension of *C. burnetii* is deposited on a slide and mixed with one drop of a dilution of the serum under test. In a serological survey, a single dilution of 1:20 is adequate for detecting positive samples. The slide is incubated at 37°C in a humid chamber for 12 hours, after which it is dried and stained with Giemsa. The preparation is examined microscopically for the presence of clumps of organisms.

For an allergic test, an inactivated, purified suspension of the organism is inoculated into the skin or into the eyelid. This test can be used for epidemiological surveys, but it is not much used now (3).

In a capillary agglutination test (CAT) (10), antigen is introduced into a capillary tube followed by the serum under test. After 18 hours the tube is examined for the presence of visible agglutination.
An enzyme-linked immunosorbent assay (ELISA) (2) can be used to monitor antibodies against *C. burnetii* in mass screening of animals when Q fever is a suspected biohazard.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

The vaccine against Q fever is an inactivated vaccine produced from a standard strain of *C. burnetii* which has been cultured in the yolk sacs of embryonated chicken eggs. When grown in eggs it is easy to inactivate. The vaccine may or may not incorporate an adjuvant; an annual booster dose is usually required. Vaccine strains should be analysed and carefully selected since recent studies with restriction endonucleases, as well as serological investigations with different antigens of *C. burnetii*, have demonstrated an antigenic heterogeneity independent of phase variation (13).

1. **Seed management**

a) **Characteristics**

The standard strain used for vaccine production is often the Nine Mile strain (ATCC VR 146). This is of bovine origin in phase II and is of lower virulence for laboratory personnel than are the more recent phase I strains.

b) **Culture**

Culture in embryonated chicken eggs usually diminishes the virulence of *C. burnetii* strains by converting them to phase II. On the other hand, passage in animals may restore any loss of virulence and maintain them in phase I. Stocks of seed *C. burnetii* in the form of a yolk sac extract are either lyophilised or placed in ampoules in the liquid state and stored at -80°C.

c) **Validation as a vaccine**

The vaccine is injected subcutaneously, behind the shoulder in sheep or goats, and into the neck or dewlap of cattle. The oil adjuvant produces some reaction after inoculation, particularly in heavily infected flocks or herds. This is principally a local inflammatory reaction to the adjuvant rather than an allergic reaction. The protocol of vaccination usually consists of one subcutaneous injection, preferably 15 days before mating. In heavily infected environments, a second inoculation may be given a month later. An annual booster dose is required.

Vaccination reduces but does not abolish the discharge of *C. burnetii* after parturition (1, 3, 4). In an infected herd of vaccinated cattle, there is a considerable reduction in the number of cases of endometritis, infertility and weak calves (7, 11, 12, 14, 15). The best results are obtained by the combination of vaccination and antibiotic treatments (12).
2. **Manufacture**

The eggs used for vaccine production may be commercial eggs, but they must originate from hens that have not been fed an antibiotic (such as oxytetracycline), residues of which would suppress any multiplication of *C. burnetii*.

After 5-6 days' incubation, embryonated chicken eggs are inoculated via the yolk sac and harvested between 12-15 days, by which time many of the embryos will have died. Infected yolk sacs have a characteristic straw yellow colour. Embryos which die between 5-10 days of incubation are discarded.

The virulent strain used for egg inoculation is a homogenate of yolk sac in PBS diluted 1:100 containing penicillin (500 IU/ml) and streptomycin (0.5 mg/ml). The yolk sacs are pooled and one part is homogenised in an airtight homogeniser (Sorvall type) with three parts of PBS. The homogeniser must be airtight to prevent dissemination of infection.

The suspension is inactivated with 1.6% formaldehyde for 24 hours at 37°C and then stored at 4°C until required. When the vaccine has passed the tests for innocuity, the supernatant fluid is decanted and the remainder centrifuged at low speed for 10 minutes. The supernatant fluid is decanted and the process is repeated. The suspension of *C. burnetii* is diluted in PBS to provide 20 doses of vaccine per egg, or 20 ml per egg in the case of an oil emulsion vaccine used in a dose of 2 ml. Merthiolate (thiomersal) is added as a preservative to a final dilution of 1:5,000.

Q fever vaccine may be used by itself or in combination with *Chlamydia* vaccine, which is a suitable combination for sheep, cattle and goats (non-pregnant females). Inactivated Q fever vaccines are usually of the oil emulsion type, prepared from one part of inactivated *Coxiella* suspension in PBS, and one part of the oil phase, such as Montanide 80, Marcol 82 and a water-dispersible emulsifier. The aqueous phase is added slowly, whilst stirring vigorously, to the oil phase in an airtight container. Stirring is continued for 10 minutes.

3. **In-process control**

The abundance of *C. burnetii* and the absence of bacterial contaminants during manufacture is verified microscopically from a smear of yolk sac stained according to Stamp.

4. **Batch control**

a) **Sterility**

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.
b) Safety tests

The detection of *C. burnetii* in a stained smear is sufficient to confirm that the relevant culture is the Q fever agent. This also provides information on its numbers.

The inactivated yolk sac homogenate is inoculated into the yolk sac of batches of ten 5-day embryonated eggs. The eggs are opened one week later, at which time the yolk sacs should reveal no abnormality. A second subculture is carried out. In addition, a double dose of vaccine is inoculated into two animals of the target species.

c) Potency tests

It is difficult to titrate homogenised yolk sac suspension by egg inoculation because embryonic mortality is not directly related to the degree of dilution. Microscopical observation provides an indication of the strength of a suspension.

**Laboratory animals:** Potency for laboratory animals is assessed by the titre and kinetics of the production of complement-fixing antibodies to *C. burnetii*. Rabbits are generally used for this purpose. Samples of blood are taken from 5 to 10 rabbits, which then receive 2 ml of vaccine (or one sheep dose). A second blood sample is taken after 2-3 weeks, which should show an increase in CF antibody titre of the order of 1:20-1:80 (8). The appearance of complement-fixing antibodies after the vaccination of the target animal can be irregular.

d) Duration of immunity

The protocol of vaccination usually consists of one subcutaneous injection, preferably 15 days before mating. In heavily infected environments, a second inoculation may be given a month later. An annual booster dose is required (15).

e) Stability

Fluid inactivated vaccine that is ready for immediate use can be stored for 2 years at 2-6°C.

REFERENCES


Rabies is a major zoonosis for which diagnostic techniques have been standardised internationally. As there is no gross pathognomonic lesion for rabies, diagnosis can only be made in the laboratory. Laboratory techniques are preferably conducted on central nervous tissue previously removed from the cranium. The hippocampus (Ammon's horn) and the medulla oblongata are the tissues of choice.

Identification of the agent: This is preferably done by using the fluorescent antibody test (FAT). A drop of immune serum prepared against viral nucleocapsid and previously conjugated with fluorescein isothiocyanate is added to a brain tissue smear made preferably from the hippocampus. For a large number of samples, as in an epidemiological survey, the immunoenzyme technique can provide rapid results (the rapid rabies enzyme immunodiagnosis, or RREID). Both the FAT and RREID provide a reliable diagnosis in 98-100% of cases.

To demonstrate infected neuronal cells, a smear of fresh hippocampal tissue on a microscope slide can also be stained by Seller's method, or a histological section stained by Mann's method. Both procedures will reveal aggregates of red staining viral material, the Negri bodies. Nevertheless these techniques have the disadvantage of producing a proportion (up to 15%) of false negative results.

Since a negative test on fresh material does not rule out the possibility of infection, mouse inoculation tests should be carried out simultaneously. Newborn mice or mice are inoculated intracerebrally with a suspension of hippocampal tissue, and then kept under observation for 28 days. For any that die between 5 and 28 days the cause of death should be confirmed by FAT.

Alternatively, a monolayer culture of neuroblastoma cells is inoculated with the same material as used for mice. FAT carried out 18-24 hours later will demonstrate the presence or absence of viral antigen. Under certain circumstances, this may require longer than 48 hours.

The identification of the agent can be supplemented in specialised laboratories by identifying any variant virus strains by the use of monoclonal antisera, specific nucleic acid probes, or the polymerase chain reaction (PCR) followed by DNA sequencing of genomic areas. Such techniques can distinguish between field and vaccine strains, and possibly identify the geographical origin of the field strains.

Serological tests: Virus neutralisation (VN) assays may be made in mice, a rapid fluorescent focus inhibition test (RFFIT) can be conducted in cell
cultures, or else some other test carried out that is known to correlate with the VN, notably enzyme immunoassay (ELISA). Results are expressed in international units or equivalent units relative to a standard antiserum.

Requirements for biological products: Rabies vaccines for use in animals contain either live virus attenuated for the target species (such as Flury, SAD or Kelev), or virus inactivated by chemical or physical means, or are recombinant vaccines. The virus is cultivated in the central nervous tissue of newborn animals, in embryonated eggs, or in cell cultures.

Rabies vaccines are usually lyophilised but inactivated virus vaccines may be used in fluid form, preferably with an adjuvant.

Before newly developed vaccines can be licensed, the duration of immunity resulting from their use should be determined in vaccinated animals of the target species. All vaccines should remain stable for at least one year.

For live virus vaccines the minimum virus content which will guarantee virus replication in the host, and therefore elicit an adequate immune response, has to be established.

The potency of inactivated virus vaccines is established by mouse vaccination followed by intracerebral challenge. Tests formulated by the National Institutes of Health (NIH) in the USA and the European Pharmacopoeia employ graded doses of vaccine, capable of protecting a variable percentage of mice against a challenge with a fixed dose of virus lethal for unvaccinated mice. A reference vaccine enables results to be expressed in units. In vitro techniques which have been shown to correlate with the above test, can also be used for in-process control. As a minimum requirement the final products of both types of vaccine are subjected to tests for innocuity and absence of toxicity.

For live vaccines which are prepared for oral vaccination of wild (or domestic) animals, innocuity and efficacy in target animals and safety in non-target species must be demonstrated.

A. Diagnostic Techniques

Rabies is caused by a neurotropic virus of the genus *Lyssavirus* of the family *Rhabdoviridae*, and is transmissible to all warm-blooded animals. Since it is transmissible to man by inoculation or inhalation of infectious virus, all suspected infected material must be handled under the appropriate safety conditions specified by WHO (8).

Four serotypes can be distinguished within the genus *Lyssavirus* by cross-protection tests (3, 13), namely the true rabies virus itself (serotype 1), Lagos bat virus (serotype 2), Mokola rhabdovirus (serotype 3) and Duvenhage rhabdovirus
(serotype 4). The European bat lyssaviruses (EBL), subdivided into two biotypes, EBL1 and EBL2, are also members of the Lyssavirus genus but are not yet classified into serotypes. Viruses of serotypes 2-4 and EBL are known as rabies-like viruses. The use of monoclonal antibodies directed against viral nucleocapsid or glycoprotein antigens and sequencing of defined genomic areas has made possible the definition of numerous subtypes within each serotype.

As no clinical signs or gross post-mortem lesion can be considered as pathognomonic, the diagnosis of rabies has to rely on laboratory facilities. Serological evidence of infection is rarely obtained because of the high mortality rate of host species, although such evidence may be used in some epidemiological surveys.

1. **Identification of the agent**

Methods for identifying rabies virus which have been standardised by WHO vary in their efficiency, specificity and reliability (8). They are all applied to brain tissue, the virus being particularly abundant in the hippocampus (Ammon's horn) and the medulla oblongata, and this involves removal of the whole brain. In some cases (e.g. large epidemiological surveys) a simplified method of sampling through the occipital foramen (5) of the orbital cavity can be used (10).

There are essentially three procedures:

a) **Identification of infection in neurons**

A direct tissue smear of hippocampus on a slide is stained by Seller's method. Evidence of infection is provided by the presence of intracytoplasmic viral material in the form of Negri bodies. Ideally, tissue from several areas of the brain should be examined. The hippocampus is usually chosen because it has larger neurons in which Negri bodies are easily seen. Alternatively, tissue can be fixed and histological sections stained with Mann's stain. There has been a tendency to abandon these methods, notably the Seller's method, because they may yield up to 15% or more false negative results. Their advantage lies in their rapidity in yielding results within 24-48 hours, the low cost of the laboratory equipment, and the avoidance of any need to keep specimens cold for histological examination, which is an advantage under tropical conditions. They are generally used to complement other available methods.

b) **Infectivity for animals or cell cultures**

The best animals are specific pathogen free (SPF) newborn mice. A litter of newborn mice, 48 hours old, is inoculated intracerebrally with 0.015 ml of a 20% (w/v) suspension of hippocampal tissue in an isotonic medium, such as Hanks', containing antibiotics. If possible, suspensions of tissue from other parts of the brain (medulla oblongata, cerebellum, cortex) should be examined, as they may contain virus even when none is present in the Ammon's horn. Needles measuring 0.45 x 12 mm are used. The mice are kept under observation until the 21st day. If no deaths have occurred on the fifth day, one
baby mouse is sacrificed and immunofluorescence is done on the brain on days 5, 7, 9, and 11. On the 21st day all the surviving baby mice are killed and submitted to immunofluorescence testing. This test can also be performed on SPF Swiss mice, 3-4 weeks old, used in a group of five. These are inoculated intracerebrally under light anaesthesia with 0.03 ml of the same suspension. The mice are kept under observation for at least 21 days, and rabies is confirmed if any die showing paralysis or other nervous disorders from the fifth day. All cases should be confirmed by specific immunofluorescence (see below).

The optimum cells for virus isolation are neuroblastoma cells (N2a cells identified as CCL 131 in the ATCC) cultured in an atmosphere of carbon dioxide in an appropriate medium such as Dulbecco modified minimum essential medium containing calf serum. The cultures are inoculated with the same material as the mice. After 18-24 hours the presence of rabies viral nucleocapsid antigen can be demonstrated by specific immunofluorescence (see below).

c) Specific immunofluorescence

The fluorescent antibody test (FAT) carried out by conventional fluorescence microscopy is the best current method; it gives reliable results within a few hours in 98-100% of cases, depending on the animal species involved and the type of virus present (7). It should be carried out on fresh specimens. A drop of specific rabies antiserum is applied to a smear of the tissues under test, preferably hippocampus. This serum is directed against rabies nucleocapsid antigen. It is available commercially, already conjugated with fluorescein isothiocyanate. Aggregates of nucleocapsid may be identified by their fluorescence. If the antiserum is conjugated with an enzyme, such as a peroxidase, a specific reaction can be observed by light microscopy. A variation of this technique (11) consists of placing the clarified supernatant of the suspect tissue homogenised in buffer solution in the well of the plate previously coated with specific antibody. Any bound virus is revealed by an immunosorbent assay (the rapid rabies enzyme immunodiagnosis or RREID test). Results can be read by eye, or by spectrophotometry that can be automated for large-scale epidemiological surveys. In certain cases formaldehyde-treated tissues can be examined after controlled enzymic digestion with pepsin or trypsin (4).

The tests above (a, b, c) may be complemented in specialised laboratories (such as OIE or WHO reference laboratories) using monoclonal antisera, nucleic probes, or the polymerase chain reaction (PCR) followed by DNA sequencing of genomic areas for typing the virus (7). This enables a distinction to be made between vaccine virus and a field strain of virus, and possibly the geographical origin of the latter.

2. Serological tests

Serological tests are rarely used in epidemiological surveys, due to the low percentage of animals surviving the disease and therefore having post-infection
antibodies. Serological tests used to assess the potency of the vaccine are listed in point 4c below.

The VN tests are recommended and can be performed either in mice or cell culture.

a) Virus neutralisation in mice

The principle of this test is the neutralisation in vitro of a constant amount of rabies virus (50 LD$_{50}$/0.03 ml of "Challenge Virus Standard" [CVS] strain) by varying quantities of the serum to be titrated, during an incubation of 90 min at 37°C. The virus-serum mixture (0.03 ml) is inoculated into the brain of 3-week-old mice. The serum titre is the final dilution of serum in the virus-serum mixture which protects 50% of the mice (mortality is 100% in the absence of neutralisation). This titre can be expressed in international units (IU) by comparing it with the neutralising dilution of a reference serum under the same experimental conditions.

To perform the test, thaw an ampoule of CVS virus and prepare a suspension containing 100 LD$_{50}$/0.03 ml (because this will be diluted twofold by addition of the same volume of test serum before being injected). The amount of virus actually used during the test (permissible limits: 30-300 LD$_{50}$/0.03 ml) is checked by titrating four dilutions of the viral preparation, which are each inoculated into five mice. The test sera are heated at 56°C for 30 min to inactivate any complement.

A reference serum must be included in order to check the titration conditions. The maximum allowable difference between its expected neutralising capacity and that measured during the titration, is $10^{0.5}$. The greatest dilution must not neutralise the virus. The diluent is the same as that used for the viral preparation.

To each serum dilution is added an equal volume of the viral preparation containing 100 LD$_{50}$/0.03 ml. The mixtures are incubated in a 37°C waterbath for 90 min. The reaction is stopped by immersion in melting ice. During inoculation, the tubes that are not used immediately are kept at +4°C.

For each dilution, five mice are inoculated intracerebrally with 0.03 ml of the serum-virus mixture. Mortality is recorded during 21 days after inoculation, although deaths occurring during the first four days are regarded as non-specific (due to shock, infection, etc.). The serum titre can be calculated in IU by comparison with an international reference serum.

b) Virus neutralisation test in cells
(Rapid fluorescent focus inhibition test, RFFIT)

The principle of the test is the neutralisation in vitro of a constant amount of rabies virus (the CVS strain previously adapted to cell culture) before inoculating intact cell monolayers derived from cells susceptible to rabies virus
(e.g. baby hamster kidney [BHK] cells).

The serum titre is the dilution which inhibits half of the number of fluorescent fields established by an unneutralised suspension of test virus. This titre can be expressed in IU by comparing it with the neutralising dilution of a reference serum under the same experimental conditions.

To perform this test it is necessary to prepare a 75 ml flask of cells the day before titration, and from this a suspension of $10^5$ cells per ml, placed in plastic chambers for cell culture, attached to microscope slides ('Labtek').

On the day of titration an ampoule of CVS virus is thawed. The test dilution should provide 20 fluorescent foci readily visible in 20 microscope fields. In order to verify the dose of virus actually used, the virus preparation is titrated in four dilutions, each inoculated into two chambers. The viral suspension should have infected 80% of the cells after 24 hours of incubation.

Inactivation of complement and dilution of the test serum and control serum, followed by virus neutralisation, is performed as for mouse inoculation (section 2a above). A volume of 0.2 ml of each serum-virus mixture is placed in contact with an intact cell monolayer at the base of the previously empty 'Labtek' chamber. After incubation for 30 min at room temperature, 0.3 ml of medium is added to each chamber, and the slides are kept at 37°C for 48 h. The walls of the 'Labtek' chambers are then removed and the slides are carefully dried in air, then fixed in acetone at -20°C for 30 min. An antinucleocapsid fluorescent conjugate (as used in the fluorescent antibody test) is then applied to the slide for 30 min. The slides are rinsed in tap water by slow immersion (to avoid detachment of the cells), and then mounted in glycerol.

The result is read 30 min later at a magnification of 100 by examining 20 microscope fields for each chamber. The number of foci for each dilution is noted. A dilution which gives no more than 50% of the number of fluorescent foci observed in the virus-only control is calculated by linear interpolation or by a computerised neoprobit procedure (1). Serum titres are calculated by comparison with an international standard reference serum (or a sub-standard serum).

There is also a variation of the RFFIT technique performed on a plate of 96 wells (microtitration). The plate is stained and read directly using an inverted microscope.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

Subtypes of rabies virus may vary considerably in their pathogenicity. They can be classified according to origin as vulpine, canine, etc. However, with the exception of serotype 3, their immunogenicity provides almost complete cross-protection. Thus vaccines prepared from the original 1885 strain of Pasteur and its derivatives (strains Pasteur Virus, Challenge Virus Standard, Pitman-Moore, etc.) and strains
isolated more recently (Flury, Street Alabama Dufferin [SAD], Vnukovo and Kelev) protect against all strains so far isolated of serotype 1. The principles governing the preparation of inactivated rabies vaccines are identical whether they are to be used in man or animals, although an adjuvant may be added to vaccines for animal use.

For animals live vaccines are also effective by the oral route and can be distributed in baits in order to immunise wild (or domestic) animals (9). Live recombinant vaccine (e.g. rabies glycoprotein expressed in the vaccinia virus) has also proven to be effective.

Different standards apply to vaccines containing live virus modified by passage in animals, eggs or cell cultures to reduce its virulence for the target animal, and to vaccines prepared from virus inactivated by chemical agents. Both types of vaccine have their advantages and disadvantages (3), but they can both be used to immunise animals for periods of between one and three years. They are not to be relied on to protect previously unvaccinated animals which have been exposed to infection (6). Man is the only species in which the efficiency of post-exposure treatment with vaccine alone has been proven.

All handling of the virus during manufacture and testing of vaccines must conform with the strict safety precautions specified by WHO (8, 13).

1. **Seed management**

Whatever the type of vaccine, except for recombinant vaccine, the initial step in vaccine production is to inoculate the virus into eggs, cell cultures or animals.

a) **Characteristics**

Any strain belonging to serotype 1, which has been proved to protect against field rabies viruses (currently found in the country where the vaccine is recommended) is suitable. The strain of virus used must be of very well known biological (e.g. pathogenicity) and serological properties (typing by monoclonal antibodies).

b) **Culture**

A master cell stock of the seed virus should be prepared and kept deep frozen. Sub-culture from this stock will be used for vaccine production. Virus multiplication is verified by titration during growth of the seed virus.

c) **Validation as a vaccine**

Before a vaccine is licensed, duration of immunity should be established by the challenge of vaccinated and control aniams of the target species. Antibody kinetics are also determined in order to establish the correlation between antibody titre and resistance to challenge.
Duration of immunity is not tested for every batch, but is usually deduced from the antigenic value for inactivated vaccines, or from the virus titre for live vaccines (see point 4c below).

For the purpose of licensing a vaccine, duration of immunity should be determined with precision by tests in each species for which the vaccine is recommended, and by antibody kinetics measured by a neutralisation test in mice or in cell cultures (see point A2).

The correlation between potency in the target species and antigenic value as estimated in mice should be established (see point B4c).

Vaccine stability is ascertained by testing batches after prolonged storage, usually 1-2 years. A process of accelerated aging, by storage at 37°C for a week, is sometimes used. The storage life claimed by the manufacturer is checked by the national licensing authority. In general it is 12-18 months for fluid vaccines and possibly 24 months for lyophilised vaccines.

2. **Manufacture**

Whatever method is adopted, close attention must be paid to the quality of the substrate: both animals and eggs should be of specific pathogen free (SPF) origin and the cell cultures, such as baby hamster kidney (BHK) cell lines, must conform to international standards of sterility and innocuity.

a) **In animals**

The virus is inoculated intracerebrally and the nervous tissue harvested when the animal has been sacrificed in the terminal stages of rabies. The virus is inactivated by physical methods, such as irradiation with ultraviolet light, or by chemical methods such as the addition of phenol, β-propiolactone, or an imine. The vaccines should be prepared in young animals (mice, lambs, etc.) for high yield of virus. In certain cases the virus is not inactivated entirely, as for example in the phenol-treated vaccines of the Fermi type, but such vaccines are not currently recommended.

b) **In eggs**

A modified strain of virus is inoculated into SPF embryonated chicken eggs which are then incubated at 38°C for 5-6 days. The virus is harvested in the form of infective embryo tissues, and is usually lyophilised and used as a live vaccine. Examples of such vaccines include those that contain the Flury low-egg-passage (LEP) or the more desirable high-egg-passage (HEP) variant strain, which is safer for some animal species (e.g. the cat).

c) **In cell cultures**

Cultures are infected with virus and incubated at 35-36°C. These may then be used as live virus vaccines (as in Flury and SAD vaccines), or as inactivated
vaccines after the addition of phenol (Semple vaccine) or some other chemical like β-propiolactone.

During manufacture the multiplication of the virus in one of the substrates mentioned above is monitored, followed by harvesting at the most appropriate time, usually 4-6 days after inoculation of animals, eggs or cell cultures. The virus harvest is suspended in a buffer solution at a dilution which will provide an optimum antigenicity of the end product. If required, the suspension is either inactivated or lyophilised. An adjuvant is recommended for vaccines prepared from inactivated virus, as well as for other vaccine antigens which may be incorporated in polyvalent vaccines.

Cell culture can also be used to grow the vector viruses (e.g. vaccinia virus) harbouring the gene coding for the expression of rabies virus glycoprotein (9).

3. **In-process control**

This consists of monitoring virus growth to provide an optimum titre and ensure the absence of undesirable microbial contamination.

**In live virus vaccines**, kinetics of virus growth should be established, in order to ensure a final titre of virus correlated to the desired protection in target species.

**In inactivated virus vaccines**, immunogenic properties of the final product may be evaluated by *in vitro* techniques (e.g. ELISA, agar gel immunodiffusion, antibody binding tests or infected cell staining). These evaluations will indicate the best time for harvesting the virus in cell cultures.

4. **Batch control**

a) **Sterility tests**

Tests for sterility and freedom from contamination of biological materials may be found in the Chapter on General Information.

b) **Safety tests**

For the purposes of licensing a vaccine safety tests should be conducted in the target species. In the case of *live virus vaccines* (including recombinant vaccines), safety tests should also be carried out on those target species which live in the area of vaccination (3).

Safety tests for batches of *inactivated virus vaccines* are carried out by inoculation in cell culture or intracerebrally into mice to detect viable virus. Currently *live virus vaccine* batches are not tested for safety.

c) **Potency tests**

For *live virus vaccines*, these are carried out by titrating the amount of virus
present in the vaccine. Once a correlation has been established between the activity of the vaccine in the target species and virus titres, virus titrations become reliable indicators of vaccine efficacy. This is carried out using cell cultures or by the intracerebral inoculation of suckling mice.

For inactivated virus vaccines, correlation between potency in the target species and antigenic value as estimated in mice provides a reliable indicator of vaccine activity (see point B1c). The potency of the vaccine is established by the National Institutes of Health (NIH) test or the European Pharmacopoeia test.

Groups of at least 10 mice, aged 3-4 weeks, are inoculated with single, decreasing doses of vaccine in accordance with the European Pharmacopoeia (12), or with 2 doses, a week apart, according to the NIH test (8). A sufficient number of dilutions of vaccine are compared to estimate the dilution at which 50% of the mice are protected against intracerebral challenge 14 days later (8, 12).

A WHO international reference vaccine is available so that the results of testing for antigenicity can be expressed in international units (IU). The test is not valid unless:
- for both the vaccine to be examined and the reference preparation the PD50 (protective dose) lies between the largest and smallest doses given to the mice;
- the titration of the challenge suspension shows that 0.03 ml of the suspension contained at least 10 ID50;
- the confidence interval (P = 0.95) is not less than 25% and not more than 400% of the estimated potency;
- the statistical analysis shows a significant slope and no significant deviations from linearity or parallelism of the dose-response lines.

The vaccine passes the test if the estimated potency is not less than 1 IU in the smallest prescribed dose.

A simplified test can also be used for the purpose of anticipating which vaccines are likely to be of an antigenic value ≥ 1 IU (2).

REFERENCES


Paratuberculosis is a chronic enteritis of ruminants caused by Mycobacterium paratuberculosis (johnei).

Identification of the agent: The diagnosis of paratuberculosis is divided into two parts: the diagnosis of clinical disease, and the detection of sub-clinical infection, which is essential for control of the disease at farm, national or international level.

Diagnosis of paratuberculosis is made on clinical grounds confirmed by the demonstration of Mycobacterium paratuberculosis in the faeces by microscopy, culture or by the DNA probes which are being developed. Serology may also be used in assessing the presence of paratuberculosis in clinically suspect cases. Diagnosis is made at necropsy by the finding of the pathognomonic lesions of the disease in the intestines either grossly or histologically and by isolation of M. paratuberculosis in culture.

The detection of subclinical infection depends on the demonstration of delayed-type hypersensitivity to johnin or to avian tuberculin, detection of specific antibodies by serology, or culture of M. paratuberculosis from faeces. The choice of test depends on the circumstances and the degree of sensitivity required at individual animal or herd level.

Cultures of M. paratuberculosis may be obtained from faeces or tissues after treatment to eliminate contaminants, by inoculation onto artificial media with and without the specific growth factor, mycobactin, which is essential for the growth of M. paratuberculosis.

Serological tests: Enzyme linked immunosorbent assays (ELISA) are the most sensitive tests for serum antibodies to M. paratuberculosis. Although lacking in the specificity of faecal culture, they provide a cheap and quick means of assessing this parameter, particularly in the case of high bacterial load. Dot immunoblotting assays are of comparable efficacy to the ELISA procedures. They are simple, fast, and cheap, and have the potential for field use. The agar gel immunodiffusion and complement fixation test are also useful for assessing suspected clinical cases. However, both tests lack the sensitivity of the ELISA for bovine paratuberculosis. For ovine paratuberculosis, however, the agar gel immunodiffusion test is quite sensitive.

Requirements for biological products: Vaccines for paratuberculosis may be live attenuated or killed bacteria either incorporated with an adjuvant or lyophilised and adjuvanted on reconstitution. Bacterial counting is difficult and bacterial content of vaccines may be based on weight, while vaccine potency may be judged by batch tests for sensitising ability in guinea pigs.
Vaccine safety or abnormal toxicity may also be tested in guinea pigs.

*Johnin,* for diagnostic skin tests, is a purified protein derivative of a heat-treated culture of *M. paratuberculosis.* *Johnin* is standardised for content of purified protein derivative by chemical assay and its biological activity is identified in guinea pigs sensitised with *M. paratuberculosis.*

### A. DIAGNOSTIC TECHNIQUES

Paratuberculosis is a chronic enteritis of ruminants (principally cattle, sheep, goats, deer, buffalo and camels) affecting adults of the species. Clinical signs are a slowly progressive wasting and the occurrence of diarrhoea which is intermittent at first and becomes progressively more severe until it is constantly present (3). Diarrhoea is less common in small ruminants. It is caused by *Mycobacterium paratuberculosis* (*johnei*). Infection is usually acquired by ingestion of *M. paratuberculosis*-infected faeces from clinical cases or sub-clinically infected carriers, or fomites contaminated with such faeces. Intra-uterine infection occurs in the offspring of a proportion of infected dams. Early lesions occur in the walls of the small intestine and the draining mesenteric lymph nodes and infection is confined to those sites at this stage. As the disease progresses gross lesions occur in the terminal small intestine, caecum and colon and in the mesenteric lymph nodes. *M. paratuberculosis* is present in the lesions and, terminally, throughout the body. The intestinal lesions are responsible for a protein leak and protein malabsorption which lead to the muscular wasting. Clinical signs usually first appear in young adulthood (after the second calving in cattle), but the disease can occur at any age over 1 to 2 years.

Within a few weeks of infection there is a phase of multiplication of *M. paratuberculosis* in the intestinal walls. Depending on the resistance of the individual this infection is eliminated or the animal remains infected as a healthy carrier. The proportions in these categories are unknown. A later phase of multiplication of the organisms in a proportion of these carriers leads to the extension of lesions, interference with gut metabolism and clinical signs of disease. Sub-clinical carriers excrete small numbers of *M. paratuberculosis* in the faeces. Larger numbers of organisms are excreted in the majority of cases as clinical disease develops. Delayed-type hypersensitivity (DTH) is intermittently or constantly present in a proportion of the sub-clinically infected carriers but as the disease progresses DTH wanes and may be absent in clinical cases. Serum antibodies are detectable later than DTH. They may also be present in carriers which have recovered from infection. Initially, serum antibodies are detected only intermittently but are present more constantly and are of higher titre as lesions become more extensive, reflecting the amount of antigen present.

Other mycobacterial diseases and infections including mammalian and avian tuberculosis cause DTH and the presence of serum antibodies. It follows therefore that these must be differentiated from *M. paratuberculosis* infection and paratuberculosis, clinically and by the use of specific diagnostic tests.

Animals vaccinated against paratuberculosis develop both DTH and serum
antibodies. Vaccination prevents clinical disease but not necessarily infection. Thus only tests to detect *M. paratuberculosis* in the faeces can be used if it is necessary to attempt a diagnosis of infection in vaccinates.

In individual animals, especially from a farm in which the disease has not previously been diagnosed, a tentative clinical diagnosis must be confirmed by laboratory tests. However, a definitive diagnosis is warranted on clinical grounds alone if the clinical signs are typical and the disease is known to be present in the herd. Diagnosis of paratuberculosis depends on the finding of either gross or microscopic pathognomonic lesions and the isolation in culture of *M. paratuberculosis*.

To confirm the presence of paratuberculosis in an individual clinically suspect animal a number of laboratory tests can be used – faecal smears, faecal culture, DNA probe in faeces, serology and necropsy.

Herd tests to detect subclinical infection are carried out to determine the prevalence of infection usually so that control measures can be instituted. As no test is 100% sensitive, control of the disease by the disposal of positive reactors depends on repeated tests at six-monthly or yearly intervals over a number of years and the elimination of reactors to serological tests or faecal shedders, but even these procedures are not always successful.

1. **Identification of the agent**

   a) **Necropsy**

   Paratuberculosis cannot be diagnosed on superficial examination of the intestines for signs of thickening. The intestines from the duodenum to the rectum should be opened to expose the mucosa. There is not a close correlation between the severity of clinical signs and the extent of intestinal lesions. The mucosa, especially of the terminal ileum, is inspected for the pathognomonic thickening and corrugation. Early lesions are seen by holding the intestine up to the light, when discrete plaques can be seen. The mesenteric lymph nodes may be enlarged and oedematous. Smears from the affected mucosa and cut surfaces of lymph nodes should be stained by Ziehl-Neelsen's method and examined microscopically for acid-fast organisms having the morphological characteristics of *M. paratuberculosis*. However, acid-flats are not present in all cases. Diagnosis is therefore best confirmed by the collection of multiple intestinal wall and mesenteric lymph node samples into fixative (10% formol saline) for subsequent histology. Haematoxylin and eosin and Ziehl-Neelsen stained sections should be examined. The pathognomonic lesions consist of infiltration of the lamina propria, Peyer's patches and the cortex of the mesenteric lymph nodes with large, pale-staining epitheloid cells and multi-nucleated Langhans' giant cells in both of which clumps or singly disposed acid-fast bacilli are usually but not invariably found. Langhans' giant cells are not invariably present.

In deer the lesions of both *M. tuberculosis* and *M. avium* infections may be similar to those of *M. paratuberculosis*. Sheep and goats infected with
non-pigmented strains of *M. paratuberculosis* that are difficult to cultivate have also been observed with caseonecrotic mesenteric lymph node lesions resembling lesions produced by *M. tuberculosis* or *M. avium*.

b) Faecal smears

Ziehl-Neelsen-stained smears of faeces are examined microscopically. A diagnosis of paratuberculosis can be made if clumps (3 or more organisms) of small, strongly acid-fast bacilli are found. The presence of single acid-fast bacilli in the absence of clumps does not warrant a definitive diagnosis. The disadvantages of this test are that only about one third of cases can be confirmed on microscopic examination of a single faecal sample, and other acid-fast objects occur in faeces.

c) Bacteriology

*M. paratuberculosis* is a small, acid-fast bacillus which grows slowly (4-8 weeks to visible growth) at 37-39°C in complex laboratory media which must contain a growth factor, mycobactin, extracted from mycobacteria. Colonies are small (1-2 mm in diameter), rough and off-white. The uncommon, bright yellow pigmented sheep strain is difficult to grow on artificial media (up to 9 months for visible colonies to develop). It has been reported that unpigmented sheep strains grow less well than cattle strains, and no cultures should be discarded as negative without prolonged incubation.

For identification of *M. paratuberculosis* small inocula of suspect colonies should be subcultured on the same medium with and without mycobactin. Reference 14 describes the laboratory preparation of a crude mycobactin.

Mycobactin can be obtained commercially (Mycobactin J) from Allied Laboratories Inc., 540 N. Traver Trail, Glenwood Springs, CO 81601, USA, or Rhône Mérieux IFFA Laboratories, 254 rue Marcel Mérieux, 69007 Lyon, France.

Media

Examples of suitable media are:–

1. **Herrold's egg yolk medium with mycobactin.** For one litre of agar: 9.0 g protease peptone, 4.5 g sodium chloride, 15.3 g agar (Difco), 27 ml glycerol, 2 mg mycobactin (Allied Laboratories), 5.1 ml of a 2% aqueous solution of malachite green, 50 mg chloramphenicol, 100,000 U penicillin, 50 mg amphotericin B, 4.0 g sodium pyruvate, 870 ml distilled water, 6 egg yolks.

Protease peptone, sodium chloride, agar and glycerol are dissolved in distilled water. The mixture is heated to 100°C. After adding chloramphenicol the medium is sterilised by autoclaving at 115°C for 15 min. The medium is cooled to 56°C in a waterbath and penicillin, amphotericin B, sodium pyruvate, mycobactin, a sterile aqueous solution of malachite green and 6 egg yolks are
added and mixed well. While dispensing the medium has to be constantly stirred at 56°C.

2. Modified Dubos medium. This is essentially Williams Smith medium (14). A litre of the medium is prepared from the following formula:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Difco casamino acids</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Di-sodium hydrogen phosphate</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Potassium di-hydrogen phosphate (anhydrous)</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Magnesium sulphate (crystalline)</td>
<td>0.6 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>25 ml</td>
</tr>
<tr>
<td>Tween 80 (1%)</td>
<td>50 ml</td>
</tr>
<tr>
<td>Agar (to 1.5% final conc)</td>
<td>15 g</td>
</tr>
</tbody>
</table>

Each salt is dissolved in turn with minimum heat in distilled water and made up to 800 ml. After adding mycobactin to a final concentration of 2 mg/l the mixture is heated to 100°C by free-steaming. Chloramphenicol (0.05 g) is then added and the medium sterilised by autoclaving at 115°C for 15 min. After cooling the medium to 56°C in a waterbath, 200 ml of bovine serum is sterilised by filtering through a Seitz 'Ex' pad and inactivated by heat at 56°C and the antibiotics penicillin (100,000 U) and amphotericin B (50 mg) are added and mixed well. The medium is kept thoroughly mixed and then dispensed in 5-6 ml quantities into sterile, screw-capped bottles and allowed to set in the sloped position. Twenty-four hours before use the water of condensation is poured off the slopes which are cotton-wool stoppered and placed in an incubator or warm room to dry. An advantage of this medium is that it is transparent, which facilitates the early detection of colonies.

Middlebrook 7H11 medium (Difco) enhanced with mycobactin at the same proportion as for Herrold's medium is also used.

Sample preparation

Samples usually require decontamination before they can be cultured. However, in some cases portions of ileal wall or ileo-caecal lymph nodes may be taken aseptically by using biopsy under local anaesthesia. Such samples do not need to be decontaminated.

Mesenteric lymph nodes: 2 g of tissue are homogenised in 10 volumes of 0.75% hexadecyl pyridinium chloride (HPC Sigma). The homogenate is then allowed to stand at room temperature for one hour and then mixed well before making four 10-fold dilutions in modified Dubos medium salt base. From each dilution 0.1 ml is used to inoculate one or more slants of medium. Undiluted homogenate may also be inoculated onto the slants.

Intestinal walls: the intestine is opened and washed under a tap to remove the intestinal contents. The mucosa is removed by scraping the intestinal wall
between a glass microscope slide and a flat surface. The mucosal pulp is macerated in a blender with distilled water and decontaminated with 0.75% aqueous solution of HPC. The homogenate is then treated as described above for mesenteric lymph nodes. In cases where extensive autolysis of the gut mucosa has occurred, the intestinal contents may be used directly rather than washed (as the villus tips frequently have the highest bacterial numbers and may otherwise be lost).

**Faeces:** one gram of faeces is suspended in 30 ml distilled water and allowed to settle for 1 h. Five ml of the faecal suspension is transferred to a round-bottomed tube containing 40 ml HPC (0.75% aqueous solution). After standing overnight the deposit is inoculated onto the surface of up to 6 slopes each of egg-based medium with, and 3 without, mycobactin. After the inocula have dried the slopes are tightly sealed and incubated for up to 6 months at 37°C and inspected for growth periodically. Some strains are pyruvate-intolerant. Tubes of Herrold's medium without pyruvate should be included to detect these. Variations in the above methods have been described (5, 6, 9, 11, 19, 20). The test is too slow for use with clinical cases, and during the sub-clinical stage of the disease is not sensitive enough to detect infection in all cases.

d) **DNA probes**

DNA probes are being developed which offer a means of detecting *M. paratuberculosis* in diagnostic samples (10) and of rapidly identifying bacterial isolates. Because of their unique specificity, they discriminate between *M. paratuberculosis* and other mycobacteria, especially those of the *M. avium* complex.

Only 1.2% base substitution differentiates *M. avium* and *M. paratuberculosis*, and most randomly isolated DNA fragments hybridise the DNA of these species equally. McFadden and co-authors (7, 8) have identified a sequence, termed IS900, which appears to be an insertion sequence specific for *M. paratuberculosis*. The use of IS900 as a DNA probe for specific identification of *M. paratuberculosis* in faecal samples from cattle by enzymic amplification of DNA using the polymerase chain reaction (PCR) has been reported (17). A commercial diagnostic test based on detection of IS900 sequences following isolation of mycobacteria from faecal samples and enrichment of a DNA fraction from IS900 sequences by the PCR has been developed. The test is available in kit form suitable for use in laboratories from IDEXX Corp, Portland Maine, ME 04101 USA.

2. **Serological tests**

a) **Complement fixation test (CFT)**

The complement fixation (CFT) test was for many years the test used for the serological diagnosis of paratuberculosis. The test is of value as a confirmatory test in clinical cases, when a sensitivity and specificity of around 90 percent
might be expected. However, the test is of limited value in the detection of subclinical infection especially in the young (1-2 year old) animal. The low sensitivity of the test is due to the fact that antibodies detectable by CFT only develop long after infection and even then may be detectable only intermittently. The poor specificity of the test is due to antibodies to other mycobacteria, principally \textit{M. avium}, and to other micro-organisms e.g. \textit{Corynebacterium}, \textit{Nocardia} and \textit{Dermatophilus} spp.

Despite its shortcomings, many countries accepting animals for importation require an export certificate certifying the animal has been screened serologically by the CFT and is negative at a 1:10 dilution.

A microtitre method for performing the CFT is as follows. The antigen is prepared as an acetone extract of Johne's bacilli which is then filtered, resuspended in distilled water and boiled for 60 minutes under a reflux condenser. The optimum dilution of antigen is calculated using a checkerboard titration against a positive control serum.

Sera are diluted 1:5 and inactivated at 60°C for 30 minutes. Red deer, sheep and goat sera are inactivated at 63°C to avoid anticomplementary reactions.

All dilutions and reagents are prepared in CFT barbitone buffer (CFTB). Fifty microlitre volumes of each reagent are used in 96-well round-bottom microtitre plates.

Five twofold serial dilutions of test sera are dispensed into rows 1-5 of the plate starting at 1:10 and a volume of the 1:10 serum is placed in row 6 to act as the serum anticomplementary control. One volume of the antigen diluted to the optimum concentration determined by titration, is added to rows 1-5 and one volume of CFTB added to row 6.

Guinea pig complement is diluted to contain one minimum haemolytic dose (MHD) as calculated by titration against the antigen and positive control serum. One volume is added to all wells including the anticomplementary serum controls in row 6. The plate is covered with sealing tape, shaken on a plate shaker for one minute and incubated at 37°C for 3 hours.

The following controls are also prepared:
- complement controls in duplicate containing working strength antigen and 2, 1, 0.5, 0.25 or 0 MHD of complement per well;
- antigen control in duplicate consisting of working strength antigen and CFTB only.

Rabbit haemolytic serum is diluted in CFTB to contain 5 MHD and is used to sensitisce 3% washed sheep erythrocytes (SRBC). Equal volumes of these are mixed together several times and incubated at 37°C in a waterbath for 30 minutes so that one volume can be added to each of the wells of the test plate at the end of its 3-hour incubation.
The plate is sealed once again and after shaking incubated for a further 30 minutes. The plate is then centrifuged for about one minute at 200 g to concentrate any remaining SRBC and facilitate reading. Plates are read as fixation of complement as follows: 4+ = 100% fixation, 3+ = 75% fixation, 2+ = 50% fixation, 1+ = 25% fixation and 0 = complete haemolysis. The titre of test sera is given as the reciprocal of the highest dilution of serum giving 50% fixation.

b) Enzyme linked immunosorbent assay (ELISA)

The ELISA is at present the most sensitive and specific test for serum antibodies to *M. paratuberculosis* (22). Its sensitivity is comparable with that of the CFT in clinical cases (90%) but is greater than that of the CFT in sub-clinically infected carriers. Seroconversion to the ELISA takes place within 6 to 9 months of faecal shedding detectable by culture (12). The specificity of the ELISA can be increased by *M. phlei* absorption of sera (see below) and the use of more *M. paratuberculosis*-specific antigens (15).

A micro ELISA plate is washed with ELISA wash buffer (EWB; 0.05 M PBS pH 7.4 and 0.05% Tween 20). The plate is then coated by adding 100 μl (10 μg/well) of antigen (PPA (paratuberculosis protoplasmic antigen), Allied Laboratories, USA) suspended in ELISA coating buffer (ECB; 0.05 M carbonate bicarbonate buffer pH 9.6) to all wells except those in column 1 (blank) which are filled with 100 μl of plain coating buffer. The plate is then sealed and incubated at 4°C overnight. After the adsorption stage the plate is again washed (x 3) with EWB. Working volumes are 100 μl/well and EWB is used for dilution of sera. Plain EWB is added to wells in the blank column. Positive control serum doubly diluted (1:20 to 1:2,560) is added in duplicate wells in columns 2 and 3. Negative control serum diluted to 1:40 is added to the wells in column 4 and test sera diluted 1:40 to the rest of the wells in duplicate. The plate is sealed and incubated at 37°C for one hour.

After washing in EWB, 100 μl/well of 1:200 dilution of alkaline phosphatase (AP) conjugated anti-species IgG in EWB is added to all wells and the plate is then sealed and incubated at 37°C for one hour.

For the colour reaction, the plate is again washed in EWB and 100 μl/well of 1.0 mg/ml AP-substrate solution (p-nitrophenyl phosphate; PNPP) in 10% diethanolamine added. The plate is then left for one hour at room temperature and further colour development stopped by adding 50 μl/well of 1.0 M NaOH.

The plate is read in an ELISA reader using a 405 nm filter. A positive to negative cut-off of absorbance figures is obtained from a checkerboard titration of a positive, standard serum. Alternatively the absorbance of the positive standard can be regarded as 100 and the absorbances of test samples related to that as a percentage.

Absorption treatment of test sera can be carried out as follows. A 50 μl sample of each test serum is mixed with 950 μl of a heat-killed saline suspension (5 mg
dry wt/ml) of *M. phlei*. The reaction mixture is allowed to react at room temperature for one hour and then it is centrifuged for 30 min at 4°C at 2,000 g. This procedure improves the specificity of the test at the cost of a slight drop in sensitivity (12).

c) Agar gel immunodiffusion (AGID) test

The AGID test is useful in the confirmation of the disease in clinically suspect sheep and goats (13).

Agarose is dissolved in 8.5% NaCl solution and buffered to pH 9.0 with 0.01 M Tris (hydroxymethyl) aminomethane to give a final agarose concentration of 0.75%. Sodium azide is added to a concentration of 0.02%. This agarose suspension is heated in a boiling water bath until dissolved and the gel is stored at 4°C in 25 ml aliquots. When needed, the gel is liquefied in a boiling water bath and poured into a 9 x 9 cm template over an agarose-coated polyester film, resulting in solidified gel 5 mm thick. Reagent wells of 4 mm diameter are punched in the gel and aspirated in a hexagonal pattern of 6 peripheral wells for serum samples and a central well for antigen. The distance from the centre of the antigen well to the centre of each peripheral well is 1 cm.

The antigen employed is a crude protoplasmic extract of *M. paratuberculosis* prepared by disruption of cells in a hydraulic press cell fractionator. Disrupted cells are centrifuged at 40,000 g for 2 hours to remove cell wall debris, and the supernatant fraction is retained and lyophilised. This antigen is resuspended in water at a concentration of 10 mg/ml.

Antigen and serum wells are filled using capillary tubes. A *M. paratuberculosis*-positive bovine serum is placed in wells adjacent to test sera. After the wells are filled, gels are incubated in covered, moistened glass dishes to avoid drying. Gels are examined for precipitation lines after 24 and 48 hours’ incubation. The appearance of one or more clearly definable precipitation lines before or at 48 hours constitutes a positive test result. A single line is recorded as a 1+ positive test, 2 lines as a 2+ positive test, and 3 lines as a 3+ positive test. Absence of any precipitation lines is recorded as a negative test result. Non-specific lines may occur, emphasising the need for well controlled positive standards.

d) Dot immunoblotting assay (DIA)

The DIA is of comparable efficacy to the ELISA and has the advantages of simplicity, rapidity and cheapness, and the potential for field use (16). Purified protoplasmic extracts (Allied Laboratories) with antigen in the range 62 ng to 4 μg/ml are prepared. Nitrocellulose sheets with a printed grid are pre-wetted with Tris buffered saline (TBS) (10 mM Tris-HCl and 150 mM NaCl, pH 8.0). The antigen (0.1 μl volumes) is spotted onto the nitrocellulose strips which are allowed to dry, then washed in 10% horse serum in TBS containing 0.05% Tween 20 (TBST). The antigen-treated strips can be stored at 4°C for future use.
The nitrocellulose strips are cut and placed in plastic containers with 8 mm wide troughs. Each strip is incubated for 45 min with 0.5 ml of the optimum dilution of serum found from preliminary titrations with control positive and negative sera. Sera are diluted in TBST which contains 1% bovine serum albumin (BSA). After 3 washes in TBST the strips are incubated for 45 min with alkaline phosphatase-conjugated affinity-purified rabbit anti-bovine IgG heavy chain (0.1 mg/ml) diluted 1:4,000 in TBS containing 1% BSA. The strips are again washed and exposed to the colour development solution (0.40 mM nitro blue tetrazolium (NBT) and 0.38 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in a solution of 100 mM Tris, 100 mM NaCl and 5 mM MgCl₂ adjusted to pH 9.5). The NBT and BCIP are prepared at a concentration of 50 mg/ml in dimethylformamide. The colour develops within 15 min after which the reaction is stopped by several washes in distilled water. The dot blots are read visually or by densitometry.

3. **Delayed-type hypersensitivity (DTH)**

The test is carried out by the intradermal inoculation of 0.1 ml volumes of avian PPD tuberculin (0.5 mg/ml) or johnin (0.5 mg/ml) (avian tuberculin and johnin are of comparable sensitivity and specificity) into a clipped or shaven site, usually on the side of the middle third of the neck. The skin thickness is measured with calipers before and 72 h after inoculation. Increases in skin thickness of over 3 mm should be regarded as indicating the presence of DTH. It should be noted that positive reactions in deer may take the form of diffuse plaques rather than discrete circumscribed swellings thus making reading of the test more difficult. The presence of any swelling should be regarded as positive in this species. Tests for DTH are of limited value. Sensitisation to the *M. avium* complex is widespread in animals and neither avian tuberculin nor johnin are highly specific. A herd test gives only an indication of the number of sensitised animals and may thus be used only as a preliminary test prior to the initiation of a control programme.

Avian tuberculin and johnin can be obtained from the MAFF Central Veterinary Laboratory, Weybridge, Surrey, KT15 3NB, UK, or from the Institut Rhône Mérieux, 254 rue Marcel Mérieux, 69007 Lyon, France.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

**Vaccines**

Types of vaccines used are live, attenuated, incorporated with oil and pumice; lyophilised, live attenuated which may be adjuvanted with e.g. oil after reconstitution; and heat-killed bacterins. Vaccines may be prepared from one strain of *M. paratuberculosis* or as many as three strains may be used. The information below applies to a live, attenuated vaccine adjuvanted with oil and pumice.

**Diagnostic products**

Johnin purified protein derivative (PPD) is a preparation of the heat-treated
products of growth and lysis of *M. paratuberculosis*. It is used, by intra-dermal injection, to reveal delayed-type hypersensitivity as a means of identifying animals infected or sensitised with *M. paratuberculosis*.

1. **Seed management**

a) Characteristics

**Vaccine**
Seed strains should be of a prevalent type which may be checked by biotyping or genetic analysis. They should have been demonstrated to be innocuous when administered by the recommended route of vaccination to intended target species.

**Johnin**
Strains of *M. paratuberculosis* used to prepare seed cultures should be identified as to species by biotyping or genetic tests. They should be shown to be free of contaminating organisms.

b) Culture

**Vaccine**
Seed cultures may be made on potato slants partly immersed in a suitable medium such as Reid's synthetic medium (18). Cultures may be stored lyophilised. Active cultures are normally incubated at 37°C.

**Johnin**
The culture substrate should be shown to be capable of producing a product free from substances known to cause toxic or allergic reactions. A suitable medium for seed culture is that of Reid (18), solidified with 1.75% agar, in screw cap tubes. Cultures may also be stored lyophilised.

c) Validation

**Vaccine**
Purity tests should be carried out on seed cultures and final harvest by stained smears.

The vaccine should be used as part of a control programme and will not on its own provide complete protection against disease caused by *M. paratuberculosis* (21). There is usually good control of clinical disease but sub-clinical infection persists in vaccinated herds albeit at a reduced level. Vaccine should be administered only to animals early in life, e.g. calves in their first month of life. It should be inoculated subcutaneously and causes a small inflammatory swelling. This is gradually replaced by a cold, painless, fibro-caseous nodule which varies in size and which may persist for years.

The use of vaccines may interfere with the outcome of diagnostic skin tests for tuberculosis and this should be borne in mind when planning a control
Johnin
Cultures should be checked by staining of smears for the presence of contaminating organisms.

To test for lack of sensitising effect, 3 guinea pigs that have not previously been treated with any material that could interfere with the test, are each injected intradermally, on each of three occasions at 5-day intervals, with 0.01 mg of the preparation under test in a volume of 0.1 ml. Fifteen to 21 days after the third injection, each guinea pig, together with each of 3 control guinea pigs that have not been injected previously, is injected intradermally with the same dose of the same johnin. The reactions of the 2 groups of guinea pigs should not be significantly different when measured 24-48 hours later.

2. Manufacture

Vaccine
The organisms may be grown for vaccine batches on a liquid synthetic medium such as that of Reid (18). The organisms grow as a pellicle on the liquid surface. To ensure a good surface area it is convenient to use vessels such as conical flasks containing one third of their nominal volume of liquid medium. These flasks may be seeded directly from potato slant cultures, but with some strains one or more passages on liquid medium may be necessary to ensure adequate pellicle growth for the final, vaccine batch, passage. Such passaging should usually take place at two-week intervals as longer periods may result in over-maturation and sinking of the pellicle. Incubation is at 37°C.

To prepare the vaccine the pellicle growth from two-week cultures of each strain to be included may be separated from the liquid medium by decantation, filtration and pressing between filter paper pads. The moist *M. paratuberculosis* culture is blended with an adjuvant such as liquid paraffin, olive oil and pumice (2).

Johnin
Johnin for skin test diagnosis is a purified protein derivative prepared from one or more strains of *M. paratuberculosis*. It may be prepared by the following method.

*M. paratuberculosis* strains are grown as a pellicle on liquid Reid’s medium. Production cultures are usually inoculated from liquid seeding cultures rather than directly from seed on solid medium. Production cultures may be grown in penicillin flasks containing half of their nominal volume of medium. These are incubated at 37°C for 10 weeks.

At the end of the incubation period the culture medium has a pH of about 5 and little or no johnin will be obtained unless the pH is raised, using sodium hydroxide, to about 7.3 before steaming. After thorough mixing the cultures are free steamed. The bulk of the killed organisms is removed by coarse filtration and the filtrate clarified by further filtration. Protein in the filtrate is precipitated chemically, washed and redissolved. The product is sterilised by filtration. An antimicrobial
preservative that does not give rise to false positive reactions, such as phenol (not more than 0.5% w/v), may be added. Glycerol (not more than 10% w/v) may be added as a stabiliser. The product is dispensed aseptically into sterile glass containers which are sealed.

3. **In-process control**

**Vaccine**

Adequate growth of culture and cultural purity need to be checked. Presence of contaminating organisms may be detected by conventional sterility tests on harvests. Tests for pathogenic mycobacteria are carried out by injection of moist culture, taken prior to blending with adjuvant and diluted tenfold in saline, into two guinea pigs, each receiving 1 ml. These are observed for 8 weeks, killed humanely, and examined for any abnormal lesions.

**Johnin**

After final filtration the sterility of each filtrate of the purified protein derivative (PPD) solution is checked.

Sterile filtrates are tested for protein content by a Kjeldahl method (1). The protein content is adjusted to give between 0.475 and 0.525 mg/ml of protein in the final product. The pH is adjusted to keep in the range 6.5 to 7.5.

4. **Batch control**

**a) Sterility**

Tests for sterility and freedom from contamination may be found in the Chapter on General Information. The vaccine organism will not normally grow to a detectable level in conventional sterility tests.

**b) Safety**

**Vaccine**

These tests are normally performed in laboratory animals although multiple dose tests in target animals would also be satisfactory. A typical laboratory animal test would be as follows.

Each of two guinea pigs is inoculated by the subcutaneous route with a fraction of the cattle dose of the vaccine, previously determined to give a nodule but no overt necrosis at the injection site with an acceptable batch of vaccine. Animals are observed for 8 weeks, killed humanely and examined for any abnormal lesions.

**Johnin**

Two guinea pigs should each be injected subcutaneously with 0.5 ml of the johnin under test. No significant local or systemic lesions should be seen within 7 days (1).
Tests on johnin for living mycobacteria may be performed either on the material immediately before it is dispensed into final containers or on samples taken from final containers themselves. A sample of at least 10 ml should be taken and this should be injected intraperitoneally or subcutaneously into at least 2 guinea pigs, dividing the volume to be tested equally between the guinea pigs. It is desirable to take a larger sample, say 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea pigs are observed for at least 42 days and examined post-mortem. Any macroscopic lesions are examined microscopically and culturally.

c) Potency

Vaccine
Since protection tests appear to be impractical, a test of sensitising ability may be used. This may then be related to bacterial content based on weight. A typical test would be as follows.

Guinea pigs are sensitised by intra-muscular injection of 0.5 ml of a hundredfold dilution in liquid paraffin of the vaccine under test. Six weeks after sensitisation, skin tests are performed using intradermal inoculations of 0.2 ml of at least three serial dilutions of a *M. paratuberculosis* antigen such as johnin PPD, the dilutions being chosen to give expected skin reactions of from 8 mm to 25 mm diameter. Each guinea pig receives several dilutions per flank, their distribution being chosen by a Latin square design. After 24-48 hours, skin reactions are measured. A reference preparation for tests of this type has not yet been fully established. Avian tuberculin PPD of known international unitage may be used as a, usually less sensitive, skin test antigen in tests of this type to ensure that the vaccine is capable of producing adequate sensitisation.

Johnin
The potency of johnin is currently determined by chemical assay for protein using a Kjeldahl method. A PPD content of 0.5 ± 0.025 mg/ml of final product is recommended (1).

The identity of the material should be confirmed by injecting small doses intradermally into guinea pigs sensitised by injections of killed *M. paratuberculosis* six weeks previously.

It is possible to perform a potency test using dilutions of johnin in guinea pigs sensitised with *M. paratuberculosis*, similar to such tests for the potency of bovine and avian tuberculin, but a reference preparation for this type of test has not yet been fully established.

d) Stability

Vaccine
The vaccine may be stored at 2-8°C for nine months without loss of potency. It should not be frozen. Organisms sediment during storage and vigorous shaking is needed to resuspend them.
Johnin
Johnin should be protected from light and stored at 2-8°C. Under these conditions it should retain its potency for at least 5 years.

REFERENCES


**SUMMARY**

Bovine anaplasmosis may result from infection with any of three species of *Anaplasma* – *A. marginale*, *A. centrale*, and *A. caudatum* – but *A. marginale* is responsible for most outbreaks of clinical disease.

The organism is classified in the genus *Anaplasma* belonging to the family *Anaplasmataceae* of the order *Rickettsiales*.

Anaemia and jaundice are characteristic signs of anaplasmosis but the clinical disease can only be confirmed by identifying the organism. Once infected, cattle may remain carriers for life and identification of these animals depends on the detection of specific antibodies using serological tests or parasite DNA using specific nucleic acid probes.

**Identification of the agent:** Microscopic examination of blood or organ smears stained with Giemsa stain is the most common method of identifying *Anaplasma*. In these smears *A. marginale* appear as dense, rounded, intra-erythrocytic bodies approximately 0.3 to 1.0 μm in diameter, with most situated on or near the margin of the erythrocyte. *A. centrale* is similar in appearance but most of the organisms are situated away from the margin of the erythrocyte. In Giemsa stained smears, *A. caudatum* is indistinguishable from *A. marginale*, but by using other staining techniques the projections which are characteristic of this species can be demonstrated.

It is important that smears be well prepared and free of foreign matter. Smears from live cattle should preferably be prepared from blood drawn from the jugular vein or another large vessel. For post-mortem diagnosis, smears should be prepared from internal organs (including liver, kidney, heart and lungs) and from blood retained in peripheral vessels. The latter are particularly desirable if post-mortem decomposition is advanced. Direct fluorescent antibody staining is also a useful technique in these circumstances.

**Sero logical tests:** The most widely used serological tests are the complement fixation test and the card agglutination test. Both are reasonably satisfactory, provided reagents and conditions are carefully controlled and standardised. An enzyme-linked immunosorbent assay (ELISA) has recently been developed. Other tests which may be useful under certain conditions are the indirect fluorescent antibody test, the capillary tube agglutination test and radio-immunoassay.

A nucleic acid probe has recently been developed and been shown to be capable of detecting the presence of low level infection in carrier cattle and in tick vectors. It is currently limited to use as a research tool, but shows promise as a future adjunct to serology, providing a standardised test which should be
useful in epidemiological studies.

Requirements for biological products: Living and non-living vaccines are used in several countries to protect cattle against A. marginale infection. Vaccine consisting of live A. centrale is most widely used and gives partial protection against challenge with virulent A. marginale.

A. centrale vaccine is provided in chilled or frozen forms. Frozen vaccine is preferred as it allows for thorough post-production control of each batch. The risk of contamination of this blood-derived vaccine makes thorough quality control essential but puts production beyond the means of some countries in the endemic regions.

A. centrale vaccine is not entirely safe. A practical recommendation is to limit its use as far as possible to calves since non-specific immunity will minimise the risk of vaccine reactions. When older animals are vaccinated, the risk of reaction warrants close surveillance and treatment with tetracycline if reactions occur. Partial immunity develops in six to eight weeks and lasts for several years after a single vaccination.

A. DIAGNOSTIC TECHNIQUES

Outbreaks of bovine anaplasmosis are usually due to infection with Anaplasma marginale. A. centrale is capable of producing a moderate degree of anaemia but clinical outbreaks in the field are extremely rare. A third bovine species, A. caudatum, can apparently cause mild to severe disease but it generally occurs in mixed infections with A. marginale and its pathogenicity has therefore not been well documented (14).

Anaplasma marginale occurs in most tropical and sub-tropical countries and in some more temperate regions. A. centrale was first described from South Africa and has since been imported by some other countries – including Australia and some South American countries – for use as a vaccine against A. marginale. A. caudatum has been reported in the USA and Mexico. Its distribution elsewhere has not been widely studied.

Anaplasma spp. were originally regarded as protozoan parasites but later research showed they had no significant attributes to justify this opinion. Since 1957 they have been classified in the family Anaplasmataceae of the order Rickettsiales. This family has four genera: Anaplasma, Aegyptianella, Haemobartonella and Eperythrozoon (23).

Anaplasma spp. are transmitted either mechanically or biologically by arthropod vectors. A review based on a careful study of reported transmission experiments lists 14 tick species as capable of transmitting A. marginale experimentally (15). These are Argas persicus, Ornithodoros lahorensis, Boophilus annulatus, B. decoloratus, B. microplus, Dermacentor albipictus, D. andersoni, D. occidentalis, D. variabilis, Hyalomma excavatum, Ixodes ricinus, Rhipicephalus bursa, R. sanguineus
and *R. simus*. The authors concluded that some of these reports, including those incriminating *R. bursa, H. excavatum* and *O. lahorensis*, were not entirely convincing and that the ticks identified as *A. persicus* were probably either *A. sanci* or *A. radfius*. In addition, *Rhipicephalus e. evertsi* and *Hyalomma m. rufipes* have been incriminated as experimental vectors in South Africa (20). While a few cases of successful transovarial transmission have been described, it seems that stadal or transstadiol transmission is the usual method, even in the one-host *Boophilus* species. Experimental demonstration of vector competence does not necessarily imply a role in transmission in the field. However, *Boophilus* species are clearly important vectors of anaplasmosis in countries such as Australia and Africa, and there is fairly good evidence that some species of *Dermacentor* are natural vectors in the USA.

Various other biting arthropods have been incriminated as vectors, particularly in the USA. Experimental transmission has been demonstrated with a number of species of *Tabanus* (horseflies) and with mosquitoes of the genus *Psorophora* (22). Transmission is mechanical in all cases. The importance of biting insects in the natural transmission of anaplasmosis has not been well documented but appears to vary greatly from region to region. *Anaplasma marginale* can also be readily transmitted during vaccination against other diseases unless a fresh needle is used for injecting each animal. Similar transmission by means of unsterile surgical instruments has been described.

The main vectors of *A. centrale* appear to be multi-host ticks peculiar to Africa, including *Rhipicephalus simus*. The common cattle tick (*Boophilus microplus*) has not been shown to be a vector. This is of relevance where *A. centrale* is used as a vaccine in *B. microplus*-infected regions.

The most marked clinical signs of anaplasmosis are anaemia and jaundice, the latter occurring late in the disease. Haemoglobin anaemia and haemoglobinuria are not present and this may assist in the differential diagnosis of anaplasmosis from babesiosis, which is often endemic in the same regions. The disease can, however, only be confirmed by identification of the organism.

1. **Identification of the agent**

Samples from dead animals should include air-dried thin smears from the liver, kidney, heart and lungs and from a peripheral blood vessel. The latter is particularly desirable should there be a significant delay before post-mortem examination as, under these circumstances, bacterial contamination of organ smears frequently makes identification of *Anaplasma* equivocal (7). Brain smears, which are useful for the diagnosis of some forms of babesiosis, are of no direct value for diagnosing anaplasmosis (12) but should be included for differential diagnosis where appropriate.

Blood from organs rather than organ tissues *per se* is required for smear preparation, as the objective is to be able to microscopically examine intact erythrocytes for the presence of *Anaplasma*. Organ smears will store satisfactorily at room temperature for several days (12).
Samples from live cattle should include thin blood smears and blood collected into an anticoagulant. Air-dried thin blood smears will keep satisfactorily at room temperature for at least a week. The blood sample in anticoagulant should be held and transferred at 5°C unless it can reach the laboratory within a few hours. This sample is useful for preparing fresh smears if those submitted are not entirely satisfactory. In addition a low packed cell volume and/or erythrocyte count can help to substantiate the involvement of *Anaplasma* when only small numbers of the parasites are detected in smears, such as may occur in the recovery stage of the disease.

In contrast to *Babesia bovis*, *Anaplasma* do not accumulate in capillaries so blood drawn from the jugular or other large vessel is satisfactory. Because of the rather indistinctive morphology of *Anaplasma* it is essential that smears be well prepared and free of foreign matter, as specks of debris can confuse diagnosis. Thick blood films as used for the diagnosis of babesiosis are not appropriate for the diagnosis of anaplasmosis, as *Anaplasma* are difficult to identify once they become dissociated from erythrocytes.

Both blood and organ smears are fixed in absolute methanol for one minute and stained in 10% Giemsa stain for 30 minutes. After staining, the smears are rinsed three or four times with tap water to remove adhering stain and are then air-dried. Smears are examined under oil immersion at a magnification of between 700-1000 times.

In Giemsa-stained smears *A. marginale* appears as dense, rounded and deeply stained intra-erythrocytic bodies, approximately 0.3 to 1.0 μm in diameter. The majority of these bodies are located on or near the margin of the erythrocyte. This feature distinguishes *A. marginale* from *A. centrale*, as in the latter most of the organisms have a more central location in the erythrocyte. *A. caudatum* is indistinguishable from *A. marginale* in Giemsa-stained smears. The appendages which give this species its name and take various forms – such as tails, loops and rings – can, however, be demonstrated using fluorescent antibody techniques, by staining with new methylene blue or by phase contrast microscopy of water-lysed erythrocyte preparations (14).

The percentage of infected erythrocytes varies with the stage and severity of the disease. Maximum parasitaemias in excess of 50% may occur with *A. marginale*. Multiple infections of individual erythrocytes are common during periods of high parasitaemias.

During the course of disease the parasitaemia approximately doubles daily for up to about 10 days and then decreases at a similar rate. Quite severe anaemia may persist for some weeks after parasites have become virtually undetectable in blood smears. Following recovery from initial infection most cattle remain latently infected for life.

Fluorescent antibody staining may be used as an alternative to Giemsa staining. Its major application is for detecting *Anaplasma* in smears taken post-mortem, as it
has been shown to be more sensitive than Giemsa staining for this purpose (12). A direct fluorescent antibody procedure for the post-mortem diagnosis of anaplasmosis has been described in detail by Johnston et al. (12). Briefly, thin blood and organ smears are fixed in acetone for 10 minutes and air dried. Fluorescein isothiocyanate-labelled globulins prepared from serum taken from cattle that have recovered from a pure Anaplasma infection are applied to the smear, which is incubated at 29°C and high relative humidity for 30 minutes. The smear is then given two 5-minute washes in phosphate buffered saline, pH 7.2, 0.012 M. A cover-slip is applied while the smear is still wet and the smear examined by fluorescence microscopy.

An expensive procedure, but one which may occasionally be justified to confirm infection, particularly in latently infected cattle, is the inoculation of blood from the suspect animal into a splenectomised calf. A quantity (up to 500 ml) of the donor's blood in anticoagulant is inoculated intravenously into the splenectomised calf, which is then smeared at least every second or third day. If the donor is infected, Anaplasma will be observed in smears from the splenectomised calf generally within 4 weeks, but this period may extend up to 8 weeks.

Use of a DNA probe to diagnose A. marginale infection in carrier cattle has recently been described (10). Parasitaemias as low as 0.000025% could be detected using this technique. Infected ticks could also be identified in this way (11). The technique, while experimental at present, holds promise for use in future epidemiological studies.

2. Serological tests

Anaplasma infections usually persist for the life of the animal. However, except for occasional small recrudescences of parasitaemia, Anaplasma cannot readily be detected in blood smears after the initial parasitaemic episode. A number of serological tests have thus been developed to detect latently infected animals.

It must be emphasised that there is a high degree of cross-reactivity between Anaplasma spp (such as between A. marginale and A. centrale) in serological tests. While the infecting species can sometimes be identified by utilizing antigens from homologous and heterologous species, equivocal results are obtained on many occasions.

a) Complement fixation (CF) test

This test is undertaken using standard CF test procedures. Antigen consists of anaplasma bodies which have been separated from erythrocytes by lysis (e.g., in a French Pressure Cell) and then washed free of most haemoglobin and cellular debris. A microtitre technique is now most commonly used as it requires only very small quantities of reagents. It is essential that all reagents be correctly standardised and the test properly controlled. Detailed manuals for the conduct of both the standard (3) and the microtitre technique (4) for the CF test for anaplasmosis have been produced.
b) Card agglutination test (CAT)

The advantages of this test are that it is sensitive, may be undertaken either in the laboratory or in the field, and a result is available within a few minutes. The CAT antigen is a suspension of *A. marginale* particles. Splenectomised calves are infected by intravenous inoculation with blood containing at least $10^9$ *Anaplasma*-infected erythrocytes. When the parasitaemia exceeds 50% the animal is exsanguinated, the infected erythrocytes are washed, lysed and the erythrocyte ghosts and *Anaplasma* particles pelleted. The pellets are sonicated, washed and then resuspended in a stain solution to produce the antigen suspension.

The test procedure is as follows (1, 2):

i) Ensure all test components are at room temperature (25-26°C) before use (this constant temperature is critical for the test).

ii) On each circle of the test card (a clear perspex/plastic or glass plate marked with 18 mm diameter circles), place next to, but not touching each other, 10 µl bovine serum factors (BSF), 10 µl test serum, and 5 µl CAT antigen. Negative and low positive control sera must be tested on each card.

BSF is serum from a selected beast with high known conglutinin level. If the conglutinin level is unknown, fresh serum from a healthy beast known to be free of *Anaplasma* can be used. The Jersey breed is often suitable. The BSF must be stored at -70°C in small aliquots, a fresh aliquot being used each time tests are performed. The inclusion of BSF improves the sensitivity of the test.

iii) Mix well with a glass stirrer. After mixing each test wipe the stirrer with clean tissue to prevent cross-contamination.

iv) Place test card in a humid chamber and rock at 100-110 rpm for 7 min.

v) Read immediately against backlight. A positive result is characteristic clumping of the antigen (graded from 1+ to 3+). A negative result is no characteristic clumping.

c) Enzyme-linked immunosorbent assay (ELISA)

An ELISA based on the use of a normal red cell antigen (negative antigen) and an *A. marginale*-infected red cell antigen (positive antigen) has been found to be extremely reliable for the detection of *A. marginale*-positive sera (9). Although more cumbersome than tests using only one antigen, this test eliminates those sera which have high levels of non-specific activity due to isoantibodies to normal red cell components. It is also able to detect single infections for up to three years after infection.
'Negative' antigen: Blood is collected into Na$_2$EDTA from a splenectomised 3-month-old calf. The cells are washed three times with phosphate buffered saline (PBS) and centrifuged at 5,000 g for 10 min at 5°C. After the initial centrifugation, white blood cells are removed by diluting cells 10 times in PBS and passing them through a Whatman CF11 cellulose column. Following further centrifugation, one volume of cells is diluted in two volumes of PBS and stored as 2.5 ml aliquots in the vapour phase of liquid nitrogen. As required, an aliquot is thawed at room temperature and then sonicated for 30 seconds at 100 W with a small probe at 0°C. The whole sonicate is then diluted as required for use in the ELISA.

'Positive' antigen: The calf previously used to produce negative antigen is infected with *A. marginale* by blood inoculation. When the parasitaemia reaches 80-85%, 500-1,000 ml of blood is collected, processed and stored as described above. When required, aliquots are sonicated as before and diluted appropriately.

Test procedure: Flat-bottomed 96-well microELISA plates are used. Standard antigen checkerboard titrations are performed with each new batch of antigen, both positive and negative, to determine the working range, which is usually 1 in 400 (150-200 µg protein/ml). Diluted antigen (200 µl) is added to each well on the plate, one half of the plate for positive antigen, the other half for negative antigen. The plates are sealed with plate sealers and incubated at 4°C overnight. After incubation the antigen solution is removed and the plates washed 3 times in PBS containing 0.1% Tween 20 (PBT), and then blocked with a 1% solution of normal horse serum in PBS for 60 min at 37°C.

After blocking, the plates are washed 5 times in PBT prior to the addition of 200 µl of test sera. (Test sera are diluted 1:400 to 1:800 in PBT containing 1% horse serum.) The plates are sealed with plate sealers and incubated for 2 h at 37°C. The wells are then washed 5 times with PBT and 200 µl of conjugated second antibody, at a dilution of 1:400 in PBS containing 1% horse serum, is added. The plates are then sealed and incubated for 2 h at 37°C. After incubation the conjugate is removed and the wells washed 5 times in PBT prior to addition of 200 µl of freshly prepared substrate.

Substrate is prepared as follows: recrystallised 5-amino salicylic acid is dissolved at 1 mg/ml in phosphate buffer pH 6.8 at 37°C; 2 µl of 30% hydrogen peroxide is then added to each ml of solution. After addition of substrate, the plates are sealed and gently agitated for 30 min at 22°C. Plates are then immediately read at 492 nm with a plate reader. Column one of each plate is always used as a blank.

Net readings are calculated for each sample by subtracting the negative antigen result from the positive antigen result. A group of 20 negative sera and a standard positive *A. marginale* serum are routinely tested with each batch of assays. A 'threshold' is calculated as the mean net absorbance reading plus two standard deviations of the 20 reference negative sera. Ratios of all other net
absorbances are determined against the 'threshold' as unity. The positive serum is used to determine that each assay batch was performing normally.

d) **Capillary tube agglutination test**

This test is based on an assessment of the degree of agglutination occurring when *Anaplasma* antigen (consisting of partially purified *Anaplasma* bodies) is mixed with test serum in a capillary tube (21). The test was developed as an alternative to the much more complex complement fixation test but has largely been superseded by the more rapid card agglutination test.

e) **Radio-immunoassay**

A radio-immunoassay utilising a similar technique to that described for the ELISA has been reported (25). Because this assay requires more sophisticated laboratory equipment the ELISA, which appears to have about the same sensitivity and specificity, would be preferred under most circumstances.

f) **Indirect fluorescent antibody (IFA) test**

Because of the limitations on the number of IFA tests that can be performed by an operator in one day, other serological tests are generally preferred to the IFA test. The IFA test can be useful as a research tool. The test is performed as described elsewhere in this Manual for babesiosis (B11), except that *A. marginale* infected blood is used for preparation of antigen smears. Non-specific fluorescence is the main problem encountered with the test. Antigen made from blood collected as soon as adequate parasitaemia (5-10%) occurs is most likely to be suitable.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

Several methods of immunisation have been used to protect cattle against anaplasmosis in endemic countries, but none is ideal (16). Use of the less pathogenic *A. centrale*, which gives partial cross-protection against *A. marginale*, is the most widely accepted. In Australia approximately 700,000 doses of *A. centrale* vaccine are used annually. Another effective method involves the use of a strain of *A. marginale* attenuated by passage in non-bovine hosts, such as deer or sheep (24). A non-living vaccine is also available commercially in the USA, but production difficulties and the problem of isoantibody formation in pregnant cattle has prevented widespread acceptance of this vaccine (16).

In this chapter, the production of live *A. centrale* vaccine is described. It involves infection of a susceptible, splenectomised calf and use of the blood as vaccine. Detailed accounts of the production procedure are available and reference should be made to these publications for details of the procedures outlined here (5, 6).

*A. centrale* vaccine can be provided in either frozen or chilled form depending on
the demand, transport networks and the availability of liquid nitrogen or dry ice supplies. Frozen vaccine is preferred in most instances as it allows for thorough post-production quality control of each batch. It is, however, more costly to produce and more difficult to transport than chilled vaccine. The risk of contamination makes post-production control essential but probably puts production of this vaccine beyond the means of some anaplasmosis endemic countries.

1. **Seed management**

a) **Characteristics**

*A. centrale* was isolated in 1911 in South Africa and has been used as vaccine in South America, Australia, Africa, the Middle East and Southeast Asia. It affords partial but adequate protection in regions where the challenging strains are of moderate virulence (e.g. Australia) (5). In the humid tropics where *A. marginale* appears to be a very virulent parasite, the protection afforded by *A. centrale* may be inadequate to prevent disease in some animals.

*A. centrale* usually causes benign infections, especially if used in calves under nine months of age. Severe reactions following vaccination have been reported when adult cattle are inoculated.

Rapid passage of *A. centrale* in splenectomised calves appears to reduce its virulence (5).

b) **Preparation and storage of stabilate**

Infective material is readily stored as frozen stabilates of infected blood in liquid nitrogen or dry ice. Dimethyl sulphoxide (DMSO) is the recommended cryopreservative as it allows for intravenous administration after thawing of the stabilate. A detailed account of the freezing technique is reported elsewhere (5) but briefly involves the following: infected blood is collected, chilled to 4°C and cold cryoprotectant (4 M DMSO in PBS) added slowly with stirring to a final blood/protectant ration of 1:1 to give a final concentration of 2 M DMSO. The entire dilution procedure is carried out in an ice-bath and the diluted blood dispensed in suitable containers (e.g. 5 ml cryovials) and frozen as soon as possible in the vapour phase of a liquid nitrogen container.

c) **Validation as a vaccine**

The suitability of *A. centrale* as vaccine is determined by inoculating cattle, monitoring the subsequent reactions and then challenging the animals and susceptible controls with a virulent local strain of *A. marginale*. Both vaccine and challenge reactions can be judged by monitoring parasitaemias in stained blood films and the depression of packed cell volumes of inoculated cattle.
2. **Manufacture**

a) **Production of frozen vaccine**

Five to 10 ml quantities of frozen stabilate are thawed by immersing the vials in water preheated to 40°C. The thawed material is kept on ice and used as soon as possible to infect a susceptible, splenectomised calf by intravenous inoculation (within 30 min).

The parasitaemia of the donor calf is monitored daily by examining stained films of jugular blood, and blood is collected for vaccine when suitable parasitaemias are reached. A parasitaemia of $1 \times 10^8$/ml (approximately 2% parasitaemia in jugular blood) is the minimum required for production of vaccine. If a suitable parasitaemia is not obtained, passage of the strain by subinoculation of 100-200 ml of blood to a second splenectomised calf may be necessary.

Blood from the donor is collected by jugular or carotid cannulation using heparin as anticoagulant (5 IU heparin/ml blood).

In the laboratory, the parasitised blood is mixed in equal volumes with 3 M glycerol in PBS supplemented with 5 mM glucose at 37°C (final concentration of glycerol 1.5 M). The mixture is then equilibrated at 37°C for 30 min and dispensed in suitable containers (e.g. 5 ml cryovials). The vials are cooled at approximately 10°C/min in the vapour phase of liquid nitrogen and, when frozen, stored in the liquid phase (8).

DMSO can be used as cryoprotectant in the place of glycerol. This is done in the same way as outlined for the preparation of stabilate (17, 18).

If glycerolised vaccine is to be diluted, the diluent should consist of PBS with 1.5 M glycerol and 5 mM glucose (13). Vaccine cryopreserved with DMSO should be diluted with diluent containing the same concentration of DMSO as in the original cryopreserved blood (19).

b) **Production of chilled vaccine**

Infecive material for chilled vaccine is prepared in the same way as for frozen vaccine, but it must be issued and used as soon as possible after collection. The infective blood can be diluted to provide $1 \times 10^7$ parasites per dose of vaccine. A suitable diluent is 10% sterile bovine serum in a balanced salt solution containing the following quantities of salts per litre:

- NaCl 7.00 g
- MgCl$_2$.6H$_2$O 0.34 g
- Glucose 1.00 g
- Na$_2$HPO$_4$ 2.52 g
- KH$_2$PO$_4$ 0.90 g
- NaHCO$_3$ 0.52 g
If diluent is not available, acid citrate dextrose (20% v/v) or citrate phosphate dextrose (20% v/v) should be used as anticoagulant to provide the glucose necessary for survival of the organisms.

c) Use of vaccine

In the case of frozen vaccine, vials should be thawed by immersion in water, preheated to 40°C, and the contents mixed with suitable diluent to the required dilution. If glycerolised vaccine is prepared, it should be kept cool and used within 8 h (8). If DMSO is used as cryoprotective, the prepared vaccine should be kept on ice and used within 15 to 30 min (18).

Chilled vaccine should be kept refrigerated and used within six days of preparation.

The strain of *A. centrale* used in vaccine is of reduced virulence but is not entirely safe. A practical recommendation is, therefore, to limit the use of vaccine to calves, when nonspecific immunity will minimise the risk of vaccine reactions. When older animals have to be vaccinated, there is a risk of severe reactions. These reactions occur infrequently, but valuable breeding stock or pregnant animals obviously warrant close attention, and should be observed between the fourth and sixth week after vaccination. Clinically sick animals should be treated with oxytetracycline or imidocarb at dosages recommended by the manufacturers.

Protective immunity develops in six to eight weeks and usually lasts for several years.

Anaplasmosis and babesiosis vaccines are often used concurrently but it is not advisable to use any other vaccines at the same time.

3. In-process control

a) Source and maintenance of vaccine donors

A source of calves free of natural infections of *Anaplasma* and other tick-borne diseases should be identified. If a suitable source is not available, it may be necessary to breed the calves under tick-free conditions specifically for the purpose of vaccine production.

The calves should be maintained under conditions which will prevent exposure to infectious diseases and to ticks and biting insects. In the absence of suitable facilities, the risk of contamination with the agents of infectious diseases present in the country involved should be estimated, and the benefits of local production of vaccine weighed against the possible adverse consequences of spreading disease (5).
b) **Surgery**

Donor calves should be splenectomised to allow maximum yield of parasites for production of vaccine. This is best done under general anaesthesia in young calves. Details of the technique, including pre- and postoperative procedures, are reported elsewhere (5).

c) **Screening of vaccine donors before inoculation**

Donor calves should be examined for all blood-borne infections prevalent in the country, including *Babesia, Anaplasma, Theileria* and *Trypanosoma*. This can be done by routine examination of stained blood films after splenectomy and preferably also by serology. Any calves showing evidence of natural infections of any of these agents should be rejected. The absence of other infective agents should also be confirmed. The testing procedures will depend on the diseases prevalent in the country and the availability of tests but should involve serology of paired sera at the very least and, in some cases, virus isolation (8, 18).

d) **Monitoring of parasitaemias following inoculation**

It is necessary to determine the concentration of parasites in blood being collected for vaccine. There are accurate techniques for determining the parasite count (5) but, in the absence of these, the parasite concentration can be estimated from the erythrocyte count and the parasitaemia (percentage of infected erythrocytes).

e) **Collection of blood for vaccine**

All equipment should be sterilised (e.g., by autoclaving) before use. The blood is collected in heparin using strict aseptic techniques when the required parasitaemia is reached. This is best done if the calf is sedated and with the use of a closed circuit collection system. Xylazine (Rompun, Bayer) is a suitable sedative.

Depending upon demand, up to 3 litres of heavily infected blood can be collected from a six-month-old calf. If the calf is to be saved, the transfusion of a similar amount of blood from a suitable donor is indicated. Alternatively, the calf should be euthanised immediately after collection of the blood.

f) **Dispensing of vaccine**

All procedures are performed in a suitable environment, such as a laminar flow cabinet, using standard sterile techniques. Penicillin (500,000 IU/litre) and streptomycin (370,000 µg/litre) should be added to the vaccine before dispensing. Use of a mechanical or magnetic stirrer will ensure thorough mixing of blood and diluent throughout the dispensing process.
4. Batch control

The potency, safety and sterility of vaccine batches cannot be determined in the case of chilled vaccine, and specifications for frozen vaccine depend on the country involved. The following are the specifications for frozen vaccine produced in Australia.

a) Sterility and freedom of contaminants

Standard tests for sterility are employed for each batch of vaccine and diluent.

The absence of contaminants is determined by doing appropriate serology of cattle inoculated for infectious agents which could potentially contaminate the vaccine. Cattle inoculated during the test for potency are suitable for the purpose. These agents include the causative organisms of enzootic bovine leukosis, infectious bovine rhinotracheitis, mucosal disease, ephemeral fever, Akabane disease, Aino virus, bluetongue, parainfluenza, foot and mouth disease, lumpy skin disease, rabies, Rift Valley fever, rinderpest, contagious bovine pleuropneumonia, Jembrana disease, heartwater, pathogenic *Theileria* and *Trypanosoma* spp., and *Brucella abortus*, *Coxiella* and *Leptospira*. (8).

b) Safety

Vaccine reactions of the cattle inoculated in the test for potency (see point 4c) are monitored by measuring parasitaemia, fever and depression of packed cell volume. Only batches with pathogenicity levels equal to or lower than a pre-determined standard are released for use.

c) Potency

Vaccine is thawed and diluted 1:50 with a suitable diluent (13). The diluted vaccine is then incubated for 8 h at 30°C, and five cattle are inoculated with 2 ml doses subcutaneously. The inoculated cattle are monitored for the presence of infections by examination of stained blood smears. All should become infected. If the batch proves to be infective at 1:50, it is recommended for use at a dilution of 1:5 with isotonic diluent.

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SUMMARY

Babesiosis is a tick-borne disease of cattle caused by the protozoan parasites Babesia bovis, Babesia bigemina, Babesia divergens, and others. The vectors, Boophilus spp., are widespread in tropical and subtropical countries. The major vector of B. divergens is Ixodes ricinus. Other important vectors include Haemaphysalis and Rhipicephalus.

Identification of the agent: Smears of blood, brain, kidney, liver and lung are suitable for demonstration of parasites in dead animals, provided decomposition is not advanced. The smears are fixed with methanol, stained with 10% Giemsa for 20-30 min and examined under oil immersion. In the case of live animals, thick and thin films of capillary blood should be taken.

Serological tests: The indirect fluorescent antibody (IFA) test is the most widely used test for the detection of B. bovis and B. divergens. The enzyme-linked immunosorbent assay (ELISA) is suitable for antibody detection.

The IFA test has been used for the serology of B. bigemina, but serological cross reactions make species diagnosis difficult.

Requirements for biological products: Vaccines consisting of live, attenuated strains of B. bovis and/or B. bigemina or B. divergens are produced in several countries from the blood of infected calves. The vaccines are provided in frozen or chilled forms. Frozen vaccine is usually preferred as it allows for thorough post-production control of each batch. The risk of contamination of this blood-derived vaccine makes thorough quality control essential but puts production beyond the means of most countries in the endemic regions.

Live Babesia vaccines are not entirely safe. A practical recommendation is to limit their use to calves when non-specific immunity will minimize the risk of vaccine reactions. When older animals are to be vaccinated, the risk of reaction warrants close surveillance and treatment with a babesiacide if reactions occur.

Protective immunity develops in three to four weeks and lasts for several years after a single vaccination.

A. DIAGNOSTIC TECHNIQUES

Bovine babesiosis is caused by protozoan parasites of the genus Babesia of the order Piroplasmida of the phylum Apicomplexa. Of the species affecting cattle, two – B. bovis and B. bigemina – are of major importance. Both of these species are
widely distributed and are especially important in Africa, Asia, Australia, and Central and South America. In north west Europe \( B. \textit{divergens} \) is of great importance.

The vector of \textit{Babesia} is the tick. The most important vector in the tropics is \textit{Boophilus microplus} and for \( B. \textit{divergens} \) it is \textit{Ixodes ricinus}. Other important vectors include \textit{Haemaphysalis} and \textit{Rhipicephalus}. Generally, \( B. \textit{bovis} \) is more pathogenic than \( B. \textit{bigemina} \), although the latter has a higher rate of transmission. \( B. \textit{bovis} \) infections are characterised by high fever, ataxia, anorexia, and general circulatory shock, often accompanied by nervous symptoms as a result of sequestration of infected erythrocytes in cerebral capillaries. In acute cases, the maximum parasitaemia (percentage of infected erythrocytes) in circulating blood is less than 1%. This is in contrast to \( B. \textit{bigemina} \) infections where the parasitaemia may exceed 10% and may be as high as 30%. In \( B. \textit{bigemina} \) infections the major symptoms include fever, haemoglobinuria, haemoglobinaemia, and anaemia. Intravascular sequestration of infected erythrocytes does not occur with \( B. \textit{bigemina} \) infections. \( B. \textit{divergens} \) is less pathogenic than \( B. \textit{bovis} \). The parasitaemia and clinical appearance are somewhat similar to \( B. \textit{bigemina} \).

Once an animal has been infected with one of these species it is immune for life against subsequent reinfection with the same species. There is some evidence also that some degree of cross protection is experienced by \( B. \textit{bigemina} \)-immune animals against subsequent \( B. \textit{bovis} \) infections. Young animals from endemic areas are more resistant to infection, calves fed colostrum from an immune or a non-immune dam being equally resistant (5).

1. **Identification of the agent**

Samples from dead animals should consist of smears from (in order of preference) blood, cerebral cortex, kidney, liver, lung and bone marrow. Organ smears are made by pressing a clean slide on to a freshly cut surface of the organ or by crushing a small sample of the tissue between two clean microscope slides drawn lengthwise to leave a film of tissue on each slide. The smear is then air dried, fixed for 5 min in absolute methanol and stained for 20-30 min in 10% Giemsa. This method is especially suitable for the diagnosis of \( B. \textit{bovis} \) infections but is unreliable if death has occurred more than 24 h previously. However, parasites can often be detected in blood from veins in the lower limb region one or more days after death (1).

Samples from live animals should consist of both thick and thin blood films. In the case of \( B. \textit{bovis} \) it is preferable to obtain blood from capillaries, such as those in the tip of the ear or tip of the tail, as this parasite is more common in capillary blood. \( B. \textit{bigemina} \) and \( B. \textit{divergens} \) parasites are uniformly distributed throughout the vasculature. If it is not possible to make fresh smears from capillary blood, sterile jugular blood should be collected into an anticoagulant such as EDTA or heparin and the sample kept cool, preferably at 5°C, until delivery to the laboratory, again preferably within hours of collection. Thin blood films are made by placing a very small droplet of blood near the end of a clean microscope slide. A second slide is then placed against the droplet at a 45° angle and pushed to the opposite end of the
first slide. The smear is air dried, fixed in absolute methanol for one minute and stained in 10% Giemsa stain for 20-30 min. Thick films are made by placing 1 μl of blood onto a clean glass slide. This droplet is then formed into a 4 mm diameter circle, air dried and fixed in absolute acetone for five minutes and then stained in 5% Giemsa for 20-30 min.

All stained smears are examined under oil immersion using (as a minimum) x6 eyepieces and x60 objective lens. B. bovis is a small parasite, usually centrally located in the erythrocyte. It measures about 1-1.5 μm long and 0.5-1.0 μm wide and is often found as pairs which are at an obtuse angle to each other. B. divergens is also a small parasite very similar morphologically to B. bovis; however, the form in which pairs occur at an obtuse angle to each other is often located at the rim of the erythrocyte. B. bigemina is a much longer parasite, and is often found as pairs at an acute angle to each other. B. bigemina is typically pear-shaped but many diverse single forms are found. It is 3-3.5 μm long and 1-1.5 μm wide, and paired forms often have two discrete red staining dots in each parasite (B. bovis and B. divergens always have only one). In acute cases the parasitaemia of B. bovis seldom reaches 1% but with B. bigemina and B. divergens much higher parasitaemias are the norm. Thick blood films are especially useful for the diagnosis of low level B. bovis infections, as are organ smears (1).

Further confirmation of infection can be made by taking about 100 ml of jugular blood in anticoagulant from a suspected carrier animal, or kidney or liver homogenate from a fresh carcass. This material can be injected subcutaneously (in the case of organ homogenates) or intravenously into a splenectomised calf known to be Babesia free. B. divergens can infect Mongolian gerbils. Confirmation of infection should be made within 7-14 days by the presence of parasites in blood films.

2. **Serological tests**

a) **Indirect fluorescent antibody test**

Antigen slides are made from jugular blood, ideally when the parasitaemia is between 2-5%.

Blood is collected into a suitable anticoagulant (sodium citrate or EDTA) and is then washed at least three times in 5-10 volumes of phosphate buffered saline (PBS) to remove contaminating plasma proteins, and in particular host immunoglobulins. After washing, the infected red cells are resuspended in 2 volumes of PBS to which 1% bovine serum albumin (BSA) has been added. The BSA is used to adhere red cells to the glass slide. By preference, single layered blood films are made by placing a drop of blood on a clean glass slide, which is then spun in a cytocentrifuge. This produces very uniform smears. Alternatively, thin blood films may be made by the cytocentrifuge. The films are air dried and then fixed for 5 min in acetone at room temperature. Fixed blood films are then wrapped in aluminium foil and stored at -70°C until required (9).
Sera are serially diluted in two or three-fold steps, starting with an initial dilution of 1:90-1:100. Sera are regarded as positive above this point. Sera may be used with or without heat inactivation at 56°C for 30 min. The slides are marked into 8-10 divisions, each 1 cm square, with an oil pen. To each test square is added 5-10 Μl of each serum dilution, using a fine pipette. The preparations are then incubated at room temperature for 1-2 h or at 4°C overnight, in a moist chamber. For controls appropriate dilutions of positive and negative sera and a conjugate control are used with each test. After incubation, the slides are gently rinsed twice with PBS and given two 10-min washes with PBS. An appropriate dilution of anti-bovine IgG antibody labelled with fluorescein isothiocyanate (which is commercially available) is then added to each test square. Every new batch of conjugate must be titrated, the working range usually being between 1:1,000-1:3,000. After a further incubation of the slides with the conjugate at 4°C overnight, the slides are again given three 5-min washes in PBS. The wet slides are mounted in 10% glycerol in PBS with coverslips and then examined by standard fluorescence microscopy. A competent operator can examine about 150 samples per day.

b) Enzyme-linked immunosorbent assay

ELISA is more suitable for processing large numbers of serum samples.

Antigen is prepared by the following procedure as described by Waltisbuhl et al. (18). Infected blood (usually 5-10% parasitaemia) is collected from splenectomised calves into EDTA. The blood is washed three times in 5 volumes PBS and is then subjected to concentration of infected cells by differential lysis of uninfected cells in hypotonic saline solution (12). Infected cells are more resistant to lysis in hypotonic saline solutions than are uninfected cells. A series of hypotonic saline solutions are prepared, ranging from 0.35-0.50% NaCl, in 0.025% increments. Five volumes of each saline solution is then added to 1 volume of packed red cells, which are gently mixed and allowed to stand for 5 min.

The mixtures are then centrifuged and the supernatant aspirated. An equal volume of plasma (retained from the original blood) is added to each tube containing packed red cells and mixed. Thin blood films are made from each of these resuspended blood cell mixtures, which are fixed in methanol and stained with Giemsa. These films are examined by microscopy to determine which saline solution produced >95% intact infected erythrocytes by differentially lysing the uninfected red cells. Generally, the optimal differential lysis step is induced by 0.400-0.425% saline solutions.

The bulk of the infected blood is then differentially lysed with the optimal saline solution and centrifuged. The sediment (>95% infected red cells) is resuspended in 1-2 volumes PBS and sonicated in appropriate volumes using medium power for 60-90 seconds. The sonicated material is ultracentrifuged (105,000 g, 5°C, 60 minutes) and the supernatant retained. Haemoglobin is removed from the supernatant by mixing CM sephadex in 0.05 PBS, pH 5.5. The relatively colourless supernatant is mixed with equal volumes of glycerol and stored in 2-5 ml aliquots at -20°C.
The ELISA test is performed as follows:

Antigen is diluted in 0.1 M carbonate/bicarbonate buffer, pH 9.6, and 200 µl aliquots are applied to 96-well polystyrene microtitre ELISA plates. The plates are covered and incubated either at room temperature (26°C) for 3 h or at 4°C overnight. The antigen solution is then aspirated and the plates blocked with 250 µl of 0.5% gelatin in carbonate buffer, pH 9.6, for 1 h at room temperature. After 5 washes with PBS + 0.05% Tween 20, 200 µl of each test serum diluted in PBS is added. The plates are then incubated for 3 h at 26°C and again washed 5 times in PBS/Tween. Finally 200 µl of enzyme-conjugated antibovine IgG diluted in PBS is added to each well. The plates are then washed 5 times with PBS/Tween and an appropriate substrate added and the plates sealed. During substrate incubation the plates are agitated with a microtitre plate agitator. At the end of the substrate incubation time, the reaction is stopped with 50 µl of 1 M NaOH and the plates read on a microtitre plate reader. To control for inter-plate variation, positive and negative serum controls are included in each plate (2, 16).

Each new batch of antigen and conjugate should be retitrated using a checkerboard layout. In general terms, a working dilution of antigen would be between 1:400-1:1,600 and commercially available conjugate 1:1,000-1:3,000. As a routine, sera should be tested within a range of 1:200-1:800. A strong positive serum is included and the substrate reaction allowed to continue until its absorbancy value is 10. Sera are then ranked from 1-10 relative to the positive control (value 10) using absorbency values of test samples. Negative sera should rank 2 or less. The most suitable enzyme-labelled conjugate is horseradish peroxidase, whilst the safest and most stable substrate is 5-amino salicylic acid.

With this test it is possible to detect antibodies at least four years after a single infection. There should be 100% positive reactions with *B. bovis*-immune animals, 1-2% false positive reactions with negative sera and 1-2% false positive reactions with *B. bigemina*-immune animals.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

Cattle develop a durable, long-lasting immunity after a single infection with *B. bovis, B. divergens* or *B. bigemina*. This feature has been exploited by several countries to immunise cattle against babesiosis. Most of these live vaccines contain specially selected strains of one or two species and are produced in government-supported production facilities as services to the livestock industries of the countries concerned. Experimental vaccines containing antigens produced *in vitro* have also been used but have resulted in only partial protection (11, 17). Parasite proteins have been characterised (8, 13) and several are candidates for use as genetically engineered immunogens. While this approach shows promise, as yet no effective subunit vaccine has been developed.

This chapter will deal only with the production of live babesiosis vaccine. It involves
infection of calves with selected strains, and use of the blood as vaccine. Detailed accounts of the production procedures have been published (1, 3, 4). Reference should be made to these publications for details of the procedures outlined here.

*B. bovis* and *B. bigemina* vaccines can be provided in either frozen or chilled form depending on demand, transport networks and the availability of liquid nitrogen or dry ice supplies. Frozen vaccine is preferred in most instances (6, 16) as it allows for thorough post-production control of each batch. However, it is more costly to produce and more difficult to transport than chilled vaccine. The potential risk of contamination of this blood-derived vaccine makes post-production control essential but probably puts production beyond the means of most countries in endemic regions.

1. **Seed management**

   a) **Characteristics**

   i) **Internationally available strains**

      Attenuated Australian strains of *B. bovis* and *B. bigemina* have been used to effectively immunise cattle in Africa, South America and Southeast Asia (6). These strains are obtainable from the Tick Fever Research Centre, Wacol, Australia. Tick-transmissible and non-transmissible strains are available. The strains of *B. divergens* used in Sweden have not been attenuated.

   ii) **Isolation and purification of local strains**

      Strains of *B. bovis*, *B. divergens* and *B. bigemina* free of contaminants such as *Anaplasma*, *Eperythrozoon*, *Theileria*, *Trypanosoma* and various viral and bacterial agents, are most readily isolated by feeding infected ticks on susceptible splenectomised cattle (1). The vectors and modes of transmission of *B. bovis* and *B. bigemina* differ and these features can be used to separate the two species (7).

      *Babesia* spp. can also be isolated from infected cattle by subinoculation of blood into susceptible splenectomised calves. A major disadvantage of this method is the difficulty of separating the *Babesia* spp. from contaminants such as *Anaplasma* and *Eperythrozoon*. Maintenance of isolated strains in *vitro* can be used to eliminate most contaminants but not to separate the two *Babesia* spp. Selective chemotherapy can be used to obtain pure *B. bovis* from a mixed *Babesia* infection while rapid passaging in susceptible calves will allow isolation of *B. bigemina* (1).

   iii) **Attenuation of strains**

      Various ways of attenuating *Babesia* spp. have been reported. The most common way of reducing the virulence of *B. bovis* involves rapid passage of
the strain through susceptible splenectomised calves. Attenuation is not guaranteed if this procedure is adopted but it usually follows after 8 to 20 calf passages (4).

The virulence of *B. bigemina* decreases during prolonged residence of the parasite in latently infected animals. This feature has been used in Australia to obtain an avirulent strain (G strain). The strain was attenuated by passaging it every three months with blood in nonsplenectomised calves, splenectomising the calves at the end of the three months and then using the ensuing relapse parasites for passaging and vaccine production (1).

Frozen stablalate of avirulent strains should be prepared for safety testing and for future use in the production of vaccine.

Irradiation has been used to attenuate *Babesia* spp. but the results have been variable. *In vitro* culture in medium containing horse serum has also been reported to reduce the virulence of *B. bovis*.

iv) Preparation and storage of stablalate

Avirulent strains are readily stored as frozen stablalate of infected blood in liquid nitrogen or dry ice. Dimethyl sulfoxide (DMSO) is the recommended cryopreservative as it allows for intravenous administration after thawing of the stablalate. A detailed account of the freezing technique is reported elsewhere (1). Briefly, it involves the following: infected blood is collected, chilled to 4°C and cold cryoprotectant (4 M DMSO in PBS) added slowly with stirring to a final blood protectant ratio of 1:1 to give a final concentration of 2 M DMSO. The entire dilution procedure is carried out in an ice-bath and the diluted blood dispensed in suitable containers (e.g. 5 ml cryovials) and frozen as soon as possible in the vapour phase of a liquid nitrogen container. The vials are stored in the liquid phase.

b) Validation as a vaccine

The suitability of a strain for use in vaccine is tested by inoculating cattle with vaccine prepared from it and then challenging them and susceptible controls with a virulent, heterologous strain. This can be done in the same way or as an integral part of Batch Control (see below).

2. Manufacture

a) Production of frozen vaccine concentrate

Five to 10 ml quantities of frozen stablalate are rapidly thawed by immersing the vials in water preheated to 40°C. The thawed infective material is kept on ice and used as soon as possible to infect a susceptible, splenectomised calf by intravenous inoculation (within 30 min).

The parasitaemia of the donor calf is monitored daily by examining stained
films of jugular blood and blood collected for vaccine when suitable parasitaemias are reached. Parasitaemias of $1 \times 10^8$/ml (approximately 2% parasitaemia in jugular blood) are the minimum required for production of vaccine. If a suitable *B. bovis* parasitaemia is not obtained, passage of the strain by subinoculation of 100-200 ml of blood to a second splenectomised calf may be necessary. Passage of *B. bigemina* is not recommended.

Blood from the donor is collected by jugular or carotid cannulation using heparin as anticoagulant (5 IU heparin/ml blood).

In the laboratory, the parasitised blood is mixed in equal volumes with 3 M glycerol in PBS supplemented with 5 mM glucose at 37°C (final concentration of glycerol 1.5 M). The mixture is then equilibrated at 37°C for 30 min and dispensed in suitable containers (e.g. 5 ml cryovials). The vials are cooled at approximately 10°C/min in the vapour phase of liquid nitrogen and, when frozen, stored in the liquid phase (6).

DMSO can be used as cryoprotectant in the place of glycerol. This is done in the same way as outlined for the preparation of stabilate (14, 15). The final concentration of DMSO should be 2 M.

If glycerolised frozen vaccine is to be diluted, the diluent should consist of PBS containing 1.5 M glycerol and 5 mM glucose (10), while diluent used with vaccine cryopreserved with DMSO should contain DMSO in PBS.

b) Production of chilled vaccine

Infecitive material for chilled vaccine is prepared in the same way as for frozen vaccine but it should be issued and used as soon as possible after collection. If it is necessary to obtain the maximum number of doses per calf, the infective blood can be diluted to provide $1 \times 10^7$ Babesia parasites per dose. A suitable diluent is 10% sterile bovine serum in a balanced salt solution containing the following quantities of salts per litre:

<table>
<thead>
<tr>
<th>Salt</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>7.00 g</td>
</tr>
<tr>
<td>MgCl$_2$·6H$_2$O</td>
<td>0.34 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.00 g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>2.52 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.90 g</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>0.52 g</td>
</tr>
</tbody>
</table>

If diluent is not required, sterile acid citrate dextrose or citrate phosphate dextrose should be used as the anticoagulant at a rate of one part to four parts blood to provide the glucose necessary for survival of the organisms. *B. divergens* may be diluted in Hanks' solution.

c) Use of vaccine

In the case of frozen vaccine, vials should be thawed by immersion in water
Bovine babesiosis (B11)

Preheated to 40°C. Glycerolised vaccine should be kept cool and used within 8 h (6), while vaccine with DMSO as cryoprotectant should be kept on ice and used within 15 to 30 min of thawing (15). If the vaccine is to be diluted, isotonic diluent should be used at a ratio determined by prior infectivity trials.

Chilled vaccine should be kept refrigerated and used within 6 days of preparation.

The strains of *B. bovis*, *B. divergens* and *B. bigemina* used in the vaccine may be of reduced virulence but will not be entirely safe. A practical recommendation is therefore to limit the use of vaccine to calves, when non-specific immunity will minimise the risk of vaccine reactions. When older animals are to be vaccinated, there is a risk of severe reactions. These reactions occur infrequently, but valuable breeding stock or pregnant animals obviously warrant due attention and should be observed daily for three weeks after vaccination. Ideally, rectal temperatures should be taken of vaccinated cattle and the animals treated if significant fever develops. Reactions to *B. bigemina* and *B. divergens* are usually seen by day 6 to 8 and those to *B. bovis* by day 10 to 16 (4).

Protective immunity develops in three to four weeks and usually lasts for several years.

Babesiosis and anaplasmosis vaccines are often used concurrently but it is not advisable to use any other vaccines at the same time.

3. **In-process control**

a) **Source and maintenance of vaccine donors**

A source of calves free of natural infections of *Babesia* and other tick-borne diseases should be identified. If a suitable source is not available, it may be necessary to breed the calves under tick-free conditions specifically for the purpose of vaccine production.

The calves should be maintained under conditions which will prevent exposure to infectious diseases and to ticks and biting insects. In the absence of suitable facilities, the risk of contamination with the agents of infectious diseases present in the country involved should be estimated, and the benefits of local production of vaccine weighed against the possible adverse consequences of spreading disease (1).

b) **Surgery**

Donor calves should be splenectomised to allow maximum yield of parasites for production of vaccine. This is best done under general anaesthesia in young calves. Details of the technique, including pre- and postoperative procedures, are reported elsewhere (1).
c) **Screening of vaccine donors before inoculation**

Donor calves should be examined for all blood-borne infections prevalent in the country, including *Babesia*, *Anaplasma*, *Theileria* and *Trypanosoma*. This can be done by routine examination of stained blood films after splenectomy and preferably also by serology. Any calves showing evidence of natural infections of any of these agents should be rejected. The absence of other infective agents should also be confirmed, including the agents of enzootic bovine leukosis, mucosal disease, infectious bovine rhinotracheitis, akabane disease, ephemeral fever, bluetongue, foot and mouth disease and rinderpest. The testing procedures will depend on the diseases prevalent in the country and the availability of tests but should involve serology of paired sera at the very least and, in some cases, virus isolation (6, 15).

d) **Monitoring of parasitaemias following inoculation**

It is necessary to determine the concentration of parasites in blood being collected for vaccine. There are accurate techniques for determining the parasite count (1) but, in the absence of these, the parasite concentration can be estimated from the erythrocyte count and the parasitaemia (percentage of infected erythrocytes).

e) **Collection of blood for vaccine**

All equipment should be sterilised (e.g., by autoclaving) before use. The blood is collected in heparin using strict aseptic techniques when the required parasitaemia is reached. This is best done if the calf is sedated and with the use of a closed circuit collection system. Xylazine (Rompun, Bayer) is a suitable sedative. Depending upon demand, up to 3 litres of heavily infected blood can be collected from a 6-month-old calf. If the calf is to be saved, the transfusion of a similar amount of blood from a suitable donor is indicated. Alternatively, the calf should be euthanised immediately after collection of the blood.

f) **Dispensing of vaccine**

All procedures are performed in a suitable environment, such as a laminar flow cabinet, using standard sterile techniques. Antibiotics (500,000 IU/litre penicillin and 375,000 μg/litre streptomycin) should be added to the vaccine before dispensing. Use of a mechanical or magnetic stirrer will ensure thorough mixing of blood and diluent throughout the dispensing process.

4. **Batch control**

The potency, safety and sterility of vaccine batches cannot be determined in the case of chilled vaccine and specifications for frozen vaccine depend on the country involved. The following are the specifications for frozen vaccine produced in Australia.
a) Sterility and freedom from contaminants

Standard tests for sterility are employed for each batch of vaccine and diluent.

The absence of contaminants is determined by doing appropriate serological testing of cattle inoculated as below (see point 4c) for infectious agents which might contaminate the vaccine. These include the agents of enzootic bovine leukosis, infectious bovine rhinotracheitis, mucosal disease, ephemeral fever, akabane disease, Aino virus, bluetongue, and \textit{Brucella abortus} and \textit{Leptospira}. Infectious diseases absent from Australia and which are not tested for include foot and mouth disease, lumpy skin disease, rabies, Rift Valley fever, rinderpest, contagious bovine pleuropneumonia, heartwater, Jembrana disease, pathogenic \textit{Theileria} and \textit{Trypanosoma} spp., and \textit{Brucella abortus}, \textit{Coxiella} and \textit{Leptospira} (6).

b) Safety

Vaccine reactions of the cattle inoculated in the test for potency (see point 4c) are monitored by measuring parasitaemia, fever and depression of packed cell volume. Only batches with pathogenicity levels equal to or lower than a pre-determined standard are released for use.

c) Potency

Vaccine is thawed and diluted 1:5 and 1:50 with isotonic diluent (6). The diluted vaccine is then incubated for 8 h at 30\degree C and two groups of 5 cattle are inoculated with 2 ml doses subcutaneously. The inoculated cattle are monitored for the presence of infections by examination of stained blood smears. If the batch proves to be infective at 1:50, it is recommended for use at a dilution of 1:5 with isotonic diluent.

REFERENCES


BOVINE BRUCELLOSIS
(B12)

SUMMARY

Bovine brucellosis is usually caused by Brucella abortus, less frequently by B. melitensis and rarely by B. suis. It is manifested by abortion, with excretion of the organisms in uterine discharges and in milk. Diagnosis depends upon the isolation of Brucella from abortion material, udder secretion or from tissues removed post-mortem. Alternatively, specific cell-mediated or serological responses to brucella antigens can be demonstrated.

B. abortus, B. melitensis and B. suis are highly pathogenic for man, and all infected tissues, cultures and potentially contaminated materials must be handled under conditions for biohazard containment.

Identification of the agent: The demonstration by modified acid-fast or immunospecific staining of organisms of brucella morphology in abortion material or vaginal discharges provides presumptive evidence of brucellosis, especially if supported by serological tests. Whenever possible, the organism should be isolated and species and biovar identified by phage lysis or oxidative metabolism tests and by cultural, biochemical and serological criteria.

Serological tests: The buffered Brucella antigen tests (rose bengal plate test and buffered plate agglutination test) are suitable for screening herds and individual animals. Positively reacting samples should be retested by the complement fixation test. Enzyme immunoassay (or radioimmunoassay) procedures can also be used for both screening and confirmation. The serum agglutination test is inferior to other tests in specificity and sensitivity and is not recommended if other procedures are available. The milk ring test performed on bulk milk samples is effective for screening and monitoring dairy cattle for brucellosis but is less reliable in large herds. Another immunological test is the brucellin skin test, which can be used for screening non-vaccinated herds, provided that a purified, standardised antigen preparation (e.g. "brucellin INRA") is available.

Requirements for biological products: B. abortus Strain 19 live vaccine should be prepared from US-derived (Ames, Iowa) seed cultures and each batch must conform to minimum standards for viability, smoothness, pathogenicity and the ability to immunise guinea pigs and/or mice against challenge with a virulent strain of B. abortus. B. melitensis Strain Rev 1 vaccine may be used to immunise cattle at risk of infection with B. melitensis. It should be prepared from approved seed cultures and each batch must conform to minimum standards similar to those for B. abortus Strain 19 vaccine. B. abortus Strain 45/20 adjuvant vaccine can be used to immunise cattle of all ages over 6 months, including pregnant animals. It must be prepared from a rough seed strain and each batch must be checked for efficacy and to ensure that it lacks toxicity and does not provoke excessive local reaction or induce agglutinating
antibody. Brucellin preparations for the intradermal test must be free of lipopolysaccharide agglutinogen and must not produce non-specific inflammatory reactions or interfere with serological tests. Diagnostic antigens must be prepared from approved smooth strains of B. abortus and comply with minimum standards for identity, purity, sensitivity and specificity. Antigens for serological tests should be standardised against reference sera calibrated against the International Standard B. abortus serum.

A. DIAGNOSTIC TECHNIQUES

Brucellosis in cattle is usually caused by biovars of Brucella abortus. In some countries, particularly in Southern Europe and Western Asia, where cattle are kept in close association with sheep or goats, infection can also be caused by B. melitensis. B. suis rarely causes infection in cattle. The disease is usually asymptomatic in non-pregnant females but adult male cattle may develop an orchitis. Following infection with B. abortus or B. melitensis adult females develop a placentitis usually resulting in abortion between the fifth and ninth month of the pregnancy. Even in the absence of abortion, profuse excretion of the organism occurs in the placenta, fetal fluids and vaginal discharges. The mammary gland and associated lymph nodes may also be infected, and organisms may be excreted in the milk. Subsequent pregnancies are usually carried to term but uterine and mammary infection recurs, with reduced numbers of organisms in cellular products and milk. Hygromas, usually involving leg joints, are a common manifestation of brucellosis in some tropical countries and may be the only obvious indicator of infection; the hygroma fluid is often infected with Brucella.

Brucellosis is readily transmissible to man, causing an acute febrile illness, undulant fever, which may progress to a chronic form and can also produce serious complications affecting the musculo-skeletal, cardiovascular and central nervous systems. Infection is acquired by the oral, respiratory, conjunctival, or possibly other routes often as a result of an occupational exposure, but ingestion of infected dairy products constitutes the main risk to the general public. Laboratory manipulation of live cultures or contaminated material from animals is hazardous and must be done under conditions of biohazard containment.

1. Identification of the agent

All abortions in cattle should be investigated with the possibility of brucellosis in mind. The clinical picture is not pathognomonic although the herd history may be helpful. Smears of placental cotyledon, vaginal discharge and fetal lung, liver and abomasal contents should be fixed with heat or ethanol and stained by the modified Ziehl-Neelsen, Kösters’, Gram or Macchiavello methods, or with a fluorochrome or peroxidase-labelled antibody conjugate. The presence of large aggregates of intracellular, weakly acid-fast organisms of Brucella morphology or immuno-specifically stained organisms is presumptive evidence of brucellosis. Care must be taken in the interpretation of results as other infectious agents may have similar morphology (e.g. Coxiella burnetii, Chlamydia) or immunological cross-reactivity (e.g. Yersinia).
Culture of placental cotyledon, vaginal discharge, fetal tissues or abomasal contents, or hygroma fluid should be made on plates of serum dextrose agar and serum dextrose agar supplemented with bacitracin 25 μg/ml, cycloheximide 100 μg/ml, nalidixic acid 5 μg/ml, nystatin 100 units/ml, polymyxin B 5 μg/ml and vancomycin 20 μg/ml. Cultures can also be made from milk or colostrum and from samples of tissues collected post-mortem, such as mammary gland, uterus, supramammary and internal iliac lymph nodes from the female, and testes, epididymides, seminal vesicles, accessory glands, external inguinal and internal iliac lymph nodes from the male. From either males or females the parotid, mandibular and retropharyngeal lymph nodes are good sources of the organism. As the numbers of brucellae in these are likely to be lower than in abortion material and as contaminating bacteria may be present in milk, colostrum and some tissue samples, enrichment is advisable in liquid medium consisting of serum dextrose broth, tryptone soya broth or brucella broth supplemented with an antibiotic mixture of amphotericin B 1 μg/ml, bacitracin 25 μg/ml, cycloheximide 100 μg/ml, D-cycloserine 100 μg/ml, nalidixic acid 5 μg/ml, polymyxin B 6 μg/ml and vancomycin 20 μg/ml. The enrichment medium should be incubated at 37°C in air supplemented with 10% v/v CO₂ for up to 6 weeks, with weekly subcultures on to solid selective medium. If preferred, a biphasic system of solid and liquid selective medium in the same bottle (Castañeda technique) may be used to minimise sub-culture.

Any colonies of Brucella type (raised, convex, circular outline, transparent, smooth surface, slow growing) should be checked by examining a Gram-stained smear. If organisms of Brucella morphology (small Gram negative coccobacilli or short rods with rounded ends and slightly convex sides) are seen, the colonies should be tested for agglutination with Brucella-specific antiserum after checking for dissociation. The latter is most easily tested by suspending colonies in 0.1% w/v aqueous acriflavine: smooth colonies form a uniform suspension, non-smooth colonies form agglutinates. If the colonies are smooth they should be checked against antiserum to smooth B. abortus, or preferably antisera monospecific for the A and M surface epitopes. In the case of non-smooth isolates they should be checked with antiserum to Brucella R antigen. Positive agglutination with a Brucella antiserum provides presumptive identification of the isolate as Brucella. Subsequent full identification is best performed by a reference laboratory. Additional identification should be done by using either oxidative metabolism tests (quantitative measurement by Warburg manometry or qualitative measurement by thin layer chromatography) or phage lysis tests to identify the species (in the latter case the phage lysis pattern is dependent upon the colonial phase of the isolate and it is essential that this be determined correctly) (7). Identification to biovar level depends upon examination for growth in the presence of basic fuchsin (p-rosaniline) and thionin at final concentrations of 20 μg/ml, production of H₂S (detected by lead acetate papers), requirement of CO₂ for growth, and agglutination pattern with A, M or R specific antisera.

Identification of the vaccine strains B. abortus Strain 19 or B. melitensis Strain Rev 1, depends upon further tests. In the first case, lack of requirement for CO₂, inhibition of growth by benzylpenicillin at 3 μg/ml, thionin blue at 2 μg/ml and i-erythritol at 2 mg/ml, a high utilisation of L-glutamate (Q₀₂N > 500) and a low
residual virulence for guinea pigs is characteristic of *B. abortus* Strain 19. In the second case, *B. melitensis* Strain Rev 1 has the normal properties of a biovar 1 strain of *B. melitensis* but grows much more slowly on ordinary media, does not grow in the presence of basic fuchsin or thionin at 20 μg/ml or benzylpenicillin at 3 μg/ml but grows in the presence of streptomycin at 5 μg/ml, has very weak urease activity and shows a reduced virulence for guinea pigs and mice. (For further details see References 1, 6 & 7.)

Worldwide, *B. abortus* biovar 1 is the type most frequently isolated from cattle, biovars 3 and 6 are common in Africa and some Asian countries, biovars 2 and 4 occur in addition to biovar 1 in North and South America, biovars 5 and 9 are rare but have been locally important in some countries in the past, e.g. Britain and Germany. In South Africa, 90% of *B. abortus* isolates are biovar 1 and 10% are biovar 2.

2. **Serological tests**

The buffered *Brucella* antigen tests (rose bengal plate test and buffered plate agglutination test) are suitable for screening herds and individual animals. Positively reacting samples should be retested by the complement fixation test. Enzyme immunoassay (or radioimmunoassay) procedures can also be used for both screening and confirmation. The serum agglutination test is inferior to other tests in specificity and sensitivity and is not recommended if other procedures are available. The milk ring test performed on bulk milk samples is effective for screening and monitoring dairy cattle for brucellosis but is less reliable in large herds. Another immunological test is the brucellin skin test, which can be used for screening non-vaccinated herds, provided that a purified, standardised antigen preparation (e.g. "brucellin INRA" or "brucellin CVT") is available.

a) **Buffered *Brucella* antigen tests**

**Rose bengal test**

Serum samples may be screened using the rose bengal plate test or card test. Serum (0.03 ml) is mixed with an equal volume of antigen on a white tile or enamel plate to produce a zone approximately 2 cm in diameter. The mixture is agitated gently for 4 minutes at ambient temperature and then observed for agglutination (1). Any visible reaction is considered positive. The test is very sensitive, especially in vaccinated animals, and positive samples should be checked by the complement fixation test or by an IgG1-specific procedure. False negative reactions occur and can be detected by retesting animals at intervals over at least 3 months.

**Buffered plate agglutination test**

Serum samples may also be screened using the buffered plate agglutination test. Serum (0.08 ml) is mixed with a 0.03 ml volume of antigen on a glass plate marked in 4 x 4 cm squares. After the initial mixing, the plate should be rotated three times in a tilting motion to ensure even dispersion of the reagents, and
then incubated for 4 minutes in a humid chamber at ambient temperature (20-25°C). The plate should be removed and rotated as above and then returned for a second 4-minute incubation. At this point, the plate should be removed and rotated and observed for agglutination (1). Any visible reaction is considered positive. Like the rose bengal test, the test is very sensitive, especially in vaccinated animals, and positive samples should be checked by the complement fixation test or by an IgG1-specific procedure. False negative reactions occur and can be detected by retesting animals at intervals over at least 3 months.

b) Complement fixation test

The most accurate confirmatory test is the complement fixation test. This is most conveniently done by the microtitration method. All dilutions are made in a buffer prepared from a stock solution of sodium chloride 42.5 g, barbituric acid 2.875 g, sodium diethyl barbiturate 1.875 g, magnesium sulphate 1.018 g, calcium chloride 1.147 g in 1 litre of distilled water and diluted by addition of 4 volumes of 0.04% gelatin solution before use (CFT buffer). The indicator system is a 3% suspension of fresh sheep erythrocytes sensitised with an equal volume of rabbit anti-sheep erythrocyte serum diluted to contain 5 x the minimum concentration required to produce 100% lysis of the erythrocytes in the presence of a 1:30 dilution of fresh guinea-pig complement. The latter is independently titrated to determine the minimum concentration required to produce 100% lysis of a sensitised erythrocyte suspension; this is defined as the unit of complement. The standard B. abortus agglutination test antigen may be used at a dilution of 1:200 in CFT buffer. Alternatively, the suspension can be diluted to a concentration which gives 50% fixation of complement (1.25 units) at a dilution of 1/220 of the second International Standard B. abortus serum (ISABS) (obtainable from the OIE Reference Laboratory for Brucellosis, Central Veterinary Laboratory, Weybridge, Surrey KT15 3NB, UK). The test sera are diluted in equal volumes of CFT buffer and inactivated by heating at 58°C for 30 minutes.

Using standard 96-well microtitre plates, volumes of 25 µl of diluted test serum are placed in the wells of the first and second rows and 25 µl unit volumes of CFT buffer added to all wells except those of the first row. Serial doubling dilutions are then made by transfer of 25 µl volumes of serum from the second row onward. Volumes of 25 µl of antigen diluted to working strength and 25 µl of complement at 1.25 units strength are added to each well. 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. The plates are incubated at 37°C for 30 minutes or at 4°C overnight and 25 µl volumes of sensitised erythrocyte suspension added to each well and the plates re-incubated at 37°C for 30 minutes with occasional agitation. The results are read after the plates have been allowed 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. Results should always be expressed in international
units, calculated in relation to those obtained in a parallel titration with a reference serum calibrated against the ISABS. In general, sera giving positive fixation at a titre equivalent to 20 ICFTU/ml are considered positive. False positive reactions may arise in the testing of vaccinated animals. Females vaccinated with Strain 19 between 3 to 6 months of age are considered positive if the sera give a positive fixation at a titre of 30 ICFTU/ml when the animals are tested at an age of 18 months or greater. False positive reactions may also occur in animals infected with organisms antigenically related to Brucella. These cause far fewer problems in the complement fixation test than in agglutination-based tests.

c) ELISA test

An ELISA test has been described by Alton et al. (1, pp. 113-122). This procedure is representative of those currently in use and employs B. abortus lipopolysaccharide as antigen. The use of secondary antibody conjugates directed against immunoglobulin isotypes and sub-classes permits these to be measured if required. An ELISA specific for IgG1 antibody detection gives results closely equivalent to the complement fixation test and may be used for testing either milk or serum samples. Use of an IgM-specific ELISA provides greater sensitivity than does the complement fixation test but with some loss of specificity.

ELISA tests may be used as screening procedures or as confirmatory tests. Progress is now being made towards achieving standardisation of the numerous variants used in different laboratories.

d) Milk ring test

In lactating animals, the milk ring test can be used for screening for brucellosis on a herd or individual basis. In large herds (>1,000 lactating cows), the sensitivity of the test becomes less reliable. The test is performed by adding one drop (0.03 ml) of standard B. abortus milk ring test antigen to a 1 ml volume of whole milk which has been stored for at least 24 hours at 4°C. If bulk tank samples from large herds are to be examined, the volume of milk is increased to 3 ml. The milk samples must not have been frozen, heated or subjected to violent shaking. Abnormal milk should not be included. The milk-antigen mixtures are incubated at 37°C for 1 hour, together with positive and negative control samples. A strongly positive reaction is indicated by formation of a dark blue ring at the top of the tube. The test is considered negative if the colour of the underlying milk exceeds that of the cream layer. False positive reactions may occur in recently vaccinated cattle or in samples containing abnormal milk (colostrum, mastitis). Positive reactions to the milk ring test should be checked by carrying out confirmatory serological tests on blood samples from all the animals in the herd. (For further details, see Reference 7.)

e) Serum agglutination test

The serum agglutination test is inferior to other tests in specificity and
sensitivity and is not recommended if other procedures are available. However, this test is still widely used for the diagnosis of bovine brucellosis. It is performed in glass or plastic tubes of about 1-2 ml total volume by placing 0.8 ml of phenol saline (0.5% w/v phenol in 0.15 M sodium chloride) in the first tube and 0.5 ml volumes of phenol saline in the remaining tubes of a series of 5 or 10. A volume of 0.2 ml serum is added to the first tube, mixed, and then 0.5 ml transferred to the next tube. Further volumes of 0.5 ml are transferred to subsequent tubes to give a series of doubling dilutions. An equal volume of standard B. abortus agglutination suspension diluted in phenol saline to working strength is then added to each tube and the latter incubated at 37°C for 20 hours.

The tests are read against opacity standards prepared by diluting the working strength antigen 1 in 4, 2 in 4 and 3 in 4 to correspond to 25%, 50% and 75% agglutination. Phenol saline is used as the 100% control and the undiluted working strength antigen as the 0% control. The results are scored as the degree of agglutination (1+ = 25%, 2+ = 50%, 3+ = 75%, 4+ = 100%) over the serum dilution. In each set of tests a positive control serum calibrated against ISABS must be included. This enables the results to be expressed in International Units (IU) and permits comparability of tests performed by different laboratories (8). Titres equivalent to 50 or more IU for unvaccinated cattle and 100 IU or more for vaccinated animals are regarded as indicative of infection. In the European Community titres equivalent to 30 IU or greater are regarded as unacceptable.

The serum agglutination test is an unsatisfactory test by contemporary standards as it fails to detect many animals in the incubation stage of the disease, as well as many in the chronic phase when the predominant antibodies belong to isotypes with weak agglutinating activity at a neutral pH. The test is also subject to false positive reactions caused by non-specific agglutinins which bind to B. abortus cells via the Fc region of their immunoglobulin heavy chain structure and by cross-reacting antibodies evoked by unrelated bacteria, especially those containing perosamine based epitopes. The former accounts for about 70-80% of false positive agglutination reactions and can be eliminated by performing the test in a diluent containing 10 mM ethylene diamine tetra-acetic acid in phosphate buffered saline, pH 7.2, instead of phenol saline.

Various modifications of the agglutination test have been used as alternatives to the complement fixation test. The 2-mercapto-ethanol test is done by diluting 0.1 ml of serum in 0.4 ml of 0.15 M sodium chloride (physiological saline) and adding 0.5 ml of 0.2 M 2-mercapto-ethanol in saline. The serum is then reduced by incubation at 37°C for 1 hour, followed by serial doubling dilutions in saline. Volumes of 0.5 ml of standard serum agglutination test antigen diluted to working strength in saline (without phenol) are then added to each tube and the test subsequently performed as for the standard test. It is more specific than the standard serum agglutination test but no more sensitive.

Other modifications of the agglutination test include the Coombs antiglobulin
test, the heat inactivation test and the Rivanol (ethacridine) agglutination test. The first two do not provide diagnostically accurate information when applied to bovine sera. The Rivanol test, which is widely used in the USA, gives results very similar to those produced by the 2-mercapto-ethanol test.

f) Brucellin test

The low sensitivity of the brucellin test limits detection of individual infected animals. Therefore, this test alone cannot be recommended as an official diagnostic test. It is done by injecting intradermally 0.1 ml of a standardised, defined brucellin preparation such as "Brucellin INRA" into the caudal fold or the skin of the flank or the side of the neck. The test is read after 48 hours and a positive reaction is indicated by local swelling and induration. It is recommended that the skin thickness at the injection site should be measured with vernier calipers before injection and at re-examination 48 hours later. Strong reactions are easily recognised, but borderline reactions require careful interpretation. The diagnosis should not be made solely on the basis of positive intradermal reactions given by a few animals in the herd but should be confirmed by a reliable serological test, such as the complement fixation test. Poorly defined brucellin preparations which may contain lipopolysaccharide agglutinogen should not be used for the intradermal test as these will interfere with subsequent confirmatory serological tests.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Antigen preparations made from smooth \textit{B. abortus} Strains 99 or 1119-3 are used as whole cell suspensions in the various agglutination tests and in the complement fixation test. Similar preparations, but stained with haematoxylin, rose bengal, or brilliant green/crystal violet, are used in the milk ring, rose bengal and buffered plate agglutination tests, respectively.

\textit{B. abortus} Strain 19 is used as a live vaccine for the prevention of brucellosis in cattle. It is normally given to female calves between 3 and 6 months old as a single subcutaneous dose of $5-8 \times 10^{10}$ viable organisms. A reduced dose of $3 \times 10^8$ to $3 \times 10^9$ organisms can be administered to beef or dairy cattle between 4 and 12 months old but 5-10\% of the animals will develop persistent antibody titres. Alternatively, it can be administered to cattle of any age as two doses of $5-10 \times 10^9$ viable organisms given by the conjunctival route; this produces protection without a persistent antibody response. Requirements for Strain 19 vaccine have been defined by WHO (13).

\textit{B. abortus} Strain 19 vaccine induces good immunity to moderate challenge by virulent organisms. The vaccine must be prepared from USDA-derived seed and each batch checked for purity, viability, smoothness and absence of toxicity or virulence. Other \textit{Brucella} strains, including the rough Strain \textit{B. abortus} 45/20, have been used as vaccines. \textit{B. abortus} Strain 45/20 vaccine is prepared by suspending killed cells in an oil adjuvant. It is normally given as two doses 6-12 weeks apart followed by an annual booster to maintain immunity. The degree of protective
Immunity produced by it is probably less than that conferred by Strain 19 vaccine. Batch variation in immunogenicity and a tendency to stimulate antibodies reactive with smooth Brucella antigens is a major problem and careful control of these factors is required. No international standards have been developed for this vaccine although recommendations have been made for its preparation and testing (3, 5, 9). In most countries where herd vaccination appears to be useful, reduced doses of Strain 19 are now used instead of Strain 45/20 vaccine.

Brucellin INRA is a lipopolysaccharide-free extract of rough B. melitensis B115 cells and contains 50-75% proteins and 15-30% carbohydrate. It does not provoke inflammatory responses in unsensitised animals and does not stimulate antibodies reactive in tests with lipopolysaccharide antigens. It is given to cattle in doses of 100 μg by the intradermal route and provokes a local delayed hypersensitivity reaction visible at 48-72 hours in sensitised animals. Positive reactions can be given by vaccinated as well as by infected animals.

1. Seed management

a) Characteristics

_B. abortus_ Strain 99 (Weybridge) or _B. abortus_ Strain 1119-3 (USDA) should be used for diagnostic antigen production. Original seed should be obtained from the Central Veterinary Laboratory, Weybridge, Surrey KT15 3NB, UK, or the USDA, National Veterinary Services Laboratories, 1800 Dayton Avenue, Ames, Iowa 50010, USA. The strains must be completely smooth and non-agglutinable in saline and 0.1% w/v acriflavine. They must be pure cultures and conform to the characteristics of CO₂-independent strains of _B. abortus_ biovar 1. The original seed cultures should be propagated to produce a seed lot which must conform to the properties of these strains and should be preserved by lyophilisation or by freezing at liquid nitrogen temperature.

_B. abortus_ Strain 19 original seed for vaccine production must be obtained from the USDA, National Veterinary Services Laboratories (address above), and used to produce a seed lot which is preserved by lyophilisation or by freezing at liquid nitrogen temperature. The properties of this must conform to those of a pure culture of a CO₂-independent _B. abortus_ biovar 1 which is, additionally, sensitive to benzylpenicillin, thionin blue and _i_ -erythritol at recommended concentrations, and displays minimal pathogenicity for guinea pigs.

_B. melitensis_ Strain B115 used to produce brucellin should be obtained from a source such as INRA, Station de Pathologie de la Reproduction, Nouzilly, Tours, France. The original seed should be propagated to produce a seed lot which should be preserved by lyophilisation or freezing at liquid nitrogen temperature. It should conform to the properties of a pure culture of a rough strain of _B. melitensis_ and must not produce smooth Brucella lipopolysaccharide. It should produce reasonable yields of a mixture of protein antigens reactive with antisera to smooth and rough Brucella strains.
b) Culture

For antigen production the *B. abortus* Strain 99 or 1119-3 seed culture is used to inoculate a number of potato infusion agar slopes that are incubated at 37°C for 48 hours. The growth is checked for purity and resuspended in sterile phosphate buffered saline, pH 6.4, and used to seed layers of potato infusion agar or glycerol dextrose agar in Roux flasks which are subsequently incubated at 37°C for 72 hours with the inoculated surface facing downwards. Each flask is checked for purity by Gram staining samples of the growth, and the organisms are harvested by adding 50-60 ml of phenol saline to each flask, followed by gentle agitation and decantation of the suspension. The organisms are killed by heating at 95°C for 1 hour.

Alternatively, the cells may be produced by batch or continuous culture in a fermentor, using a liquid medium containing D-glucose 30 g, a high grade of peptone 30 g, yeast extract (Difco) 10 g, sodium dihydrogen phosphate 9 g, and disodium hydrogen phosphate 3.3 g per litre of distilled water. The initial pH is 6.6 but tends to rise to pH 7.2-7.4 during the growth cycle. Care should be taken to check batches of peptone and yeast extract for capacity to produce good growth without formation of abnormal or dissociated cells. Vigorous aeration and stirring is required during growth and pH adjustment by addition of sterile 0.1 M HCl may be necessary. The seed inoculum is prepared as described above and the culture is incubated at 37°C for 48 hours in the case of batch culture. Continuous culture runs can be operated for much longer periods but more skill is required to maintain them. The culture is harvested by centrifugation to deposit the organisms which are then resuspended in phenol saline. They are killed by heating at 80°C for 90 minutes, followed by a viability check and subsequent storage at 4°C. In-process checks should be made on the growth from either solid or liquid medium to ensure purity, an adequate viable count and freedom from dissociation to rough forms. The packed cell volume of the killed suspensions can be determined by centrifuging 1 ml volumes in Wintrobe tubes at 3,000 g for 75 minutes.

*B. abortus* Strain 19 for vaccine production is grown on medium free of serum or other animal products under conditions similar to those described for *B. abortus* Strain 99 or 1119-3.

*B. melitensis* Strain B115 is best grown in the liquid medium described for fermentor culture. It may be grown in a fermentor or in flasks agitated on a shaker. Purity checks should be made on each single harvest and the organisms must be in the rough phase.

c) Validation as a vaccine

Numerous independent studies have confirmed the value of *B. abortus* Strain 19 as a vaccine for protecting cattle from brucellosis. The organism behaves as an attenuated strain when given to sexually immature cattle. In rare cases it may produce localised infection in the genital tract.Persisting antibody responses
are likely to occur in a substantial proportion of cattle vaccinated with the standard dose as adults. Some of the cattle vaccinated as calves may later develop arthropathy, particularly of the femorotibial joints (4). The vaccine is safe for the vast majority of animals if administered to calves between 3 and 8 months of age. It produces lasting immunity to moderate challenge with virulent strains but the precise duration of this is unknown. The vaccine strain is stable and reversion to virulence is extremely rare. It has been associated with the emergence of i-erythritol-utilising strains when inadvertently administered to pregnant animals. The organism behaves as an attenuated strain in the guinea pig and even large inocula are rapidly cleared from the tissues. It has some virulence for man, and infections have occasionally followed accidental inoculation with the vaccine. Care should be taken in its preparation and handling and a hazard warning should be included on the label of the final containers.

2. Manufacture

Antigens

Rose bengal plate test antigen is prepared by depositing killed *B. abortus* Strain 99 or 1119-3 cells at, e.g., 23,000 g for 10 minutes at 4°C and uniformly resuspending in phenol saline at the rate of 1 g to 22.5 ml. To every 35 ml of this suspension, 1 ml of 1% w/v rose bengal (CI No. 26600) in distilled water is added and the mixture stirred for 2 hours at room temperature. The mixture is filtered through cotton wool, centrifuged to deposit the stained cells which are then uniformly resuspended at the rate of 1 g cells to 7 ml of diluent (21.1 g sodium hydroxide dissolved in 353 ml of phenol saline, followed by 95 ml lactic acid and then adjusted to 1,056 ml with more phenol saline). The pH of this suspension should be 3.65 ± 0.05. After filtration through cotton wool it is filtered twice through a Sartorius No. 13430 glass fibre pre-filter, adjusted to a packed cell volume of 8% pending final standardisation against reference sera and stored at 4°C in the dark. (Note: if sodium carboxymethyl cellulose is used as the sedimenting agent during preparation of the cell concentrate, insoluble residues of this must be removed by filtering the suspension through an AMF-CUNO Zeta-plus pre-filter (Type CPR 01A) before staining.)

Antigen for the buffered (*Brucella*) plate agglutination test is prepared from *B. abortus* Strain 1119-3 according to the procedure described by Angus and Barton (2). The following description has been summarised from ref. 1 (Chapter 2).

Two staining solutions are required: a solution of brilliant green certified biological stain in distilled water (2 g/100 ml) and a solution of crystal violet certified biological stain in distilled water (1 g/100 ml). Once prepared, the two solutions should be stored separately for a period of 24 hours and then mixed together in equal parts in a dark bottle and stored in a refrigerator for a period of not less than 6 months before use. The mixed stain may be used between 6 and 12 months after initial preparation, after which it should be discarded.

Buffered diluent is prepared by slowly dissolving 150 g of sodium hydroxide in
3-4 litres of sterile phenol saline. Lactic acid (675 ml) is added to this solution and the final volume is adjusted to 6 litres by adding sterile phenol saline. The pH of the solution should be 3.63-3.67.

*B. abortus* packed cells are diluted to a concentration of 250 g/l in phenol saline. Six ml of stain are added per litre of the cell suspension and the mixture is shaken thoroughly before being filtered through sterile absorbent cotton. The cells are then spun down using a refrigerated centrifuge. The packed cells are then resuspended at a concentration of 50 g/100 ml in buffered diluent (as described above) and the mixture is shaken thoroughly for 2 hours. The mixture is further diluted by the addition of 300 ml of buffered diluent per 100 ml of suspended cells (i.e. final concentration of 50 g packed cells/400 ml buffered diluent). This mixture is stirred at room temperature for 20-24 hours before the cell concentration is adjusted to 11% w/v in buffered diluent. This suspension is stirred overnight before testing. Pending final quality control tests, the antigen is bottled, labelled with an expiry date of one year, and stored at 4°C until required for use.

**Milk ring test antigen** is prepared from concentrated, killed *B. abortus* Strain 99 or 1119-3 cell suspension by depositing the cells at, e.g., 23,000 g for 10 minutes at 4°C followed by resuspension in haematoxylin staining solution. This is prepared by adding 100 ml of 4% w/v haematoxylin (Cl No. 75290) in 95% ethanol to a solution of 9 g ammonium aluminium sulphate in 100 ml distilled water plus 30 ml glycerol, followed by 2 ml of freshly prepared 10% w/v sodium iodate solution. After standing for 30 minutes at room temperature, the deep purple solution is added to 940 ml of 10% w/v ammonium aluminium sulphate in distilled water. The pH of this mixture is adjusted to 3.1 and the solution stored at room temperature in the dark for 3 months.

Before use, the staining solution is shaken and filtered through cotton wool. The packed cells are suspended in the staining solution at the rate of 45 g per litre and stained at room temperature for 48 hours. The stained cells are then deposited by centrifugation and washed three times in a solution of 6.4 g sodium chloride, 1.5 ml 85% lactic acid and 4.4 ml of 10% sodium hydroxide in 1.6 litres of distilled water, final pH 4.0. The washed cells are resuspended at the rate of 1 g in 27 ml of a diluent consisting of phenol saline adjusted to pH 4.0 by addition of about 2.5 ml of 0.1 M citric acid and 1 ml of 0.5 M disodium hydrogen phosphate and held at 4°C for 24 hours. The mixture is filtered through cotton wool, the pH checked and the packed cell volume determined and adjusted to 4%. The sensitivity of the antigen is then checked against that of a previously standardised batch using *Brucella*-negative milk in which the ISABS has been diluted (11). The antigen must be stored at 4°C and not frozen. Variations on this manufacturing procedure (1, 10) yield a similar product.

**Standard antigen suspension for the serum agglutination test** is prepared from killed *B. abortus* Strain 99 or 1119-3 concentrate by diluting this in phenol saline to a packed cell concentration of 2%. This is then diluted in phenol saline and titrated against the ISABS or an equivalent reference serum and the cell concentration adjusted to give the appropriate sensitivity. Currently, the Weybridge antigen at
working strength is adjusted to give 50% agglutination with a 1-in-650 dilution of the ISABS. The stock bulk suspension is stored at 10 x the working strength at 4°C pending distribution into its final containers.

Antigen for the *B. abortus* complement fixation test can be prepared by special procedures (1, 10), or the standard agglutination test antigen can be used after diluting the stock suspension 1 in 200 in complement fixation test buffer.

**Vaccines**

For production of *B. abortus* Strain 19 vaccine the procedures described above (in 1b) can be used except that the cells are collected in phosphate buffered saline, pH 6.3, and sedimented by centrifugation or by addition of sodium carboxymethyl cellulose at a final concentration of 1.5 g/litre. The yield from one fermentor 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 a final bulk which is used to fill the final containers of a batch of vaccine. Before pooling, each single harvest must be checked for purity, cell concentration, dissociation and identity. A similar range of tests must be done on the final bulk which should have a viable count of between 8 and 24 x 10⁹ colony forming units per ml. Adjustments in concentration are made by the addition of phosphate buffered saline for vaccine to be dispensed in liquid form, or stabiliser for freeze-dried vaccine. If the latter is to be used, loss of viability on freeze-drying should be allowed for. This should not be much in excess of 50%. The final dried product should not be exposed to a temperature exceeding 35°C during the process and the residual moisture content should be 1-2%. The contents must be sealed under vacuum immediately after drying and stored at 4°C.

*B. melitensis* Strain B115 is best grown in the liquid medium described for fermentor culture. It may be grown by the batch or continuous method in a fermentor or in flasks agitated on a shaker. Purity checks should be made on each single harvest and the organisms must be in the rough phase. They are collected by centrifugation at 9,000 g for 15 minutes at 4°C, washed in cold sterile distilled water and killed by dispersing a dense aqueous suspension into at least a twenty-fold excess of acetone at -20°C.

After repeated washing in cold acetone, followed by a final rinse in diethyl ether, the cells are dried over calcium chloride and held at 4°C. The dried cells are then subjected to a viability check. They are resuspended in sterile 2.5% NaCl to a final concentration of 5% w/v and agitated for 3 days at 4°C. The bacteria are deposited by centrifugation and the supernatant concentrated four-fold on a Diaflo PM10 ultrafiltration membrane and precipitated by addition of three volumes of ice-cold ethanol. The mixture is held at 4°C for 24 hours and the precipitate recovered by centrifugation, redissolved in sterile water and dialysed to remove ethanol. After centrifugation at 105,000 g for 6 hours at 4°C, the supernatant material comprising the unstandardised brucellin, is subjected to assays for protein and carbohydrate. It may be freeze-dried as the bulk material or after dispensing into its final containers.
3. **In-process control**

Cells for the preparation of milk ring, rose bengal plate, buffered plate, agglutination or complement fixation test antigens should be checked for purity and smoothness at the stage of harvesting. After killing, they must form stable suspensions in physiological saline solutions and show no evidence of auto-agglutination. A viability check must be performed on the suspensions after killing and no growth must be evident after 10 days' incubation at 37°C.

After staining and washing, the milk ring and rose bengal plate test antigens should not contain free stain in the supernatant of a centrifuged sample. The pH of the milk ring test antigen should be between 3.3 and 3.7, its colour dark blue appearing almost black in bulk, and the packed cell volume must be 4%. The pH of the rose bengal plate test antigen should be 3.65 ± 0.05, its colour an intense pink and the packed cell volume should approximate 8% before standardisation. The pH of the buffered plate antigen itself should be 3.7 ± 0.03, and the pH of a serum:antigen mixture of 8:3 should be 4.02 ± 0.04. The 11% stained cell suspension should be blue-green in colour. The packed cell volume of the concentrated antigen suspension for the agglutination and complement fixation tests should be 2%. The phenol concentration must not exceed 0.5% and the pH should be between 6 and 7. The appearance of the antigen when diluted 1 in 10 in phenol saline must be that of a uniform, dense white suspension with no visible aggregation or deposit after incubation at 37°C for 18 hours.

Strain 19 vaccine should be checked for purity and smoothness during preparation of the single harvests. The cell concentration of the bulks should also be checked. This can be done by opacity measurement but a viable count must be performed on the final filling lots. The identity of these should also be checked by agglutination tests with antiserum to *Brucella* A antigen. The viable count of the final containers should be not less than 50 x 10^9 per dose after freeze-drying, if this is to be done, and at least 95% of the cells must be in the smooth phase.

The crude brucellin extracts should be checked for sterility after acetone extraction to ensure killing of the *Brucella* cells and again at the end of the process to check for possible contamination. The pH and protein concentration should be determined and potency and identity tests should be performed on the bulk material before filling into the final containers.

4. **Batch control**

a) **Sterility**

Tests for sterility and freedom from contamination of biological materials may be found in the Chapter on General Information.

b) **Safety**

The only safety test needed for antigen preparations is the viability test
performed on the heated cell suspensions before staining and/or standardisation, to ensure that all the brucellas are dead.

A safety test on Strain 19 vaccine may be performed in guinea pigs. Groups of at least ten animals are given intramuscular injections of doses of vaccine diluted in phosphate buffered saline, pH 7.2, to contain $5 \times 10^9$ viable organisms. They should show no obvious adverse effects and there must be no mortality. After 10 days the animals are killed, and their spleens and blood samples collected. The spleen tissue must not contain more than $5 \times 10^5$ brucella organisms per g and the serum agglutinin titre with standard *B. abortus* antigen must not exceed 1,000 IU/ml. In practice, batches of vaccine which fail this test are encountered extremely infrequently and its value is uncertain.

Samples of brucellin from the final containers should be subjected to the standard sterility test. Doses equivalent to 100 cattle test doses should be injected subcutaneously or intramuscularly into pairs of normal guinea pigs that have not been exposed previously to brucella organisms or their antigens. There should be no local or generalised reaction to the injection and the animals should not become seropositive to the standard tests for brucellosis (buffered *Brucella* antigen test, agglutination or complement fixation tests). Similarly, after intradermal injection of graded doses of brucellin up to and including $100 \times$ the optimum guinea pig skin test reactive dose, normal guinea pigs should not develop delayed hypersensitivity responses.

c) Potency

The rose bengal plate test antigen should give a clearly positive reaction with 1-in-45 and 1-in-47.5 dilutions but not 1-in-50 dilutions of the ISABS in saline. It should also give a pattern of positive and negative reactions with a panel of sera similar to that produced by a previously standardised batch. Each batch of buffered plate antigen should be checked by testing at least 10 weakly reactive sera and comparing the results with one or more previous batches of antigen. If possible, the antigen batches should be compared to the reference antigen prepared by the National Veterinary Services Laboratories of the US Department of Agriculture.

Standardised milk ring test antigen should react to the appropriate level of sensitivity with "positive milk" prepared by diluting the ISABS in negatively reacting milk.

The standard serum agglutination test antigen should show an appropriate level of sensitivity with the ISABS or a reference serum calibrated against this. The same antigen can also be used for the complement fixation test after checking that it does not produce anticomplementary effects at the working strength for this test.

The potency of Strain 19 vaccine is determined routinely in guinea pigs or mice. In the former case groups of guinea pigs are injected subcutaneously with 1/15th of the standard subcutaneous cattle dose of the test vaccine or a
reference preparation, followed 6 weeks later with an intramuscular challenge of virulent *B. abortus* strain, such as 544 or 2308. This can be given at the single dose level of 5,000 viable organisms but it is preferable to use a graded challenge of 500, 5,000 and 50,000 organisms. After a further 6 weeks, the animals are killed and the spleen counts of viable *B. abortus* determined. The protection index (organisms/gram of spleen in vaccinated compared with unvaccinated) relative to the reference preparation is then calculated (12). The virulence and infectivity of the challenge strain must be such that the ID$_{50}$ is less than 100 organisms and that five weeks after inoculation of guinea pigs with 5,000 organisms the mean spleen: body weight ratio is not less than 0.30% and the mean serum agglutination titre is not less than 600 IU/ml.

In the mouse potency assay, graded doses of vaccine are injected into 5-week-old mice (preferably CD1) weighing 21-25 g. Thirty days later the mice are challenged by the intraperitoneal injection of 200,000 viable *B. abortus* Strain 544 organisms and the spleen counts determined 15 days subsequent to this. Parallel tests are conducted with a reference vaccine and the protection index calculated.

There is some dispute as to the validity of mouse or guinea pig assays for determining the potency of brucella vaccines. However, provided that statistically valid numbers of animals are used (at least 30 per group) and simultaneous comparison is made with a reference vaccine, the results do appear to differentiate between effective and ineffective vaccines.

The potency of brucellin preparations is determined by intradermal injection of graded doses of brucellin into guinea pigs sensitised by inoculation with brucella antigen in Freund's complete adjuvant from 1 to 6 months previously. The erythematous reactions are read and measured at 24 hours and the optimum dose determined. Multiples of this dose (usually in the region of 100 fold) are checked by intradermal injection into the neck of brucella-sensitised cattle. The reactions, in this case marked by local swelling, are read at 72 hours. No reaction must be produced in unsensitised or uninfected animals. Guinea pigs or cattle infected with brucella can be used for the test but the level and duration of sensitivity tends to be more variable than in those sensitised by injection of the antigen in Freund's complete adjuvant.

REFERENCES


SUMMARY

Genital campylobacteriosis is a venereal disease of cattle caused by Campylobacter fetus subsp. venerealis.

In the bull, the organism localises in the prepuce, on the glans penis and in the distal urethra, but causes no pathological lesions. In the cow, it infects the vagina, cervix, uterus and oviducts. This may cause failure of conception or abortion, resulting in serious economic loss.

Identification of the agent: Genital campylobacteriosis can be diagnosed from samples taken from bulls, cows or aborted fetuses. Diagnosis is made by demonstration either of the causal organism or of a specific immune response to it. In the case of bulls, samples of semen or of preputial smegma secretions can be collected; in cows, mucus samples are obtained by suction, vaginal lavage or by use of tampons. Aborted fetuses can also be examined by similar techniques, and wet preparations of the stomach contents can be examined for the organism by dark-field and phase-contrast microscopy.

The organism can be cultured on blood agar and modified Skirrow's plates when incubated microaerobically at 37°C for 3 days. It is a Gram-negative spirally curved rod, about 1.5 μm long and 0.5 μm wide.

Immunofluorescence may also be used to identify the organism.

Serological tests: Agglutination tests on vaginal mucus provide a useful herd test, but not for identifying individual infected animals. The animals to be tested should be selected carefully, since even in infected herds some animals may have escaped infection. After infection, there is a lag phase before antibodies develop. Also, agglutinins tend to disappear at the time of oestrus.

Enzyme immunoassay, such as the enzyme-linked immunosorbent assay (ELISA), is more sensitive but still should be used as a herd test rather than for individuals.

Requirements for biological products: A vaccine may be prepared from Campylobacter fetus subsp. fetus, which shares antigens with C. fetus subsp. venerealis. This vaccine is inactivated with formalin, and may be administered in combination with an oil-emulsion adjuvant.

A. DIAGNOSTIC TECHNIQUES

Genital campylobacteriosis is a venereal disease of cattle caused by Campylobacter fetus subsp. venerealis.
In the bull, this organism is located in the prepuce, on the glans penis and in the distal end of the urethra, but it causes no pathological lesions. In the cow, it infects the vagina, cervix, uterus and oviducts; this may lead to failure to conceive or to abortion, and so cause serious economic losses.

1. Identification of the agent

Genital campylobacteriosis can be diagnosed from samples taken from bulls, cows or aborted fetuses. The causal organism is identified directly or its presence detected by a specific antibody response. Samples can be processed for direct culture, enrichment culture, or immunofluorescence techniques.

Campylobacters are microaerobic, requiring an oxygen concentration of 3-15% (optimal 4-8%). Their growth is improved by raising the carbon dioxide level to 5-10% (11). This can be achieved by using a gassed incubator or a gas-generating apparatus such as commercially available gas-generating envelopes (Campy Pak BBL): these are placed in an anaerobic jar.

a) Direct culture

For the direct culture of *C. fetus* subsp. *venerealis*, modified Skirrow's medium and 7% blood agar plates are used.

**Modified Skirrow's medium:** This consists of 40 g of blood agar base No. 2 added to 1 litre of distilled water. This is brought to the boil to dissolve the ingredients, and is then autoclaved at 121°C for 15 minutes and allowed to cool to 50°C. Two vials of *Campylobacter* growth supplement (Skirrow) are added to 50 ml lysed horse blood, and actidione (cycloheximide) added to a final concentration of 100 μg per ml. The plates are poured as usual.

**7% blood agar:** 40 g of blood agar base are dissolved in 1 litre of distilled water and brought to the boil to dissolve the ingredient. It is autoclaved at 121°C for 15 minutes and cooled to 50°C. 70 ml of sheep's blood are added and the plates poured as usual.

The plates are inoculated with suspect material and incubated at 37°C under microaerobic conditions of 5-15% oxygen, 5-10% carbon dioxide in nitrogen or hydrogen for 3 days. They are then examined for the colony growth typical of *Campylobacter*. The colonies are 1-3 mm in diameter, convex with an entire edge, and are translucent or buff coloured. On moist agar swarming may occur (11). The organism is a spirally curved Gram-negative, non-acid-fast, non-sporulating rod. It is 0.2-0.8 μm in diameter and 0.5-8.0 μm in length. It stains weakly so that it may be necessary to increase the concentration of the stain or to prolong the staining time. The rods may have one or more spirals and appear S-shaped or as "gull's wings". In older cultures, coccoid forms may predominate. Frequent subculturing in the laboratory may cause the organism to lose its curved morphology (11). It has a characteristic, darting, corkscrew motility which is best observed by dark-ground or phase-contrast microscopy (11). Normally, the organism has one flagellum on one or both poles although
non-flagellated variants and those with several flagellae can occur. It may be distinguished from other campylobacters by biotyping (11).

b) Enrichment media

There are various transport and enrichment media (TEM) that will selectively increase numbers of C. fetus organisms in culture and control contaminant growth. These include Weybridge and Australian TEM media, and, in the USA, Foley's or Clark's transport media.

Weybridge TEM (9): This requires aliquots of filtered and unfiltered samples of preputial washings to be inoculated. After 3 days' incubation at 37°C, the TEM is subcultured onto plates of blood agar and modified Skirrow's medium and incubated as before.

Australian TEM (5): This TEM requires a sample of preputial fluid to be injected via a small hole in the lid of a container holding 10 ml of enrichment medium in an atmosphere of 5% carbon dioxide, 5% oxygen and 90% nitrogen. This is transported to the laboratory within 2 days and incubated at 37°C for 4 days. Two to 3 ml of sterile physiological saline is added and mixed thoroughly and then all available liquid is examined for C. fetus by culture and immunofluorescence. This requires the correct manufacture and evacuation of the containers and storage at 4°C for about a week before use. It remains active for at least 3 months and permits a delay of up to 2 days for the transport of samples to the laboratory. The production of this medium is more complicated than for the Weybridge TEM, and its manufacture demands more laboratory equipment.

c) Immunofluorescence

This can be applied to identify the organism by the direct technique. C. fetus strains (C. fetus subsp. venerealis, C. fetus subsp. venerealis biotype intermedius and C. fetus subsp. fetus) are grown separately on 7% blood agar microaerobically at 37°C for 3 days. The organisms are harvested into phosphate buffered saline (PBS) pH 7.2 and washed twice by centrifugation. Rabbits aged 3 months are inoculated intramuscularly with 2 ml of 10^{11} organisms per ml of a C. fetus subspecies suspended in PBS and Freund's incomplete adjuvant. The inoculum is administered at four sites, 0.5 ml at each site. The animals are bled before inoculation and at weekly intervals thereafter. When the serum titres reach high levels, as estimated by C. fetus serum agglutination tests, 0.5-1.0 ml of 10^{10} viable organisms are injected intravenously. The rabbits are bled for serum 7 days later. Homologous sera are pooled. The IgG fraction is separated by precipitation with sodium sulphate. The serum is adjusted to pH 8.0 with 0.1 M PBS, and 18 g anhydrous sodium sulphate added to each 100 ml. The precipitate is collected by centrifugation and redissolved in distilled water to its original volume. It is reprecipitated by the addition of 12 g sodium sulphate per 100 ml. The precipitate is collected,
dissolved in distilled water to half its volume and dialysed against PBS pH 7.2 at 4°C until free of sulphate. The protein content is determined and the concentration adjusted to 1.0-1.5 g protein (as bovine serum albumin) per 100 ml with PBS. This is adjusted to pH 9.0 with 1 M sodium carbonate. Fluorescein isothiocyanate isomer 1 (FITC) in a minimum volume of 0.1 M sodium carbonate is added to a final concentration of 15 mg FITC per 1.0 g protein. This is stirred for 18 hours at 4°C. The mixture is adjusted to pH 7.0 with 0.1 M hydrochloric acid and dialysed against frequent changes of PBS at 4°C. Final traces of free FITC are removed by gel filtration on Sephadex G25, and the final product stored at -20°C or lower in small aliquots.

The working dilution of the conjugate is determined by testing various dilutions in PBS against smears of a C. fetus culture, and selecting twice the lowest concentration which produces brilliant fluorescence with these test organisms. Under high-power microscopy the organisms fluoresce with a typical morphology, and are most often concentrated at the edges of the smear.

The fetus: Aborted fetuses are examined for the presence of C. fetus by the standard techniques. Wet preparations of fetal stomach contents are examined by dark-ground and phase-contrast microscopy for the presence of C. fetus subsp. venerealis. These preparations will show specific fluorescence with the FITC conjugate.

Sampling: In the bull, samples of the preputial smegma secretions are collected. These may be obtained by suction, scraping, preputial lavage, or indirectly, through semen collection. To obtain preputial washings, 20 ml of PBS, pH 7.2, are used to irrigate the prepuce, which is then vigorously massaged (3). Semen is collected artificially and retained under sterile conditions. Swabs are taken of the artificial vagina where it contained the penis. The appliance is subsequently flushed through with about 40 ml PBS and the washings collected for culture and immunofluorescence. The semen is similarly cultured.

The use of a Bartlett pipette is a means of collecting preputial smegma. It is a glass pipette about 54 cm long fitted with a 60 ml rubber bulb at one end, having a 15 degree bend in the tube about 38 cm from the bulb (1). This is principally for the diagnosis of Trichomonas fetus, but it is a technique still widely used for the diagnosis of infection with C. fetus subsp. venerealis (6). Scraping the penile and preputial mucosa is another method of sampling, but this practice may cause some bleeding, although without apparent discomfort or secondary effects to the bull. This has been found to be an efficient means of collecting samples (12), but it is a technique which may be resisted by owners of highly prized bulls (6).

The samples are fixed onto glass slides, stained with the FITC conjugated antiserum and examined for the fluorescing organisms. Staining is carried out in a moist chamber at 37°C for 30 minutes. The slides are then rinsed free of conjugate, washed in 2 changes of PBS (10 minutes each wash) and mounted in buffered glycerol (80% v/v glycerol: 20% 0.1 M phosphate buffer, pH 8.0).
The coverslips are sealed to prevent drying, and the slides examined under ultraviolet light microscopy.

For direct culture, part of the sample is filtered through a 0.65 μm pore diameter filter. Aliquots of filtered and unfiltered sample are inoculated onto plates of blood agar and modified Skirrow's medium and incubated microaerobically for 3 days. They are examined for the typical colonies.

Diagnosis of infection in bulls may also be made by a test mating procedure. A virgin heifer, which has been tested and found to be free of Campylobacter infection, is permitted to be served naturally by the bull in question. The heifer is then examined 4-7 days later for the presence of the organism, and after 21 days it is tested by the vaginal mucus agglutination test. This is necessarily an expensive means of diagnosis.

Samples may be collected from cows by vaginal lavage, suction or by the use of tampons. For vaginal lavage, a length of rigid polythene tubing is prepared by burning about 16 small holes about 3 cm from the closed end (10). The open end of the tube is fitted to a 20 ml syringe, and sterile PBS drawn into both tube and syringe, excluding all air. The blocked end is inserted into the vagina as far as the cervix and the saline solution forcibly expelled into the vagina. The fluid is aspirated back into the syringe and the process repeated 4-6 times before being finally collected into a sterile container.

Samples of vaginal fluid may also be collected by suction. A tube about 45 cm long and 0.75 cm in external diameter is inserted into the vagina and suction applied by mouth (which is undesirable) or by small suction pump (13). Tampons can also be employed. This is time-consuming, for the tampons should remain in the vagina for at least 20 minutes; only then will a small amount of fluid be collected. This method is reported to give rise to false positive results in the vaginal mucus agglutination test.

The samples are then processed for examination by immunofluorescence. They are first centrifuged at 500 g for 5 minutes and the deposit discarded. The supernatant fluid is recentrifuged at 5,000 g for 15-20 minutes. This second supernatant fluid is again discarded. The deposit is resuspended in a minimum volume of PBS and smears made on glass slides. These are air-dried and fixed in 90-95% ethanol at room temperature for 10-12 minutes. After rinsing in PBS the preparations are stained with the FITC conjugate diluted to the previously determined optimal titre.

For direct culture, part of the sample is filtered through a 0.65 μm filter. Aliquots of unfiltered and filtered samples are inoculated onto plates of 7% blood agar and modified Skirrow's medium and incubated microaerobically at 37°C for 3 days, when they are examined for the typical colonies of C. fetus subsp. venerealis. For examination of enrichment medium, 1 ml of the vaginal lavage is placed in TEM and incubated at 37°C for 3 days. Blood agar and modified Skirrow's plates are inoculated from the TEM and the plates incubated microaerobically at 37°C for 3 days.
2. **Serological tests**

Tests for antibodies include the vaginal mucus agglutination test and the enzyme-linked immunosorbent assay (ELISA).

a) **Vaginal mucus agglutination test**

This test is useful in herds, but not for identifying individual animals with C. *fetus* infection. Only about 50% of infected animals are identified. The test is best done on vaginal mucus taken 37-70 days after infection, but the presence of antibodies may be delayed for up to 3-4 months. Some cows may remain positive for several years whilst others become negative within 2 months. About 50% of positive cows will become negative in 6 months (6).

Samples taken by vaginal lavage are assumed to have been diluted 1:5 with the saline wash. When samples are obtained by the use of tampons, the vaginal mucus is extracted with 7 ml of physiological saline and left overnight at 4°C. The fluid is pressed from the tampon and used as the test fluid for the vaginal mucus agglutination test.

The antigen for the vaginal mucus agglutination test is a 48-hour growth of C. *fetus* subsp. *venerealis* on blood agar. This is either subcultured onto 20-30 blood agar plates, or a suspension of the bacteria in sterile PBS is pipetted into Roux flasks of blood agar and spread over the surface of the medium by gentle rocking. The cultures are incubated for 2 days at 37°C microaerobically in 85% nitrogen, 10% carbon dioxide and 5% oxygen. The growth is harvested and suspended in 0.5% formol saline. If Roux flasks are used, 10 ml of formol saline and a few glass beads are used to remove the growth. The suspension is filtered through muslin to remove coarse debris, washed 3 times by centrifugation at 6,000 g for 20 minutes, and the final wash resuspended in 0.25% formol saline and stored for one week. To titrate the antigen, a dilution series is prepared in formol saline. Each tube should contain 0.5 ml serum and 0.5 ml antigen. The tube contents are mixed well before incubation at 37°C for 18 hours. The correct dilution of antigen should show 50% agglutination at a serum dilution of between 1:320-1:640.

Vaginal mucus samples are homogenised with 4 volumes of PBS using ground glass beads. If a vaginal lavage has been carried out, the sample is already suitable. Two ml of the homogenate are transferred to a test tube placed in a water bath at 57°C. Approximately 10 ml of molten Oxoid agar no. 1 are also placed in the same water bath. When the contents of both tubes have equilibrated to 57°C, 2 ml of the agar are pipetted into the homogenate. The mixture is shaken vigorously and quickly poured into a 45 mm wide-mouth screw-cap container. When the mixture has gelled, the lid is secured and the container incubated for 18 hours at 37°C to extract the antibody.

The clear fluid is aspirated off the agar surface and used as the test sample in a 3-tube agglutination test. The first tube is left empty, while 0.5 ml of 0.5%
phenol saline is pipetted into the others. Then 0.5 ml of the test sample is put into both the first and second tubes. The contents of the second are mixed and 0.5 ml is transferred into the third tube. The contents of the third tube are mixed and 0.5 ml are discarded. 0.5 ml of a standardised dilution of the antigen is then added to each tube and well mixed. After incubation at 37°C for 18 hours, the tests are read by oblique light against a dark background. Each dilution is scored as follows:

Water clear = ++++
75% clearing = +++
50% clearing = ++
25% clearing = +
No clearing = -

A positive control titration should be carried out at the same time, using the same method as that for determining the titre of the antigen against a known positive antiserum. The antigen is used at the same standardised dilution against dilutions of positive serum.

The positive control tubes should read:

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:160</td>
<td>++++</td>
</tr>
<tr>
<td>1:320</td>
<td>++++</td>
</tr>
<tr>
<td>1:640</td>
<td>++++</td>
</tr>
<tr>
<td>1:1,280</td>
<td>++</td>
</tr>
<tr>
<td>1:2,560</td>
<td>-</td>
</tr>
<tr>
<td>1:5,120</td>
<td>-</td>
</tr>
</tbody>
</table>

A positive result is one where agglutination has occurred in the third tube, or beyond, if more than 3 tubes are used. The reaction is doubtful if there is less evidence of agglutination. It is generally agreed that the mucus test is valuable for the diagnosis of genital campylobacteriosis, but it must be emphasised that interpretation of the results should be made on a herd basis. To use the test to its best advantage, it is necessary to select the animals with care since even in herds where widespread infection exists, it is probable that a variable proportion of the animals may have escaped infection. There is a variable lag period between infection and the development of agglutinins, and at the time of oestrus, agglutinins tend to disappear, either partially or entirely, from the mucus although they may be present in high concentrations at other stages of the cycle. In any case agglutination titres tend to decline with time.

b) ELISA

ELISA is more sensitive than the vaginal mucus agglutination test but it should also be used as a herd test rather than as an individual animal test. This is because occasional false negative results will occur. It detects agglutinating and non-agglutinating antibodies in the earlier stages of infection and avoids autoagglutination because it employs a soluble antigen extracted from disintegrated cells. It makes available a wider range of antibody binding sites (7).
B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

*Campylobacter fetus* subsp. *fetus* vaccine confers immunity against *C. fetus* subsp. *venerealis* because both strains share common antigens (2). Two antigenic groupings of *C. fetus* are recognised, the thermolabile "H" flagellar antigens and the thermostable "O" somatic antigens. In addition, a capsular or "K" antigen is present (8). The vaccine must incorporate these antigens. It is an oil-emulsion vaccine of one or more strains which have been inactivated by formaldehyde.

In the United States, the addition of a second strain of *C. fetus* subsp. *venerealis* to the biological product is widely practised (4). The presence is critical of 4-5 heat labile glycoprotein immunogens, shared by many *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* strains; the presence of such immunogens should be confirmed.

1. Seed management

   a) Characteristics

      The seed consists of a large, homogeneous batch of a culture of *C. fetus* subsp. *fetus*, thoroughly characterised as to identity and purity.

   b) Culture

      The initial growth of the seed is accomplished in semi-solid medium. This consists of basal medium with the addition of 0.16% Bacto agar. Basal medium is composed of 2.8% *Brucella* broth, 0.5% yeast extract, 1.2% sodium succinate and 0.001% calcium chloride. The initial culture is maintained for 3 days at 37°C in an atmosphere of 5% carbon dioxide, 5% oxygen and 90% nitrogen. The use of a candle jar gives adequate results. The resulting growth is transferred to additional tubes with semi-solid medium and incubated for 48 hours. The resulting growth is used for vaccine production.

   c) Validation as a vaccine

      The seed must be free of contaminating organisms.

      It is not practicable to test efficacy under laboratory conditions. It is determined in the field on the basis of epizootiological observation.

      The vaccine should be stored at 4°C.

2. Manufacture

   The final seed material is seeded into broth medium consisting of basal medium with the addition of 0.025% sodium thioglycollate. These cultures are incubated at 37°C for 24 hours while being shaken at a rate of 80 rpm. The fluids are harvested, and formaldehyde added to a final concentration of 0.2%.
The vaccine is mixed with an oil-emulsion adjuvant to extend the period of immunity.

3. **In-process control**

The identity of the organism should be checked, as well as the absence of contaminating organisms.

4. **Batch control**

a) **Sterility**

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

b) **Safety**

The inactivation process must be complete; this is checked by inoculating the equivalent of one dose into the same medium under the same conditions as are used in the production process. This culture is incubated under the same conditions for 72 hours, after which there should be no evidence of bacterial growth. The final product must be shown to be free of viable bacterial and fungal contaminants, using suitable culture methods.

Two guinea pigs are inoculated with 2 ml of the final product either intramuscularly or subcutaneously. They must not have an unfavourable reaction attributable to the vaccine, during a 7-day observation period following inoculation.

c) **Potency**

Potency of the vaccine may be measured by seroconversion in rabbits. Their serum titres are measured by the tube agglutination test. Five rabbits, serologically negative at 1:100 serum dilution, are vaccinated subcutaneously on 2 occasions with half the dose used in cattle, at an interval of 14 days. Serum from at least 4 of the 5 rabbits, collected 14 days after the second vaccination must show at least a 4-fold increase in titre.

**REFERENCES**


SUMMARY

Bovine tuberculosis, caused by Mycobacterium bovis, is diagnosed in the live animal by clinical examinations and primarily on the basis of delayed hypersensitivity reactions; after death, this is done by post-mortem examination and histopathological and bacteriological examinations.

In a less advanced infection there may be no clinical signs of the disease. In advanced tuberculosis the characteristic signs include gradual emaciation, enlargement of lymph nodes and a cough.

Characteristic tuberculous lesions occur most frequently in the lungs and the retropharyngeal, bronchial, and mediastinal lymph nodes. Lesions are also frequently found in the mesenteric lymph nodes, liver, spleen, on serous membranes and in other organs. Any necropsy should be supported by bacteriological and/or histopathological examinations.

Identification of the agent: Bacteriological examinations may comprise:
- the demonstration of acid-fast bacilli by microscopical examination;
- the isolation of mycobacteria on selective culture media and their subsequent identification by cultural and biochemical tests; and
- a biological test based on the intramuscular or intraperitoneal injection of suspect material into guinea pigs. This test is very sensitive and even single bovine mycobacteria are capable of inducing the disease. The use of appropriate media and biochemical methods has made it less necessary to use animal inoculation on a routine basis.

Serological tests: Serological tests are of no diagnostic value.

Delayed hypersensitivity test: A delayed hypersensitivity test may be performed with bovine tuberculin alone; or preferably, with bovine and avian tuberculin simultaneously at different sites (comparative test) to differentiate between specific and non-specific reactions. The tuberculin is injected intradermally into the neck. Each injection site should first be clipped and the thickness of the skin-fold measured. After 72 hours the inoculation sites are examined and the skin swelling measured. The reactions are interpreted on the basis of an appropriate protocol.

Requirements for biological products: Vaccines are not available. There are standard methods for the production of bovine tuberculin. Tuberculins used for performing the tests specified should be prepared in accordance with the Requirements for Tuberculins, Requirements for Biological Substances No. 16, World Health Organization, Technical Report Series, 1968, No. 384, and should in particular conform to those requirements in respect of source
materials, production methods and precautions, added substances, freedom from contamination, identity, safety, potency and freedom from sensitising effect.

A. DIAGNOSTIC TECHNIQUES

Bovine tuberculosis is caused by *Mycobacterium bovis*. The bacterium infects mainly cattle but it can also reproduce the disease in man (particularly in children), pigs, goats, sheep, cats and other mammals. In zoological gardens *M. bovis* has been isolated from monkeys, antelopes, fallow deer and other wild animals. Cattle can also be infected by *M. avium*, *M. tuberculosis* and atypical mycobacteria present in the environment. These bacteria are less pathogenic for cattle than *M. bovis*, but nevertheless can induce a reaction to tuberculin which complicates diagnosis of the disease by means of delayed hypersensitivity tests.

The diagnosis of tuberculosis in the live animal is based on clinical signs and response to delayed hypersensitivity tests. Diagnosis post-mortem is based on pathological changes and histopathological findings.

1. Identification of the agent

Clinical signs of tuberculosis only indicate but cannot confirm the disease. During the initial period of infection, no clinical signs are seen. In the more advanced disease there is a gradual emaciation, enlargement of lymph nodes, a cough and other signs, depending on the organs affected.

On gross examination tuberculosis may be provisionally diagnosed when caseous or calcified foci are observed. In the initial stages of the disease or when the changes are non-specific, tuberculosis can seldom be diagnosed at necropsy.

Tuberculous lesions may develop in all visceral organs, on serosal surfaces, especially the pleura, and in bones and joints. They occur most frequently in the lungs, bronchial, mediastinal and retropharyngeal lymph nodes, mammary gland, liver, spleen, and intestines. Early lesions consist of small caseous and calcified granulomas in the retropharyngeal, broncho-mediastinal and occasionally the mesenteric lymph nodes. Later, there are numerous firm, grey-white nodules, occasionally pedunculated, localised mainly on the pleura and peritoneum. These vary in size from pinhead to hazel nut. In miliary tuberculosis the lesions are the size of millet seeds, and a large number of them can be found in the lungs, liver, spleen and perhaps other organs. They are yellowish-grey in colour.

Post-mortem examinations should be supported by a histopathological examination. Samples of affected lymph nodes and other tissue are fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin. Nodules comprised of epithelioid and giant cells are not sufficient in themselves to diagnose tuberculosis. Since the lesions are not conclusive it is necessary to demonstrate the aetiological agent.
Material for bacteriological examination is taken from abnormal lymph nodes and other tissues. The samples should be conveyed in sterilised sealed containers. In those cases where the animal is a positive reactor to the tuberculin test but shows no pathological lesions, samples from the mandibular, retropharyngeal, bronchial, mediastinal, and some mesenteric lymph nodes are collected for examination.

Samples of milk may be collected where tuberculous mastitis is suspected. About 25-30 ml is drawn from each quarter under aseptic conditions towards the end of milking. In warm ambient conditions, boric acid may be added (0.5% w/v final concentration) as a bacteriostatic agent.

To eliminate microorganisms other than mycobacteria, tissue samples are homogenised and to one part of the homogenate are added 2 parts of 5% oxalic acid or 5% NaOH and mixed. The mixture is kept for 5-10 minutes at room temperature. The supernatant fluid is decanted carefully into small bottles or tubes with screw caps containing glass beads, and kept at 37°C for 15 minutes. This is centrifuged for 10 minutes at 3,000-4,000 g. The supernatant fluid is discarded and the deposit washed with sterile physiological saline and recentrifuged. The deposit is used for culture, microscopical examination and biological testing. Milk, urine or other fluid materials are also centrifuged for 10 minutes at 3,000-4,000 g. The preparations are made directly from the collected material or after its homogenisation. Acid-fast bacilli are demonstrated by the staining method of Ziehl-Neelsen. The bacilli stain red whilst other cells stain blue.

All procedures involving culture should be performed in a safety cabinet.

Homogenised material is spread on two Lowenstein-Jensen, two Petragnani and two Stonebrink media. The Lowenstein-Jensen medium is enriched with pyruvate and basic Coletosos medium added. Modified 7H11 medium (3) is recommended instead of Petragnani media as an agar-based medium containing antibiotics for suppression of contaminants. The cultures are incubated at 37°C for a minimum of 8-10 weeks and checked for evidence of bacterial growth each week. When modified 7H11 medium is used, this is reduced to 6 weeks. The media should be in tightly closed tubes to avoid desiccation. Any deficiency in the condensation fluid is replenished by adding 0.5 ml of sterile broth or physiological saline.

*M. bovis* grows more slowly than *M. tuberculosis*. Primary cultures of *M. bovis* take 5-8 weeks to appear. On solid media they may form white, moist, slightly rough and friable colonies. They do not produce any pigment. The addition of 1% sodium pyruvate stimulates their growth. Glycerol inhibits growth.

Growth of *M. tuberculosis* takes at least 3-4 weeks. The colonies are dry, solid, rough, and white, yellow or orange in colour. They adhere firmly to the medium. Glycerol stimulates their growth.

*M. avium* and related organisms take 2-3 weeks to grow. They form humid, diffuse, smooth and sparkling colonies. The optimum growth temperature is 40-42°C. Atypical mycobacteria which are photochromogenic, scotochromogenic and rapidly-growing, usually form yellow or orange colonies. Most but not all atypical
Mycobacteria grow rapidly, and may be white or have orange or yellow pigment. In some species pigment only develops in light. Colonies of so-called avian-like bacilli are similar to those of *M. avium*. The majority of atypical species grow faster than either the bovine or human tubercle bacilli.

Biological tests are performed in guinea pigs since these are the most susceptible to bovine and human mycobacteria. Usually, two guinea pigs are injected intramuscularly into the thigh or intraperitoneally with homogenised material resuspended in 1 ml of sterile physiological saline.

After 4-5 weeks avian and mammalian tuberculin are injected into the flank. The diameters of induration and the nature of the reactions are read 24 hours after injection, when a preliminary diagnosis can be made. This avoids the need for subculture of tissues to identify the type of mycobacteria i.e. avian or mammalian type, and also to rule out infections giving tuberculosis-like lesions such as *Yersinia pseudotuberculosis*.

The use of appropriate media and biochemical methods has made it less necessary to use animal inoculation on a routine basis. Furthermore, there are good biochemical and metabolic tests to differentiate *Mycobacterium* species without the use of animals, so that their use should be avoided for humanitarian reasons and to reduce the biohazards associated with the handling of infected animals. Cases in which the use of animals can be justified include heavily contaminated samples, and for the differentiation of *M. bovis* from the BCG strain. Positive cases display enlargement of the spleen which contains numerous yellowish-white tubercles, and yellowish-green necrotic foci in the liver. There is caseation of the regional lymph nodes. The organism should be reisolated on artificial media to identify the mycobacterial type.

To distinguish *M. avium* from *M. bovis* and *M. tuberculosis* is relatively easy. The guinea-pig is highly susceptible to both the mammalian types, but resistant to the avian type. *M. avium* grows faster on artificial media and its colonies possess different features (see above). It can easily be resuspended in physiological saline. The nitratase test, sensitivity to pyrazinamide and thiophen-1-carboxylic acid hydrazide (TCH), and oxygen preference are also used as discriminatory tests for human and bovine strains (2). *M. tuberculosis* is aerobic, niacin and nitratase positive, TCH (LJ medium containing TCH, 5 mg/l) resistant and pyrazinamide (modified LJ and Kirchner media containing pyrazinamide, 65 mg/l) sensitive. *M. bovis* is microaerophilic, niacin and nitratase negative, TCH sensitive and pyrazinamide resistant. *M. africonum* is microaerophilic, TCH and pyrazinamide sensitive (3).

To distinguish *M. tuberculosis* from *M. bovis* is more difficult. In this case the character of the growth on the media used for their isolation (see above) and on Wagner-Mitscherlich medium is taken into account. Human strains decompose the glycerol present in the medium and form yellow colonies that change the colour of the surrounding medium to a yellow-brown. Bovine strains do not acidify the medium and therefore do not alter its colour.
To distinguish *M. tuberculosis* from other acid-fast microorganisms, including *M. bovis*, the niacin test is commonly used. This test is performed as follows: 1 ml of 1% potassium cyanide and 1 ml of 5% chloramine are quickly added to the bacteria cultured on Lockeman medium and killed within 3 hours with streaming steam. After careful mixing the culture is maintained at room temperature for 3-5 minutes. A yellow colour indicates the presence of nicotinic acid which is produced by human bacilli (1). This test may be also performed with absorbent paper slips (Warner-Lambert Pharm. Co., Morris Plains, USA). The end of the slip is immersed in a suspension of the organisms previously grown on Lowenstein-Jensen medium in physiological saline. If the slip changes to a lemon colour within 15 minutes, the test is considered positive (6, 13).

If typing facilities are not available, biological tests are of great value in the differentiation of bovine and human strains. A rabbit injected intravenously with 0.01 mg of a semi-dried culture of *M. bovis* will die after 4-6 weeks with signs of generalised tuberculosis. *M. avium* may also prove fatal to rabbits, but generally the gross disease is neither as extensive nor as apparent as that caused by *M. bovis*. Smears of apparently normal spleens and livers from such rabbits may reveal an abundance of acid-fast bacilli arranged in clumps in a characteristic rosette formation, which is typical of the so-called Yersin (acute septicaemic) type disease associated with *M. avium*. Rabbits usually die in 3-6 weeks. A dose of 0.01 mg of a human strain culture is not usually fatal for the rabbit.

The guinea-pig is very susceptible to bovine and human strains, but is resistant to *M. avium*. A dose of 0.1 mg of a semi-dried culture of *M. bovis* or *M. tuberculosis* inoculated intramuscularly kills the animal in about 6 weeks. A similar dose of the avian organism will induce only local lesions and enlargement of the regional lymph nodes. A 3-month old chicken injected intravenously with 0.1 mg of the avian organism will die within 8 weeks. *M. bovis* and *M. tuberculosis* are not lethal to chickens.

The identification of atypical mycobacteria is made by the use of the various cultural and biochemical methods as described in the Janowiec (5) or Tsukamura (9, 10) codes. Members of *M. avium-intracellulare-scrofulaceum* group can also be identified with specific agglutinating antisera according to the method of Schaefer (8, 12).

2. **Delayed hypersensitivity test**

The tuberculin test is essential for the diagnosis of tuberculosis in the live animal. The test may be performed with bovine tuberculin alone, or, preferably, with both bovine and avian tuberculins at the same time at different sites. This permits differentiation between specific and non-specific delayed hypersensitivity.

In cattle, tuberculin PPD containing not less than 2,000 units (4) is injected intradermally in 0.1 ml or 0.2 ml into a site in the mid-neck. The injection site is first clipped and seen to be free from obvious lesions. The distance between the sites should be about 10-15 cm. A skin-fold is first measured with callipers before injection and again 72-77 hours after.
The results of the test are interpreted on the basis of a special key. A positive result is usually where there is an increase in the skin-fold thickness of at least 3 mm; or, when there is only a diffuse subcutaneous oedema, irrespective of the thickness of skin-fold. A comparative test is one in which both bovine and avian tuberculins are used simultaneously at different sites on the same side of the neck. The extent and character of the reaction to both tuberculins indicates whether or not the animal is infected with \( M. \text{bovis} \) or shows non-specific delayed hypersensitivity. In doubtful cases, the tuberculin test should be repeated after 60 days.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

No vaccines are available.

Bovine tuberculin is a preparation made from the heat-treated products of growth and lysis of \( M. \text{bovis} \). The requirements below are based on ref. 11.

1. **Seed management**

   a) **Characteristics**

   Strains of \( M. \text{bovis} \) used to prepare seed cultures must be identified as to species by appropriate tests. A record must be kept of their origins and subsequent history.

   b) **Culture**

   The culture substrate must be shown to be capable of producing a product that conforms to these norms. It must be free from ingredients known to cause toxic or allergic reactions.

   c) **Validation**

   The strains of \( M. \text{bovis} \) used as seed cultures must be shown to be free from contaminating organisms and to be capable of yielding a product that conforms to these norms.

   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 European Pharmacopoeia units of the preparation under test in a volume of 0.1 ml. Fifteen to 21 days after the third injection, each guinea-pig, together with each of three control guinea pigs that have not been injected previously, is injected intradermally 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.
During storage, liquid bovine 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. Periods of exposure to higher temperatures or to direct sunlight in practical use should be kept at a minimum.

If correctly stored, liquid tuberculin should retain its potency for at least two years and freeze-dried tuberculin for at least eight years.

2. Manufacture

Bovine tuberculin may be made by the following three methods:

a) Old tuberculin (OT)

The organism is cultivated in glycerol broth medium, killed by heating in flowing steam and filtered. The filtrate is concentrated by heat and sterilised by filtration.

b) Heat-concentrated synthetic-medium tuberculin (HCSM)

As for (a), but the glycerol broth is replaced by a synthetic medium.

c) Purified protein derivative (PPD)

As for (b), but instead of being concentrated by heat, the protein in the filtrate is precipitated chemically, 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 5% w/v), may be added. Mercurial derivatives have been found to cause such reactions. Glycerol (not more than 10% v/v), may be added as a stabiliser. The product is dispensed aseptically into sterile, neutral-glass containers which are sealed so as to preclude contamination. The product may be freeze-dried.

3. In-process control

The pH must be between pH 6.5-7.5.

One or more representative batches must be tested in naturally or artificially infected tuberculous cattle by comparison with a reference preparation of known potency. The potency relative to that of the reference preparation must not differ significantly from that declared on the label.

One or more representative batches must be tested along with a reference preparation of avian tuberculin by comparing the reactions produced in guinea pigs sensitised with \textit{M. avium} using a procedure similar to that described below under
'Potency tests'. The potency of bovine tuberculin under test relative to the potency of the avian tuberculin must not be more than 10%. In guinea pigs sensitised with *M. bovis*, the potency of the reference preparation of avian tuberculin must be shown to be not more than 10% of the potency of the reference preparation of bovine tuberculin used in the test for potency.

Each filled final container must be inspected before it is labelled and any showing abnormalities must be discarded.

4. **Batch control**

a) **Sterility**

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

b) **Safety**

Two guinea pigs, each weighing not less than 250 g and which have not previously been treated with any material which will interfere with the test, are injected subcutaneously with 0.5 ml of the tuberculin under test. No abnormal effects should occur within 7 days.

Tests 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 should be taken and this should be injected intraperitoneally or subcutaneously into at least two guinea pigs, dividing the volume to be tested equally between the guinea pigs. It is desirable to take a larger sample, say 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea pigs are observed for at least 42 days and examined macroscopically post-mortem. Any lesions found are examined microscopically and culturally.

c) **Potency**

Potency is determined by comparison with a reference preparation of bovine tuberculin in guinea-pigs sensitised with *M. bovis*.

An International Reference Preparation has yet to be established. The European Pharmacopoeia and the European Community have adopted a reference preparation in common. Other countries may need to establish their own reference preparations. These could, if desired, be calibrated in European Pharmacopoeia units.

A suitable test is as follows: Use not fewer the 9 albino guinea pigs, each weighing 400-600 g. Sensitise them by administering to each, by deep intramuscular injection, 0.0001 mg (wet weight) of living *M. bovis* culture suspended in isotonic saline solution. Between 4-6 weeks later, the test is
performed as follows. Shave the guinea pigs' flanks so as to provide space for 3-4 injections each side. Prepare at least 3 dilutions of the tuberculin under test and at least 3 dilutions of the reference 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 infection sites randomly according to a Latin square design. The dilutions are injected intradermally, reactions are measured and the results 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 66% and not more than 150% 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 the 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 bovine tuberculin should contain the equivalent of at least 20,000 European Pharmacopoeia units/ml, giving a dose for practical use of 2,000 units per 0.1 ml.

REFERENCES


Summary

Cysticercosis in farm animals is caused by the larval stages (metacestodes) of tapeworms, which in their adult stage occur in the intestine of man or dogs. Bovine and swine cysticercoses are caused by the metacestodes of the human tapeworms Taenia saginata (Taeniarhynchus saginatus) and Taenia solium respectively. Bovine cysticercosis generally is of low prevalence globally, except in certain areas, such as tropical Africa, where traditional habits exacerbate infection. Swine cysticercosis is generally rare, but it may pose major public health problems in Central and South America and parts of South and Southeast Asia.

Cysticercosis in sheep occurs in a number of forms. Taenia ovis metacestodes occur in the musculature, those of Taenia hydatigena occur in the peritoneal cavity, while the metacestodes of Taenia multiceps occur in the central nervous system, causing the disease of "gid", which is frequently fatal. With this exception, the metacestodes cause little or no clinical effect in cattle, pigs or sheep. Likewise, the adult tapeworms produce minimal effects in their final hosts (man or dogs). However, in the case of T. solium, the metacestode may occur in man and cause cerebral cysticercosis, this being responsible for serious ill health and death.

Identification of the agent: The adult stages of the Taenia species are large, several centimetres (species in dogs) to several metres (species in man) in length. They are segmented, hermaphrodite organisms, with segments increasing in maturity as they pass along the body, or strobila, of the tapeworm. The anterior end is a scolex with four suckers and a rostellum bearing a circket of hooks of varying size, shape and number, characteristic of the individual species. In the living host animal, Taenia are diagnosed by faecal examination for individually shed gravid segments or the eggs released from these.

The larval stages (metacestodes) are usually readily visible in the organs or musculature at autopsy. In the case of cysticerci of T. saginata, T. solium and T. ovis, the metacestodes are small, 0.5 cm in diameter. With T. hydatigena the cysticercus is larger, 2-3 cm in diameter, and is present in the peritoneal cavity. In the case of T. multiceps, the development stage is a coenurus occurring in the CNS, usually on the surface of the brain.

Serological tests: There are no serological tests for the diagnosis of infection with adult tapeworms. Similarly, there are no serological tests of value for individual diagnosis of cysticercosis in domestic animals.
Requirements for biological products: No biological products are in use at this
time. Chemotherapeutic agents are used for the treatment of adult tapeworm
infections.

A. DIAGNOSTIC TECHNIQUES

The larval stages (or metacestodes) of *Taenia* species tapeworms are the cause of
cysticercosis in various domestic animals and man. Infection is acquired by the
ingestion of herbage contaminated with the eggs shed from the gravid segments of
tapeworms passed from the intestinal tract of the infected final host. Diagnosis is
based on the morphology of the adult tapeworm, the presence of eggs in the faeces
of infected humans or dogs, or the presence of a metacestode and its location.

1. Identification of the agent

*Taenia saginata*: *T. saginata* (the beef tapeworm), the cause of bovine cysticercosis,
is a large worm 4 to 8 metres in length, found in the small intestine of man, where it
may survive for many years. The scolex, or head, has four suckers and no rostellum
or hooks. The gravid segments each contain a much branched uterus filled with
many thousands of eggs. In identification, the parasite should be differentiated
from *Taenia solium*, the major characteristics of which are given by Soulsby (2).

Eggs passed in the faeces or discharged from ruptured gravid segments are
subspherical to spherical, 45-50 mm in length and 40 mm in diameter, brown in
colour, and have a striated embryophore or "shell". They contain an infective larval,
or hexacanth, embryo when passed in the faeces.

Cysticerci are found as pea-sized (5 mm in diameter), semi-translucent
metacestodes in the striated muscles of cattle. When incised, they contain a single
scolex, or head, of a future tapeworm. Depending on the age of infection and host
reaction, cysts may range from viable to various stages of degeneration such as
calcification.

*Taenia solium*: *T. solium* (the pork tapeworm) is similar to, though smaller (3-5
metres in length) than, *T. saginata*. The scolex possesses a rostellum and a circle of
hooks. Eggs passed with the faeces are similar to those of *T. saginata*. Gravid
segments may be distinguished from those of *T. saginata* on morphological grounds,
i.e. worms with a smaller number of branches. Cysticerci are similar to those of
*T. saginata* and are found in the musculature of pigs. When incised they contain a
single scolex with a rostellum and hooks. Cysticerci in humans are similar to those
found in pigs, except that a racemose form without a scolex may occur in the brain.

*Taenia ovis*: *T. ovis* in the adult form in the intestine of dogs is 1-2 metres in
length. The eggs are similar to those of other *Taenia* spp. The cysticerci are
5-6 mm in diameter and are found in the musculature of sheep. Cysticerci may
degenerate quickly and are often represented by a semi-calcified, circumscribed
lesion in the muscle.
Cysticercosis (B15)

Taenia hydatigena: *T. hydatigena* is 1-5 metres in length, with a rostellum and hooks on the scolex. It occurs in the intestine of dogs and other carnivores. The eggs are similar to those of other species. The metacestodes (cysticerci) are up to 6 cm in diameter and each consists of a scolex invaginated into a long neck and surrounded by a bladder of fluid. They are found attached to the omentum in the peritoneal cavity.

Taenia multiceps: *T. multiceps* is 40-100 cm in length and occurs in the intestine of carnivores. Eggs passed in the faeces are similar to those of other species. The metacestode (a coenurus) develops in the brain and spinal cord of sheep; it reaches up to 5 cm or more in diameter and contains several protoscolices invaginated in clusters on the cyst wall.

Detailed points for the identification of these species and their eggs are given by Soulsby (2).

**Diagnosis of adult parasites in man or dog**

Detached gravid segments may appear in the faeces, either individually or in chains. Microscopic examination of these will reveal masses of *Taenia*-type eggs. Alternatively, faeces may be examined by flotation techniques; saturated salt (NaCl) solution may be used as a flotation medium (3).

There are no satisfactory diagnostic procedures for the detection of metacestodes in the living animal except for *T. multiceps*, where clinical disease may result from infection. Metacestodes are detected on autopsy. In the case of *T. multiceps* the location of the metacestode may be evident from a softening of the bones of the skull overlying the parasite. On autopsy the metacestode (coenurus) is found on the surface of the brain as a translucent bladder with white scolices appearing in a cluster.

2. **Serological tests**

There is no satisfactory serodiagnostic test available with an acceptable specificity and sensitivity for diagnosis in the individual animal. Whereas a strong serological response is evident in experimental infections, non-specific reactions confuse diagnosis of low-level naturally acquired infections (1).

With bovine cysticercosis, experimental serodiagnostic tests based on ELISA may be useful on a herd basis when cysticercosis "storms" occur. These use antigen prepared from cysticerci of *Taenia crassiceps* obtained from mice. However, there is no standardised serodiagnostic test available at present.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

No biological products are yet available for cysticercosis. Several experimental "vaccines" have been investigated for ovine and bovine cysticercosis, with promising
results. Recombinant vaccines may offer effective immunising approaches in the future.

REFERENCES


**DERMATOPHILOSIS**

(B16)

**SUMMARY**

Dermatophilosis (also known as streptothrichosis) is an exudative, pustular dermatitis which affects mainly cattle, sheep and horses, but also goats, many wild mammals, lizards and, occasionally, man.

Laboratory diagnosis of dermatophilosis depends on the demonstration of the bacterium *Dermatophilus congolensis* in material from the skin or other organs. Sites other than the skin are rarely affected.

Identification of the agent: *D. congolensis* normally affects the epidermis, causing the formation of scabs. It may be demonstrated in smears made from scabs emulsified in water or in impression smears from the bases of freshly removed adherent scabs. The organism is Gram-positive, but its morphology is more readily appreciated in smears stained with Giemsa. In stained smears, the organism is seen as branching filaments breaking up into multiple rows of cocci. This characteristic appearance is diagnostic. In wet or secondarily infected scabs, only the cocci may be present, so that staining by immunofluorescence is necessary. *D. congolensis* is demonstrated in histopathological sections by Giemsa staining or by immunofluorescence.

Isolation of *D. congolensis* from freshly plucked scabs is straightforward, but the organism is readily overgrown by other bacteria. When cultured from contaminated sites, special techniques involving filtration, chemotaxis or selective media are necessary.

Demonstration and identification of *D. congolensis* by immunofluorescence is a reliable and very sensitive method of diagnosis. Although antigenic cross-reaction with *Nocardia* spp. has been reported, this is likely to give only weak fluorescence. Ideally, a monoclonal antibody specific to *D. congolensis* should be used.

Serological tests: A variety of serological tests has been used in studies of the epidemiology and pathogenesis of dermatophilosis; however, since varying levels of antibody are found in the blood of all cattle, such tests are not of diagnostic significance.

Requirements for biological products: Inactivated antigens may be used to prepare antiserum for diagnostic purposes but cannot be used to protect against infection. A diagnostic monoclonal antibody is available.
A. DIAGNOSTIC TECHNIQUES

Dermatophilosis is an exudative, pustular dermatitis which affects mainly cattle, sheep and horses, but also goats, many wild mammals, lizards and, occasionally, man. Dermatophilosis is caused by the bacterium *Dermatophilus congolensis.* Typically, *D. congolensis* infection gives rise to the formation of dense scabs on the skin but in certain areas, such as the perineum, moist lesions with thickened, folded skin may occur - the so-called "leproid" form of the disease. Here, relatively thin scabs are found. Where lesions are exposed to prolonged wetting, with or without secondary infection with other organisms, exudative lesions may be present.

It must be remembered that both scabs and cultures can infect man.

1. **Identification of the agent**

Diagnosis can usually be made by demonstrating the causal organism in scabs from the lesions or in exudate beneath them. The organism has a characteristic microscopic appearance when its septate, branching filaments become longitudinally as well as transversely divided to form ribbons of spherical or ovoid cocci, each about 0.5 μm in diameter, in multiple rows. Provided that cocci are seen in transverse rows of four or more, this appearance is diagnostic and is readily seen in stained preparations. However, the distinctive formation can be disrupted during the preparation of smears for examination if the material is spread too vigorously over the slide.

Impression smears may be made from the moist, concave undersurfaces of freshly plucked scabs. Otherwise, thick smears are best prepared from scabs emulsified in sterile distilled water. Smears are then dried in air, fixed by heating or immersion in methanol for five minutes, and stained. Dilute carbol fuchsin or methylene blue stain the organism well but Gram’s stain or, preferably, a 1-in-10 dilution of Giemsa stain for 30 minutes, gives better differentiation in thick smears, the darkly-stained, Gram-positive *D. congolensis* contrasting with the paler or pink counterstained background of keratinocytes and neutrophils.

Wet or secondarily infected scabs often contain few, if any, intact filaments, and the organism may not stain Gram-positive. In such material the cocci cannot be differentiated morphologically from other coccoid bacteria, so that staining by immunofluorescence is required. Thin, heat-fixed smears are used. In difficult cases and when infection of organs other than the skin is suspected, histopathological examination of biopsy or necropsy material is advisable. Giemsa stain or immunofluorescence is used.

The characteristic appearance of the lesions and of the organism in smears from typical bovine dermatophilosis makes culture unnecessary in most cases. However, confirmation of the diagnosis may be made by isolating the bacterium. Cultures are made on blood agar and incubated at 37°C. Growth is accelerated under microaerophilic conditions; rough, sometimes haemolytic, greyish colonies, about 1 mm in diameter, are seen pitting the medium after 24 hours. Incubation in air
produces similar pinpoint colonies at 24 hours, which grow to about 1 mm at 48 hours. The rough colonies are formed by the branching filaments, but continued growth in air stimulates the production of the cocci, which are commonly yellow in colour. Colonies take on a smooth, often yellowish appearance. The cocci are normally vigorously motile when taken from young cultures. The colonies must be differentiated from *Nocardi*a spp. and *Streptomyces* spp., neither of which produces filaments breaking up into multiple rows of motile cocci.

For isolation, material can be streaked out directly from the moist undersurfaces of freshly plucked, uncontaminated scabs or from scab emulsions, but the relatively slow-growing *D. congolensis* is readily overgrown by other bacteria. Special isolation techniques are thus required for contaminated specimens. In most specimens free cocci, whether motile or not, will be present in emulsions of the material. Filtration of the emulsion through a 0.45 μm membrane filter is usually sufficient to reduce or eliminate contaminants and permits isolation from the filtrate, as described above. Alternatively, Haalstra's method (1) may be used. Small pieces of scab are placed in a bijou bottle containing 1 ml of sterile distilled water and allowed to stand at room temperature for 3 to 4 hours. The open bottle is then held for 15 minutes in a candle jar. Samples of the surface liquid are removed with a bacteriological loop and cultured. The method depends on the release from the scab of the motile cocci of *D. congolensis* and their chemotropic attraction towards the carbon dioxide-rich atmosphere of the candle jar. A selective medium consisting of 1,000 units/ml of polymixin B in blood agar can also be used and is effective when the contaminants are sensitive to this antibiotic.

Immunofluorescence staining of smears or tissues is the most reliable and sensitive immunological technique for the identification of *D. congolensis* antigens and for the diagnosis of dermatophilosis. Polyclonal antibody obtained from animals vaccinated with *D. congolensis* (as described below) can be used, but there is a risk of possible cross-reaction with some strains of *Nocardi*a spp. Monoclonal antibody to species-specific antigen (2) is preferable. Thin, heat-fixed smears of scab emulsions or impression smears are stained. Known positive and negative control specimens should always be included.

When other diagnostic techniques fail, animal inoculation can be used to isolate *D. congolensis*. Rabbits are clipped at the inoculation sites, which are swabbed with ether and then lightly scarified with a needle, whilst avoiding haemorrhaging. White skin should be used, if possible. A suspension of the specimen is applied to the prepared skin and observed daily. At sites inoculated with *D. congolensis*, erythema will be apparent after 48 hours. Some serous exudation may be seen and scab formation will occur after about 72 hours. These lesions will normally be uncontaminated, and *D. congolensis* can be demonstrated in the scabs or isolated from them.

2. **Serological tests**

Demonstration of antibody to *D. congolensis* in bovine sera is not of diagnostic significance as it is normally present in all but fetal blood.
B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Inactivated antigens may be used to prepare antiserum for diagnostic purposes but cannot be used to protect against infection. Vaccines are under development, but so far no commercial vaccines are available.

REFERENCES


ENZOOTIC BOVINE LEUKOSIS
(B17)

SUMMARY

Enzootic bovine leukemia (EBL) is a systemic disease of the lymphoreticular tissue. The aetiological agent is a retrovirus, bovine leukemia virus (BLV).

Infection with BLV is often subclinical and may result in either persistent lymphocytosis or lymphosarcoma.

The symptom of persistent lymphocytosis was earlier used as diagnostic criterion. Many studies have shown that on a herd basis persistent lymphocytosis indicates BLV infection, but today detection of antibodies against BLV has replaced the haematological diagnosis. The BLV genome does not code for an oncogene, and the functional role of BLV in leukaemogenesis is still unknown.

Identification of the agent: In the infected animal BLV is mainly present in the form of proviral DNA. Proviral DNA in peripheral lymphocytes can be detected with the polymerase chain reaction (PCR).

Virus isolation can be performed from live animals by cultivation of peripheral lymphocytes, followed by demonstration of BLV particles by electron microscopy or demonstration of BLV antigens by radio-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), or agar gel immunodiffusion (AGID). At autopsy of tumour-bearing animals, BLV provirus can be demonstrated by PCR or nucleic acid hybridisation techniques, or tumour cells can be co-cultivated with fetal lamb kidney or spleen cells for the demonstration of BLV or BLV antigens.

Serological tests: In research laboratories, the virus neutralisation test, syncytia inhibition test, early polykaryocytosis test, pseudotypes inhibition test and RIA can be used, but the methods widely used to detect, control and eradicate the infection are the AGID and ELISA.

All the methods mentioned can detect antibodies against BLV in individual sera. To detect low antibody titres, for example in individual milk samples, in pooled sera or in bulk milk samples, the diagnostic sensitivity of RIA or ELISA is needed.

A number of kits for AGID as well as ELISA are commercially available. A reference serum defines the lower limit of sensitivity for routine testing in both AGID and ELISA, whether testing serum or milk samples.
A. DIAGNOSTIC TECHNIQUES

Enzootic bovine leukosis (EBL) is a systemic disease of the lymphoreticular tissue. The aetiological agent is a retrovirus, bovine leukemia virus (BLV).

Under natural conditions, BLV infects cattle, sheep, zebu, water buffaloes, and capybaras. Other species can also be infected experimentally but do not develop lymphoid tumours.

The term sporadic bovine leukosis (SBL) is used for various other leukotic manifestations not associated with BLV infection, for example juvenile, thymic and cutaneous leukosis. At present no morphological criteria can definitely distinguish between EBL and SBL. The exact diagnosis of a leukotic tumour relies on the demonstration of BLV provirus, virus or antigen. Serological examination of the herd of origin of the tumour-bearing animal is used administratively to define the case as EBL or SBL.

BLV is transmitted by cells carrying the proviral DNA: vertically in utero, by colostrum and milk; and horizontally by blood-contaminated needles, equipment for ear-tagging, dehorning and castration, surgical equipment and exploration gloves etc., and by close contact of infected and uninfected animals. Blood-sucking insects may play a role under certain climatic conditions in the transmission of the infection.

1. Identification of the agent

Bovine leukaemia virus (BLV) is classified as a retrovirus. In the infected animal the virus is found mainly in the form of proviral DNA, which is detected in peripheral lymphocytes by polymerase chain reaction (PCR) techniques, and in lymphatic and tumour tissue by hybridisation techniques (2, 6).

When infected cells are cultivated in vitro, viral replication occurs. After short-term cultivation of peripheral lymphocytes, the viral particles can be detected by electron microscopy and syncytia formation in appropriate indicator cells (3, 4). Antigen can also be demonstrated by concentrating the growth medium and then testing the concentrate in the agar gel immunodiffusion test (AGID). Co-cultivation of peripheral lymphocytes, lymphatic tissue or tumour tissue can be attempted with fetal lamb kidney or spleen cells followed by the demonstration of viral particles or viral antigens by radio-immunoassays (10) or ELISA. Demonstration of tumour-associated antigen, TAA, can be performed with a monoclonal antibody, C143 (1, 9). The reagent is not commercially available.

BLV is easily transmitted to sheep and seroconversion occurs within two to three weeks after inoculation. Sheep do not develop persistent lymphocytosis but the frequency of tumour development is much higher and occurs earlier than observed in cattle.
2. **Serological tests**

BLV causes a persistent infection and demonstration of antibodies can be used to identify infected animals (provided that the animal has not been vaccinated or received colostrum/milk with antibodies against BLV).

In experimental infections seroconversion occurs from three weeks to three months post-infection, and it is generally accepted that the incubation period by natural horizontal transmission is also relatively short, except where colostral antibodies prolong the incubation period for calves infected by lymphocytes carrying proviral DNA.

Antibodies to several BLV structural proteins are present in the sera of infected cattle. In most cases, antibodies against gp51, the viral envelope glycoprotein of molecular weight 51,000 daltons, have the highest titre and appear earlier than antibodies against p24, the core polypeptide of molecular weight 24,000 daltons.

Several methods can be used to demonstrate antibodies against BLV. The methods widely used to detect, control and eradicate BLV infection are the AGID (7) and the enzyme-linked immunosorbent assay (ELISA) (8).

a) **Agar gel immunodiffusion test**

The AGID test is a double radial diffusion where antigen and antibody migrate, react and precipitate in an agar/agarose gel.

Use of the AGID test to detect antibodies against gp51 in individual blood samples has proved to be sufficient to declare a herd free from infection with BLV and the test has successfully been used to help eradicate BLV from infected herds. If blood samples have not been taken, extracts of leukotic tumours can be examined using the AGID test. Extracts from BLV-induced tumours often show antibodies against core protein p24.

Most AGID antigens contain both gp51 and p24, but the test should be standardised for the demonstration of antibodies against gp51.

**Minimal requirements**

Several commercial kits are available. The minimal requirements for these kits and for laboratories' own tests are:

i) The antigen must contain BLV glycoprotein. Standardisation is performed with reference serum E-1.

ii) In the test, reference serum E-4 diluted 1:10 in negative serum must be scored positive without any retest or concentration procedure.

E-1 originates from a cow naturally infected with BLV. In the AGID, E-1 precipitates only gp51.
E-4 is a pool of sera from three cows infected, before receiving any colostrum, with BLV-infected lymphocytes originating from a naturally infected cow. The anti-gp titre of E-4 equals a median titre of sera from naturally infected cattle. In the AGID, E-4 precipitates only gp51. In the ELISA, antibodies against p24 can also be detected.

Reference sera E-1 and E-4 are obtainable from the National Veterinary Laboratory, P.O. Box 373, DK-1503 Copenhagen, Denmark.

Procedure for standardisation of antigens

National standards should be produced using the reference sera E-1 and E-4.

i) Make a two-fold dilution of the antigen preparation.

ii) The highest dilution that, against undiluted reference serum E-1, gives a precipitation line equidistant from the antigen and the serum will contain one unit.

iii) Work with 2 units of antigen in the test.

Reagents

Antigen is prepared in a suitable cell culture system, e.g. permanently infected fetal lamb kidney cells (FLK-BLV cells, M. van der Maaten, Ames, Iowa) or bat lung cells (BLV-BAT2, J. Ferrer, University of Pennsylvania). Supernatants are harvested once a week from confluent cell layers. The supernatants are concentrated by precipitation with 30% (NH₄)₂SO₄, followed by ultrafiltration or precipitation by polyethylene glycol, followed by desalting and size separation on a polyacrylamide bead column. Fifty- to 100-fold concentration of the initial volume is needed.

Positive control serum: Serum from naturally or experimentally infected cattle is used. The control serum should not contain antibodies to other bovine viruses (e.g. bovine herpesvirus, bovine virus diarrhoea virus). The precipitation line formed should be a sharp distinct line midway between the antigen and the control serum wells.

Negative control serum: Serum from cattle free of antibodies against BLV is used.

Gel: A 0.8-1.2% solution of agar or agarose is prepared in 0.05 M Tris buffer pH 7.2 with 8.5% NaCl.

The agar/agarose is dissolved and the hot agar/agarose is poured into petri dishes (15 ml per petri dish of diameter 8.5 cm). A punch is used to cut wells in the solidified gel solution. Various well patterns can be adopted, but a hexagonal arrangement with a central well is usual. The antigen is placed in the central well and the positive control serum is placed in two or three peripheral wells. Commonly used patterns:
**Enzootic bovine leukosis (B17)**

i) Antigen well diameter       4 mm  
Serum wells diameter           6 mm  
Distance between antigen and serum wells 3 mm

ii) Antigen well diameter      7 mm  
Serum wells diameter           7 mm  
Distance between antigen and serum wells 3 mm

The test plates should be kept at room temperature (20-27°C) in a closed moist chamber, and read at 24, 48 and 72 hours.

A test should be regarded as invalid if the controls do not give the expected results.

**Reading of the test**

i) A test serum is positive if it forms a specific precipitation line with the antigen and forms a line of identity with the control serum.

ii) A test serum is negative if it does not form a specific line with the antigen and if it does not bend the line of the control serum.

iii) The reaction should be considered inconclusive if a test serum:
   - bends the line of the control serum towards the antigen well without forming a visible precipitation line with the antigen, or
   - it cannot be read either as negative or positive.

Sera giving inconclusive results can be concentrated and retested.

b) **Enzyme-linked immunosorbent assay (ELISA)**

The first step in the classical ELISA for the detection of antibodies against BLV was the coating of microplates with a crude BLV antigen. A number of false positive reactions were obtained due to reaction with non-viral components of the antigen. So far it has not been economical to use purified viral proteins as antigens and nearly all BLV ELISAs now make use of capture antibodies as a purification step.

**Principal steps in the ELISA**

Step 1: Capture reagent  
a. Monoclonal antibodies against gp51  
b. Monoclonal antibodies against p24  
c. Bovine polyclonal antibodies (gp51 + p24)

Step 2: Antigen (for indirect ELISA also control antigen)  
Usually supernatant from FLK-BLV cells

Step 3: Test serum
Step 4: Detection reagent
Indirect ELISAs
a. Monoclonal antibodies against bovine IgG
b. Polyclonal antibodies against bovine IgG
Blocking/competitive ELISAs
a. Monoclonal antibodies against gp51 (directed against other epitopes than in Step 1a)
b. Bovine polyclonal antibodies (same antibodies as in Step 1c)

Step 5: Colour development
The detection reagent in Step 4 can be either biotinylated or enzyme-labelled, e.g. with horse radish peroxidase.

On each plate positive and negative controls must be included to ensure that the test is valid.

The ELISA can detect antibody levels ten- to a hundred-fold lower than those detectable using the AGID test. Commercial kits are available and these kits and laboratories' own tests should be standardised by using reference serum E-4 (5).

Pooled serum and milk samples can be used. This enables slaughterhouse and tank milk surveys to be done.

The goal of the standardisation procedure is to ensure that an antibody-positive animal will be detected with the same probability independently of whether the screening is performed on individual serum or milk samples or on pooled serum or milk samples.

The standardisation does not take into consideration the different sensitivities of a herd milk test compared to a herd blood test.

Standardisation of tests used on individual samples

ELISA on serum
Reference serum E-4 diluted 1:10 in negative bovine serum should be positive with the cutoff used in the routine procedure.

The reference serum E-4 diluted 1:10 in negative bovine serum should always be diluted in the same buffer and tested in the same dilution as the field sera.

ELISA on milk
The only estimate available to date suggests that on average the antibody content in milk is 25 times less than in serum. Therefore, an ELISA for testing milk samples has to be at least 25 times more sensitive if the antibody-positive animal is to be detected independently of whether the analysis is performed on serum or milk.
Reference serum E-4 is diluted 1:10 in negative bovine serum, and this diluted 1:25 in milk from a seronegative herd must be scored positive in the test. (Tested in the same buffer and the same dilution, and with the cutoff used in the routine procedure.)

**Standardisation of tests used on pooled samples**

The titre of reference serum 4 determines how many samples can be included in the pool. Reference serum E-4 must be scored positive when diluted 10 times more than the dilution applied to individual sera when these are included in pools. It must be scored positive when diluted 250 times more than the dilution of individual milk samples when these are included in pools.

Reference serum E-4 is obtainable from the National Veterinary Laboratory, Copenhagen (address as above). Using reference serum E-4 a national standard serum should be produced.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

As yet no vaccine against BLV is available.

**REFERENCES**


SUMMARY

The diagnosis of haemorrhagic septicaemia depends on the isolation of the causative organism, Pasteurella multocida, from the blood or bone marrow of a dead animal by cultural and biological methods, and the identification of the organism and type by biochemical and serological methods.

Identification of the agent: Pure cultures of *P. multocida* can be obtained by streaking material onto artificial media or by the inoculation of suspect infective material into mice, and the subsequent culture of their blood in a suitable medium. Identification is made on the basis of its morphological, cultural and biochemical characteristics.

The identification of the specific serotype is done by one or more serological methods. These are rapid slide agglutination, indirect haemagglutination for capsular typing using sheep erythrocytes coated with bacterial extracts, agglutination using acid-treated cells for somatic typing, and agar gel immunodiffusion tests.

Serological tests: Serological tests for detecting specific antibodies are not normally used for diagnostic purposes.

Requirements for biological products: Vaccines against haemorrhagic septicaemia are simple formalin killed bacterins, or dense bacterins with adjuvants. The latter enhances the level and prolongs the duration of immunity.

Cultures for the production of vaccines should contain capsulated seed cultures. They are standardised as to their bacterial density on the basis of turbidity tests. Potency tests are most conveniently carried out in mice.

A. DIAGNOSTIC TECHNIQUES

Haemorrhagic septicaemia is an acute, highly fatal, septicaemic disease of cattle and buffaloes caused by certain serotypes of *Pasteurella multocida*, notably serotypes 6:B (Asian strains) and 6:E (African strains). A few countries, notably Egypt and Sudan, have recorded both serotypes. Haemorrhagic septicaemia is a primary pasteurellosis, reproducible in susceptible animals with pure cultures of the causative organism alone (2, 4).

A clinical diagnosis can be made on the basis of characteristic signs, gross pathological lesions, herd history, morbidity and mortality patterns, species susceptibility, age of affected animals, etc. A tentative clinical diagnosis is generally useful in order to instigate control measures.
A routine diagnostic procedure may follow the sequence: Animal material – mouse inoculation – heart blood culture – examination of blood smear and/or rapid slide agglutination test.

1. **Identification of the agent**

The septicaemia in haemorrhagic septicaemia is essentially terminal. Therefore, blood samples taken from sick animals before death may not always contain *P. multocida* organisms. Also, they are not consistently present in the nasal secretions of sick animals.

A blood sample or swab collected from the heart is satisfactory if this is done within a few hours of death. If the carcase is old, a long bone freed of tissue can be taken. If there is no facility for post-mortem examination, blood can be collected from the jugular vein by incision or aspiration. Blood samples in any standard transport medium should be dispatched on ice and well packed to avoid any leakage.

Blood smears from affected animals are stained with Gram, Leishman's or methylene blue stains. The organisms appear as Gram-negative, bipolar staining short bacilli. No conclusive diagnosis can be made on the basis of direct microscopical examinations alone.

A suitable medium for the growth of *Pasteurella* is casein-sucrose-yeast (CSY) agar containing 5% of blood. The composition of this medium is casein hydrolysate 3 g, sucrose 3 g, yeast extract 5 g, sodium chloride 5 g, anhydrous dipotassium hydrogen sulphate 3 g, and distilled water to 1 litre. The pH is adjusted to 7.3-7.4 and the medium is autoclaved at 1 bar for 15 minutes, after which sterile 1.5% agar is included. After cooling to 45-50°C, 5% blood of a calf (*P. multocida* antibody-free) is added.

Blood samples, or swabs eluted into 2-3 ml sterile normal saline, are cultured. Alternatively, the surface of a long bone is swabbed with alcohol, flame sterilised and split open. The marrow is extracted aseptically and cultured. Direct culture usually is satisfactory only if the material is fresh and free of contaminants or post-mortem invaders which would otherwise overgrow any *Pasteurella* present.

For biological examinations, a small volume of eluted blood swabs or a portion of bone marrow in normal saline are inoculated (0.2 ml) into mice subcutaneously or intramuscularly. The mouse usually serves as a biological 'screen' for extraneous organisms. Following inoculation, the mice die in 24-36 hours, and a pure growth of *P. multocida* can be seen in blood smears. Pure cultures of *P. multocida* can usually be grown from blood cultures of the mice, even when the original samples come from relatively old carcases. The organism can be identified by its morphological and cultural characteristics, biochemical reactions and serological tests.

Freshly isolated *P. multocida* forms smooth, greyish glistening translucent colonies, approximately 1 mm in diameter, on blood agar after 24 hours incubation at 37°C. Colonies on CSY agar are larger. Old cultures, particularly those grown on media devoid of blood, may produce smaller colonies. It does not grow on MacConkey
Haemorrhagic septicaemia organisms produce oxidase, catalase and indole, and will reduce nitrates. They do not produce hydrogen sulphide or urease, and fail to utilise citrate or liquefy gelatin. Glucose and sucrose are always fermented with the production of acid only. Most strains also ferment sorbitol. Some strains ferment arabinose, xylose and maltose, whereas salicin and lactose are almost invariably not fermented.

2. Serological tests

Several serological tests are used for the identification of the serotypes of Pasteurella multocida that cause haemorrhagic septicaemia. These consist of a rapid slide agglutination test (7), an indirect haemagglutination test for capsular typing (3), an agglutination test using hydrochloric acid-treated cells for somatic typing (8) and agar gel immunodiffusion tests (1, 5, 11).

Antisera for most serological tests are prepared against a reference strain in rabbits. Six- to 8-hour cultures in CSY broth are seeded onto CSY blood agar medium. After overnight incubation (18-20 hours) the growth is washed into physiological saline containing 0.3% formalin. The turbidity of the cell suspension is adjusted to that of Brown's tube no. 7. Rabbits are inoculated intravenously at 3-4 day intervals with 0.2, 0.5, 1.0, 1.5 and finally, 2.0 ml of this suspension. One week after the last injection, 0.5 ml of a similar but live suspension is inoculated subcutaneously or intramuscularly. The animal is bled 10 days later. The serum is stored at -20°C but small quantities for regular use are stored at 4°C with the addition of 1:10,000 merthiolate.

a) Rapid slide agglutination test

A single colony is mixed with a drop of saline on a slide, a drop of antiserum added, and the slide warmed gently. A coarse, floccular agglutination appears within 30 seconds. Old cultures may give a fine, granular agglutination and take longer to appear.

b) Indirect haemagglutination test (capsular typing)

This was originally done using human type 'O' red cells (3), but more recently with sheep red cells (10). The antigen is prepared as follows:

A 6-8 hour broth culture of a reference strain, 6:B or 6:E, is seeded onto CSY blood agar plates and incubated overnight at 37°C. The growth is harvested with 3 ml normal saline containing 0.3% formalin. This suspension is then heated at 56°C for 30 minutes, centrifuged at 3,000 g for 15 minutes at 4°C and the clear supernatant fluid is stored at -20°C. If a refrigerated centrifuge is not available, centrifugation at 1,500 g for 30 minutes gives a clear supernatant
fluid. This is used as the antigen extract.

Sheep blood is collected aseptically into an anticoagulant, and centrifuged at 500 g for 10 minutes. The packed erythrocytes are washed 3 times in sterile physiological saline. The antiserum (3 volumes) is absorbed by the addition of packed red cells (1 volume) for 2 hours at 37°C with periodic shaking. The red cells (1 volume) are sensitised by the addition of antigen extract (15 volumes) for 1 hour at 37°C with frequent shaking. The sensitised red cells are recovered by centrifugation, washed 3 times in sterile physiological saline, and made up to a final 1% suspension in physiological saline.

The test itself can be carried out in tubes or plates, and is performed in duplicate. Eight drops of normal saline and 2 of the inactivated absorbed antiserum are added to the first well, and 5 drops of normal saline to all other wells. Serum dilutions are made by mixing and transferring 5 drops to the penultimate well, leaving the last well as a control without serum. To the first row is added an equal volume (5 drops) of the 1% sensitised cell suspension, and to the second row is added a 1% suspension of unsensitised red cells as controls. The plates are shaken and left at room temperature for 2 hours. Coarse agglutination of red cells indicates a positive result; a small button of deposited cells is a negative reaction.

c) Somatic typing

The somatic 'O' antigen is prepared essentially by a method described previously (8). A 6-8 hour test culture is seeded onto CSY blood agar and incubated overnight. The growth is harvested in 2-3 ml of normal saline containing 0.3% formalin per plate, and centrifuged at 3,000 g for 15 minutes at 4°C (or 1,200-1,500 g for 30-45 minutes at room temperature). The deposited bacteria are resuspended in 25 ml normal HC1 saline to give an opacity of about Brown's opacity tube no. 6. This is incubated overnight. The suspension is again centrifuged, the supernatant fluid discarded and the cell residue washed successively in phosphate buffered saline (PBS) at pH 5.0, 6.0 and 7.0 respectively. Finally, a suspension of the residual cells, equivalent to Brown's opacity tube no. 6, is prepared in PBS at pH 7.0. Any suspensions that autoagglutinate should be discarded.

Antisera are prepared against whole bacterial cell suspensions of the reference strains 6:B (Asian HS), 6:E (African HS) and 11:B (Australian 989, non-HS). Agglutination tests are carried out on a slide with the test antigen against the 3 types of sera. A fine granular agglutination indicates a specific somatic agglutination. Tests carried out against the standard strains will facilitate reading and interpretation.

When non-specific partial agglutination occurs, tube tests carried out with 10-fold dilutions of the sera against the test and reference antigens will help to identify somatic antigen.
d) Agar gel immunodiffusion tests

Immunodiffusion tests are used for what is described as 'capsular' as well as 'somatic' typing, depending on the antigens and antisera used. The double diffusion technique is employed. Wells are punched in the solid agar in a circular pattern with one centre well and 6 peripheral wells around it.

**Capsular typing:** The gel medium is 1.0% Noble agar (Difco), or equivalent product, in 0.2 M phosphate buffer with merthiolate at a final concentration of 1:10,000 (1, 11). Antigens and antisera are the same as for capsular typing by the indirect haemagglutination method (3). The reference antiserum is placed in the centre well, and the test antigens are placed in the peripheral wells alternately with reference homologous antigen.

**Somatic typing:** The gel medium consists of special Noble agar (Difco), or equivalent product, at a concentration of 0.9% in 8.5% sodium chloride solution. For antigen preparation the growth from each plate is harvested in 1 ml of 8.5% NaCl, containing 0.3% formalin. The suspension is heated at 100°C for 1 hour, the cells sedimented by centrifugation and the supernatant fluid is used as antigen.

Antisera are prepared in chickens. Bacterin (1 ml) is injected subcutaneously in the mid-portion of the neck of 12- to 16-week old male birds. The birds are exsanguinated 3 weeks later, and the serum separated.

e) Serotype designation

Broadly, 2 typing systems are adopted. One is capsular typing by Carter's haemagglutination (3) or by immunodiffusion tests (1, 11). The other is somatic typing by the method of Namioka and Murata (8, 9), and by the method of Heddleston, Gallagher and Rebers (5). It is generally agreed that designation of serotypes should be based on a somatic-capsular combination. Two systems commonly in use are the Namioka-Carter and the Carter-Heddleston systems. By the former system, Asian and African haemorrhagic septicaemia serotypes are designated 6:B and 6:E respectively, whilst in the latter system they are designated B.2 and E.2 respectively.

### B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Three types of vaccine used are bacterins, alum precipitated vaccine (APV) and oil adjuvant vaccine (OAV). To provide sufficient immunity with bacterins, repeated vaccination is required.

1. **Seed management**

a) **Characteristics**

A local isolate of *P. multocida* representing the prevalent serotype is used. A
well capsulated, stable culture which produces large colonies of approximately 2 mm diameter on CSY blood agar must be maintained. Seed cultures should be stored as semi-solid nutrient agar stab cultures at room temperature, or as lyophilised cultures.

b) Culture

A calf is infected with the culture, and within 2-3 hours of its death blood is collected aseptically from the heart and stored at -20°C in aliquots of 1 ml. A fresh aliquot is used for each new batch of vaccine. It is permissible to sub-culture this once or twice for use, provided the colony size does not diminish. A blood aliquot is thawed, plated onto CSY blood agar, and the growth tested for agglutinability by the appropriate antiserum in a slide test. A good culture will give a coarse floccular agglutination in under 30 seconds. A poor culture will yield only a fine granular agglutination.

c) Validation as a vaccine

Purity tests must be carried out on the seed flasks and the final harvest by examination of stained smears. The seed used is checked for good capsulation, a good indication of which is the colony size after 24 hours growth in a suitable medium such as CSY blood agar. Tests for agglutinability in the appropriate serum must be performed on the seed culture plate, seed flask and the final harvest. Formalin is added to the harvest and after standing for 6 hours the harvest is plated out to check for remaining viable bacteria.

2. Manufacture

For vaccine production, dense suspensions of bacteria are necessary. They should have a minimum bacterial content of 1.5 g per litre dry weight for volume. There are 2 methods of producing dense suspensions. The first is to culture on solid media in Roux flasks and harvest in formalinised normal saline, by which means suspensions of any density can be achieved. This is laborious as each flask has to be harvested separately and tested for purity. The second, and recommended, method is the use of aerated cultures in a medium that specifically supports *P. multocida*. A suitable sterilised medium is casein hydrolysate 2 g, sucrose 6 g, yeast extract 6 g, sodium chloride 5 g, anhydrous dipotassium hydrogen orthophosphate 8.6 g, anhydrous potassium dihydrogen orthophosphate 1.36 g, and distilled water to 1 litre. A denser growth is obtained if the casein, sucrose and yeast are prepared as a concentrate, filter sterilised and transferred aseptically to the tank, and heat sterilised with the rest of the ingredients.

There are 2 types of aeration process, namely by vortexing and sparging. Sterile air is provided by a compressor. In vortex aeration the culture is stirred by an impeller shaft operating in the air stream; whereas in sparging aeration, the air is dispersed through a sparger. The more finely dispersed the air, the better is the bacterial growth. Vessels of 20-40 litres are usually employed, and incubation is at 37°C. In continuous culture systems, once a maximum density has been reached, usually within 15 hours, about 25% of the working volume is replaced hourly. The harvests
of continuous cultures are collected in relatively small volumes into separate vessels, but after several days the density diminishes, presumably through loss of capsular antigen. For this reason, batch cultures are to be preferred. If batch culture vessels are inoculated at a rate of 50 ml/litre of medium, maximum turbidity is obtained within 15-18 hours when the growth can be terminated by the addition of formalin to a final 0.5%. This procedure, where a large inoculum is employed and the growth terminated within a short period, helps to minimise the chances of contamination. The turbidity is standardised against a reference containing the equivalent dry weight/volume of 1.5 g per litre.

Oil adjuvant vaccine is made by the emulsification of equal volumes of a light mineral oil and the bacterial suspension, with 5% pure anhydrous lanoline as emulsifying agent. The mineral oil and lanoline are first sterilised, and on cooling to 40°C 0.5% formalin is added to the mixture. The bacterial suspension is added slowly and emulsification continued for a further 10 minutes. Following overnight storage, the mixture is re-emulsified, bottled and stored at 4°C for 2 weeks prior to use.

Alum precipitated vaccine is prepared by first adjusting the turbidity of the suspension to the reference standard, and diluting it with 0.5% formalinised normal saline to a final density of half that of the final product. The pH is adjusted to 6.5 and hot 20% solution of potash alum added to give a final 1% alum. After overnight storage with continuous agitation the vaccine is bottled for use.

3. **In-process control**

Proper concentration of bacterial growth, the capsulation of the bacteria, purity of culture and efficient inactivation all need to be checked.

4. **Batch control**

a) **Sterility**

Tests for sterility and freedom of contamination of biological materials may be found in the chapter on General Information.

b) **Safety**

Two seronegative cattle are vaccinated with twice the recommended dose and observed for 10-14 days for adverse effects.

Five mice are inoculated with 0.2 ml each of the vaccine intramuscularly, and observed for 5 days. The blood of any that die is cultured for *P. multocida*.

c) **Potency**

Potency tests can be carried out by any of the following methods:
i) vaccination of cattle, followed by direct challenge or passive mouse protection tests using the bovine sera. This procedure is hardly feasible since cattle take a long time to develop adequate immunity after OAV;

ii) vaccination of rabbits, followed by direct challenge or passive mouse protection tests using the rabbit sera; or,

iii) potency tests in mice, the most feasible method of the three.

Fifty mice are each vaccinated intramuscularly with 0.2 ml vaccine and again 14 days later. On day 21, they are divided into 10 groups of 5, each group being challenged with respective dilutions of a 6-8 hour broth culture of a field strain in the range of $10^{-1}$ to $10^{-10}$. Fifty unvaccinated controls are similarly challenged, and all mice observed for 5 days. The median lethal dose ($LD_{50}$) can then be calculated in order to obtain an indication of sufficient protection for cattle: vaccines prepared in the manner described give at least $10^4$ units protection in the vaccinated mice.

d) Duration of immunity

Alum precipitated vaccine provides 3-4 months immunity and OAV 6-9 months immunity after a primary vaccination. Vaccination with OAV provides adequate immunity if given at 4-6 months of age and repeated 3-6 months later, followed by annual revaccinations thereafter. A dose of 3 ml of vaccine in calves 4-6 months old provides immunity for 6-9 months. A further dose is recommended 3-6 months later; thereafter, an annual dose is required.

e) Stability

The oil adjuvant vaccine emulsion should be pure white and stick to glass like paint. If the emulsion shows signs of cracking, it should be discarded. Separation of a thin layer of oil is permissible. It can be stored at 4-8°C for 6 months without loss of potency. It must not be frozen.

REFERENCES


INFECTIONOUS BOVINE RHINOTRACHEITIS (B19)

SUMMARY

Diseases caused by herpesvirus type 1 in cattle include infectious bovine rhinotracheitis, infectious vulvovaginitis, and infectious balanoposthitis. The different strains have predilections for different tissues. In addition, strains of the virus have been isolated from aborted fetuses and cases of dermatitis, encephalomyelitis, enteritis, mastitis, conjunctivitis and lesions of the interdigital space.

Identification of the agent: The virus may be recovered from infected cattle, using swabs of affected mucosae or from infective milk, by the inoculation of tissue cultures of bovine origin. The virus is present only in very small amounts, or not at all, in the tissues of aborted fetuses; it may, however, be present in the accompanying cotyledons and placenta. Infected animals may have pyrexia and at the same time shed large quantities of the virus.

Animals with secondary infections may excrete only small amounts of virus. Infections can also be latent with no obvious clinical signs, but virus may be excreted from these cases following stress.

Serological tests: Specific antibodies may be demonstrated by virus neutralisation (VN), enzyme immunoassays, immunofluorescence and indirect haemagglutination tests. In serum surveys it may be preferable to test selected animals from different age groups to obviate having to test large numbers of samples.

Requirements for biological products: Vaccines may be of various types, such as live attenuated, ts-mutant, genetically engineered or inactivated virus vaccines.

A. DIAGNOSTIC TECHNIQUES

Strains of bovine herpesvirus 1 (BHV1) have been isolated from cases of infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV) and infectious balanoposthitis (IBP). In addition, this virus has been isolated from aborted fetuses, cases of dermatitis, encephalomyelitis, conjunctivitis, enteritis, mastitis and lesions in the interdigital space. Although these strains may differ biologically, they are nevertheless serologically identical; the exceptions are a few strains that have been isolated from the brain and which show a slight antigenic difference. This is useful in laboratory diagnosis and in the production of suitable vaccines (1, 2, 3, 4, 5, 6).

Laboratory diagnostic procedures for BHV1 infections consist of two types. The first comprises tests for virus isolation and antigen detection; the second includes
Infectious bovine rhinotracheitis (B19) tests for the detection of specific antibody. The choice of test will depend on the disease situation. In countries free of BHV1 infections but where its presence is suspected, laboratory diagnosis must be aimed at isolation of the virus; at the same time, the inoculation of susceptible cattle must be carried out. Serological examinations, including examination of paired blood samples collected at an interval of 2-4 weeks, are also useful.

1. Identification of the agent

Containers used for the transport of samples should be sterile. The isolation of BHV1 virus from cattle is performed in primary calf-cell monolayer cultures or other susceptible cell culture systems.

The recovery of virus in culture will enable specific identification to be made. Methods suitable for such identification include: enzyme immunoassay, such as the enzyme-linked immunosorbent assay (ELISA), immunofluorescence and virus neutralisation (VN) tests.

In cases of IBR, it is necessary to isolate the virus by means of gauze (not cotton) swabs collected from the mucosa of the nasal cavity. It is possible to take swabs of the conjunctival mucous membrane. The gauze is preferably held in a forceps and placed in a sterile tube after collection. This material should then be maintained at approximately 4°C and transported to a diagnostic laboratory. It is advisable to submit a serum sample at the same time, should the case involve animals where IBR virus or other viruses cannot be isolated. The serum sample may then be tested later, should a diagnosis based on serology be desirable (6).

In cases of IPV, a gauze swab is placed at the end of a pair of long forceps, inserted into the vagina, left there for at least a minute and rotated on removal. Care should be taken in parting the vulva before insertion of the forceps, so as to avoid trauma and faecal contamination. Serum samples should also be collected and transported as indicated earlier (6).

In cases of IBP, if gauze swabs are not available for swabbing the prepuce, the alternative is to take preputial washings using isotonic saline as the fluid. At artificial insemination centres, the artificial vagina can be flushed out after the semen has been collected. This method can, of course, only be used if the bulls are in the early, late or latent stages of infection. Blood samples should be taken and transported as indicated earlier.

In the case of abortion, there is little or no virus present in the tissues of the aborted fetus. To attempt virus isolation in this instance, a cotyledon, together with a portion of attached placenta, should be collected and dispatched under cool conditions to a diagnostic laboratory. A serum sample should again be included.

In other rare instances, BHV1 virus has been the cause of mastitis, responsible for the occurrence of skin lesions and for lesions in the interdigital spaces of cattle. In cases of BHV1 virus mastitis, the virus may be isolated from milk, which should be
collected as individual samples from each quarter. In cases of epithelial skin lesions, skin scrapings should be taken. Immediate cooling and proper shipment, together with a serum sample, are desirable (6).

If there are many affected animals in a herd, a limited number should be sampled, with preference being given to febrile animals showing typical signs of BHV1 infection. Otherwise, it is advisable to examine a number of animals from different age groups before making a diagnosis. Infected animals are then frequently detected where the only typical sign of illness is a high body temperature. Such animals usually shed large amounts of virus, whereas animals with secondary infections excrete only small amounts of virus. Animals with latent infections often shed infectious virus as the result of stress, usually without exhibiting any typical clinical signs of infection.

2. **Serological tests**

Where the disease is endemic, the investigation of new outbreaks should include the detection of a rise in specific antibodies in cattle sera or, where appropriate, in milk. For the purposes of control, surveillance, or certification for trade, a highly sensitive serological test should be used, e.g., a VN test with 24 hours' pre-incubation of the virus-serum mixtures, or an ELISA of similar sensitivity.

Serological methods include VN tests, ELISAs, indirect fluorescent antibody tests and indirect haemagglutination. Sampling strategies for surveillance purposes should be based on standard statistical methods. It should be noted that antibody assays used on milk samples may be less sensitive than when used for sera. For large herds, animals to be sampled should be selected from each age cohort, for example from 0-3 months, 4-6 months, 7-12 months, 13-24 months, then at yearly intervals up to 8 years of age, and finally, cattle above that age.

There is a need for international standardisation of antibody assays for BHV1. The VN and ELISA are the most widely used. Examples of suitable protocols are given below.

a) **Virus neutralisation and plaque reduction**

The test is carried out in tissue culture using microtitre plates, test tubes or (for the plaque reduction assay) petri dishes. The principle of all the methods is the same: primary or secondary cultures of bovine cells can be used, as well as permanent cell lines such as MDBK cells. The only difference is in the step in which the serum/virus mixture comes into contact with the cells. In the tube and plaque reduction test the mixtures are pipetted onto the monolayers and in the microtitre plates the cell suspension is added to the mixture.

The sera are first inactivated at 56°C for 30 minutes. Serial serum dilutions are performed either in 2-fold or 4-fold dilutions, starting with undiluted serum. Equal volumes are added of a virus suspension calculated to contain 30-100 TCID50 per unit volume. The serum/virus mixtures are incubated for 18-24
hours at 4°C or for 30 min at 37°C or for 1.5-2 hours at room temperature (22°C). The addition of complement is not necessary. Two or four test tubes or microplate wells should be used per dilution; in the plaque reduction test one petri dish per dilution is sufficient.

Controls should include a known seronegative and a titration of a known seropositive serum, together with a titration of the test virus to ensure that its titre is within acceptable limits (30-100 TCID<sub>50</sub>). For the microplate VN test a cell suspension of 3 x 10<sup>5</sup> cells per ml in growth medium (produced by trypsinisation of a suitable monolayer) is now added at 50-100 μl per well.

The cultures are then incubated at 37°C for at least 5 days. By that time the virus has usually formed plaques. These can easily be counted after staining the cells (e.g. by Giemsa stain). In the microtitre plate wells are virus positive where virus-specific cytopathic effect is observed. A serum is considered positive when no evidence of virus multiplication is observed in one or more of the wells with undiluted serum portion. Neutralisation titres are calculated by Spearman/Kärber or any similar method. The same methods are used for the calculation of plaque reduction titres.

b) ELISA

ELISA kits are now widely available. A number of laboratories also prepare their own ELISA, and many such methods have been published. The serum dilutions are prepared as prescribed by the manufacturer of the kit.

The sensitivity of the ELISA has been increased to check bulk milk samples from up to 50 cows. The handling of such samples is described in detail by the manufacturers of the kits.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

1. Seed management

a) Characteristics

The types of BHV1 virus vaccine that are currently available, or are in preparation, include live attenuated, ts-mutant, genetically engineered and inactivated virus vaccines (6).

b) Culture

Seed virus is cultured by inoculating suitable types of bovine tissue cultures.

c) Validation as a vaccine

When using live attenuated vaccines, the degree of strain attenuation must be demonstrated by the inoculation of serologically negative cattle for five
consecutive passages. For this, two animals are inoculated by spraying the vaccine onto the mucous membranes of the upper respiratory and/or genital tracts and the production of virus is monitored by collecting the mucus on swabs. Three days after vaccination, the virus is passaged into two more animals, which should preferably be 6-8 months pregnant. The mucus collected on each swab is weighed and 10-fold dilutions of this material prepared. The first (or 10⁻¹) dilution is retained to inoculate (5 ml) each animal for the passages. The infectivity titre of the remaining dilutions is determined in suitable bovine tissue cultures. This procedure is repeated (for a total of 5 passages). No clinical signs of infection should be observed, nor any abortions occur (6).

The handling of ts-mutant vaccines is the same as that described above for the live attenuated vaccines.

Genetically engineered vaccines are handled in a similar manner, but they must also be shown to be genetically stable by using suitable methods, such as restriction enzyme analysis (1).

For inactivated vaccines, suspensions of the suitable virus strains are inactivated by the appropriate methods. The inactivation process is controlled by inoculating bovine cell monolayer cultures. Two passages are carried out during which no evidence of virus growth should be detectable during an observation period of 7 days for each passage.

Lyophilised vaccine can be stored according to the results obtained in stability tests. The vaccine should be employed according to the manufacturer's instructions.

2. **Manufacture**

Each vaccine is produced by harvesting the supernatant fluid from a collection of cell monolayer cultures in which BHV1 virus growth has occurred. The monolayers may be primary or low-passage calf tissue cultures, or a continuous bovine cell line, placed in maintenance medium at 37°C under appropriate conditions. The time of harvesting is determined by the occurrence of an advanced characteristic cytopathic effect involving 70-100% of the cells. The supernatant fluid medium is clarified by low-speed centrifugation. Fluid harvests may be collected from the same cell batch on two successive days.

The cells used for vaccine production should be derived from normal, healthy tissues and should be free of bacterial, fungal, mycoplasmal and viral contamination.

3. **In-process control**

No BHV1 virus should survive neutralisation with immune serum. Virus-serum mixtures are examined for residual live virus by inoculating a series of cell cultures which must be maintained and examined under a microscope for at least 10 days.
4. **Batch control**

a) **Sterility**

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on general information.

b) **Safety**

Two animals should receive a double dose of vaccine by the appropriate route. The test fails if either animal shows signs of disease other than a mild pyrexia. Both animals must be kept under observation for 3 weeks following inoculation and must develop antibodies specific for BHV1 virus by the end of this period.

c) **Potency**

The potency of live vaccines is estimated in bovine cell cultures. The minimum infectivity titre required for the vaccine must be determined by the national authorities.

The potency of inactivated vaccines should be tested in cattle and/or suitable laboratory animals. The minimum protection rate is also determined by the national authorities.

Samples taken from ampoules of each batch of vaccine must be tested for the possible presence of bacterial, mycotic and mycoplasmal contamination.

**REFERENCES**


THEILERIOSIS
(B20)

SUMMARY

The Theileria parasites of cattle are important constraints to the improvement of the livestock industry throughout a large part of the old world. Theileria annulata and T. parva, the most economically important species, are responsible for mortality and losses in production that are only guessed at but these species are consistently recorded as the cause of major disease entities. Control of theilerioses in many countries is by control of their vectors, often as part of a general tick-borne disease control strategy. Tick control has become less reliable because acaricides are expensive, resistance has developed to many of them, regulations regarding cattle movement and quarantine are not strictly enforced, and management and maintenance of dips and spray races are often poor. More reliable systems are desirable and vaccination should provide for better control of theileriosis.

Identification of the agent: Diagnosis of disease is based upon clinical signs, a knowledge of disease and vector distribution, and examination of Giemsa-stained blood and lymph node smears. The schizont is a characteristic diagnostic feature of infections with T. parva and T. annulata in lymph node biopsy or impression smears. Animals infected with T. parva show enlarged lymph nodes, fever, a gradually increasing respiratory rate, dyspnoea and/or diarrhoea, and various post-mortem lesions; parasitised cells may be found in impression smears from all tissues. The gross pathology caused by schizonts of T. annulata resembles that of T. parva, while the piroplasm stages may also be pathogenic, causing anaemia.

Serological tests: Sero-epidemiology, particularly of T. parva, does not distinguish between the different subspecies or between different immunogenic stocks. The most widely used diagnostic test for Theileria species is the indirect fluorescent antibody (IFA) test. For the IFA test both schizont antigens and piroplasm antigens may be prepared on slides or in suspension and preserved deep frozen, except in the case of piroplasm suspension which is stored at 4°C. Test sera are diluted with bovine lymphocyte lysate and incubated with antigen, and antiovine immunoglobulin conjugate is then added. Using the test as described, the fluorescence is specific for the etiological agent. The IFA test is sensitive, fairly specific, and usually easy to perform. However, because of the problems of cross-reactivity between some Theileria species, the test has limitations for large-scale surveys in areas where these species overlap. Developments in molecular biological techniques provide possibilities for new more sensitive and specific tests.

Requirements for biological products: Reliable vaccines of known infectivity are a recent development. For T. annulata the vaccine is prepared from schizont-infected cell lines which have been isolated from cattle and attenuated in vitro. The vaccine contains schizont-infected cells and must
remain frozen until shortly before administration. Vaccination against *T. parva* is based upon a method of infection and treatment in which an aliquot of ground-up infected ticks is inoculated subcutaneously and the animals are treated simultaneously with tetracyclines; a mild or inapparent reaction occurs and the immune response controls the infection. Safety precautions must be taken in the preparation, handling and delivery of *T. parva* vaccines to protect the workers involved, to avoid contaminating the stabilates, and to avoid the possibility of introducing new isolates into an area where they may then become established through a carrier state.

A. DIAGNOSTIC TECHNIQUES

Introduction

*Theileriae* are obligate intracellular protozoan parasites which infect both wild and domestic *Bovidae* throughout much of the world. They are transmitted by ixodid ticks and have complex life cycles in both vertebrate and invertebrate hosts. There are six *Theileria* spp. which infect cattle; the two most pathogenic and economically important are *T. parva*, causing East Coast fever (ECF), Corridor disease and Zimbabwean theileriosis; and *T. annulata*, causing tropical theileriosis. *T. orientalis*, *T. taurotragi* and *T. mutans* generally cause no or mild disease and *T. velifera* is non-pathogenic, but they are common and complicate the epidemiology of theileriosis in cattle.

The most widely used method for the control of theileriosis is chemical control of ticks with acaricides. However, tick control has become less reliable because of acaricide resistance, the high cost of acaricides, poor management of tick control, and illegal cattle movement in many countries. Vaccination using attenuated schizont-infected cell lines has been widely used for *T. annulata*, while a method of infection and treatment using ground-up infected ticks as stabilates and tetracycline treatment is being implemented in a number of countries in East and Central Africa for *T. parva* control.

Chemotherapy is also widely used. Parvaquone (Clexon, Wellcome) and halofuginone (Terit, Hoechst) are used to treat *T. parva* and *T. annulata*, and recently an even more effective drug, buparvaquone (Butalex, Coopers Animal Health), has been introduced.

Most *Theileria* spp. produce a carrier infection in recovered cattle but there are no data on the role of carriers in transmission under natural conditions. Indigenous cattle in endemic areas may experience very mild disease or subclinical infection while introduced indigenous or exotic cattle are susceptible and may die when infected.

1. Identification of the agent

The schizont is a characteristic diagnostic feature of infections with *T. parva* and *T. annulata* in lymph node biopsy or impression smears stained with Giemsa stain.
Schizonts are transitory in *T. mutans* and *T. orientalis*, in which the piroplasm stage may be pathogenic. *T. taurotragi* schizonts are not readily detected in Giemsa-stained blood smears. *T. velifera* may be distinguished by a veil to the side of the piroplasm. The schizonts of *T. mutans*, if detected, are distinct from *T. parva* having larger, flattened and irregular nuclear particles. The piroplasms of *T. parva*, *T. annulata* and *T. mutans* are similar but those of *T. annulata* and *T. mutans* are generally larger and may be seen to divide. Diagnosis is based upon clinical signs, a knowledge of disease and vector distribution, and examination of Giemsa-stained blood and lymph node smears.

The schizont is the pathogenic stage of *T. parva* causing initially a lymphoproliferative and later a lymphodestructive disease. The infected animal shows enlargement of the lymph nodes, fever, a gradually increasing respiratory rate, dyspnoea and/or diarrhoea. The most common post-mortem lesions are enlarged and congested lymph nodes, interstitial pneumonia and interlobular oedema, erosions and ulceration of the abomasum, enteritis sometimes with necrosis of Peyer's patches and, in longer standing cases, lymphocytic infiltrations of the kidneys, resembling infarcts. Parasitised cells may be found in impression smears from all tissues. In animals that recover, occasional relapses occur and a nervous syndrome called "turning sickness" is sometimes seen. In this syndrome intravascular and extravascular aggregations of schizont-infected lymphocytes may be detected and accompanied by thrombosis and ischaemic necrosis.

In *T. annulata* both the schizont and piroplasm stages may be pathogenic. The gross pathology caused by schizonts of *T. annulata* resembles that of *T. parva*, while anaemia is the feature of the piroplasm pathology. Pathogenic strains of *T. mutans* also cause anaemia and in Japan and Korea strains of *T. orientalis* (referred to as *T. sergenti*) also cause anaemia. The immune response to these parasites is complicated. Cell-mediated immunity is the most important protective response in *T. parva* and *T. annulata*.

2. **Serological tests**

The indirect fluorescent antibody (IFA) test is the most widely used diagnostic test for *Theileria* spp.

a) **Preparation of schizont antigen**

i) **Schizont antigen slides**

Approximately 1 litre of either *T. parva* or *T. annulata* schizont-infected cells from cell cultures containing $10^6$ cells/ml, of which at least 90% of the cells are infected, is centrifuged at 150 g for 10 minutes. The supernatant fluid is removed and the cell pellet is resuspended in 10 ml of phosphate buffered saline (PBS) pH 7.2-7.4 and centrifuged as before. This washing procedure is repeated 3 times and after the final wash the cell pellet is resuspended in PBS (approximately 10 ml) containing 3.5% bovine albumin powder (fraction V of bovine plasma) to give a final concentration of $10^8$ cells/ml.
Thin smears of the cell suspension are made on Teflon-coated multi-spot slides (Bellco Glass Inc., Vineland, New Jersey, USA), or on ordinary slides using nail varnish for separation. The smears should give between 50-80 intact cells/field when examined under x 40 oil immersion objective. This is usually achieved by spreading approximately 200 µl of the cell suspension onto a well and then immediately aspirating the excess and transferring to the next well. This is performed from well to well until the volume is exhausted. With this method approximately 600 good quality slides containing a total of 6,000 individual antigen spots can be obtained. The smears are air-dried, fixed in acetone for 10 minutes, individually wrapped in tissue paper and then in groups of 5 in aluminium foil, and stored in airtight, waterproof plastic containers at either -20°C or -70°C. The antigens keep for a least a year at -20°C and longer at -70°C. At 4°C the antigen slides keep for up to 4 months.

ii) Schizont antigen in suspension

Five hundred ml of *T. parva* or *T. annulata* infected cells containing $10^6$ cells/ml are centrifuged at 190 g for 20 minutes at 4°C and the cell pellet obtained is washed twice in 100 ml of cold PBS. The viability of the cells is determined by eosin or trypan blue exclusion (should be greater than 90%). The cells are resuspended in cold saline, in 10 times less than the original volume. To this volume, two volumes of a cold fixative solution containing 80% acetone and 0.1% formaldehyde (0.25% formalin) in saline are added drop by drop while the cell suspension is stirred continuously. The cell suspension is kept at -20°C and allowed to fix for 24 hours. The fixed cells are then washed three times in cold PBS and centrifuged at 200 g for 15 minutes at 4°C. After the last wash the cells are resuspended in a volume of PBS which is 100 times less than the original volume. The fixed cells are distributed in aliquots of 0.5 ml. The antigen is stable at 4°C with 0.2% sodium azide as preservative for 2 weeks and keeps indefinitely at -20°C.

b) Preparation of piroplasm antigen

i) Piroplasm antigen slides

The piroplasm stage of *Theileria* spp. cannot be cultured *in vitro*, except for short-term culture, therefore the piroplasm antigen has to be prepared from infected animals. Experimental infections are induced by infecting cattle subcutaneously with sporozoites using ticks infected with either *T. parva*, *T. annulata* or *T. taurotragi*. *T. mutans*, *T. orientalis* and *T. velifera* infections are usually induced by inoculating splenectomised cattle intravenously with blood from a carrier animal, or with a blood stabilate, or by application of infected ticks. One hundred ml of the infected blood is collected from the jugular vein in a heparinised or EDTA vacutainer when the piroplasm parasitaemia is at least 5%, preferably more, and gently mixed in 2 litres of PBS. The mixture is centrifuged at 500 g for 10 minutes at 4°C; the plasma and buffy coat are removed and the red blood cells
Theileriosis (B20) are again resuspended in 2 litres of PBS and centrifugation is repeated. It is important to remove the buffy coat after each wash. This washing procedure is repeated 4 times. After the final wash an aliquot of the packed erythrocytes is used to make doubling dilutions in PBS and a 5 µl drop of each dilution is placed on slides. The dried spots are fixed in methanol and stained with Giemsa stain and the concentration of erythrocytes examined using a light microscope. The dilution which gives a single layer of erythrocytes spread uniformly on the spot is then selected, for large scale preparation of piroplasm antigen slides. Approximately 10,000 antigen slides (100,000 antigen spots) can be prepared from 100 ml of infected blood. The antigen smears are allowed to dry at room temperature before fixing in acetone for 10 minutes. The fixed smears can be stored as for the schizont antigen slides, and keep for similar periods.

ii) Piroplasm antigen in suspension

An alternative method of preparing antigens to that described above is available and has been tested for *T. parva* and *T. mutans*. In this procedure 100 ml of blood is taken from the jugular vein of an animal with a high piroplasm parasitaemia in EDTA (4.5%, pH 7.4, 10 ml to 100 ml of blood). The blood is washed three times with saline by centrifugation at 1,000 g for 15 minutes and the buffy coat is removed at every wash. The washed red blood cells (RBC) are suspended in saline and the packed cell volume (PCV) adjusted to 5%.

One volume of the RBC suspension is added to two volumes of the fixative (see above) while stirring. The cells are allowed to fix at -20°C for 24 hours. The fixed cells are then washed three times with saline and centrifuged at 1,000 g for 30 minutes. The deposit is resuspended to the original volume of blood with PBS containing 0.2% sodium azide.

The piroplasm antigen is stable at 4°C when preserved with 0.2% sodium azide for a period of at least three years.

c) Standardisation of antigen

The schizont and piroplasm antigens in suspension are mixed on a rotor mixer and titrated in PBS by doubling dilution starting from neat through to 1:16. The dilution giving a cell distribution of about 50-80 schizont-infected cells or 150-200 infected RBC per field on the slides under x 40 objective is taken as the dilution recommended for use for that batch of antigen. Using this dilution, test antigen smears are prepared on slides and these antigen smears plus the antigen slides previously frozen are thawed and tested against a panel of known strong, intermediate and weak positive and negative control sera. If the positive control sera titrate to their known titres and the negative control sera give no fluorescence, the antigen is used in the routine IFA test.

Both types of antigen preparations, acetone-fixed smears stored at either -20°C or -70°C, and antigens fixed in suspension and stored either at 4°C or at -20°C,
are used routinely in many laboratories. The sensitivity of both types of antigens is comparable. In laboratories where adequate low temperature storage facilities with a reliable supply of electricity are available the acetone-fixed slides can be used. However, such antigens can only be transported on dry ice or in liquid nitrogen. Antigens fixed in suspension have an advantage over the acetone-fixed smears because the initial method of preparation is simpler and quicker. A large batch of this antigen can be stored in one container and aliquots may be taken out as necessary from which fresh smears are prepared for the IFA test. This avoids the need for a large storage facility. The antigens fixed in suspension can also be stored at 4°C and can be safely transported at room temperature without loss of antigenicity.

d) Preparation of bovine lymphocyte lysate

The lymphocyte lysate is prepared according to the method described by Goddeeris et al. (7). Briefly, a 3-month old calf is splenectomised and maintained under tick- and tsetse fly-free conditions. To exclude the possibility of latent infection, blood smears from the animal are taken daily for a period of 4 weeks, stained with Giemsa stain and examined for parasites. The animal is euthanised and the thymus and all the accessible lymph nodes are removed. These tissues are sliced into small pieces in cold PBS containing 0.45% EDTA as anticoagulant. Cells are teased out of the tissue, separated from the debris by passing through a muslin cloth, and washed 3 times with PBS-EDTA by centrifugation at 200 g for 20 minutes at 4°C. The washed lymphocytes are resuspended in PBS without EDTA to give a final concentration of $5 \times 10^7$ cells/ml. The cells are disrupted by sonication in 100 ml aliquots on ice for 5 minutes using the 3/8 probe. The sonicated material is centrifuged at 1,000 g for 30 minutes at 4°C and the supernatant, adjusted to 10 mg protein/ml, stored at -20°C in 4 ml aliquots.

e) Method

Schizont and piroplasm antigen slides are removed from -20°C storage and allowed to thaw for 30 minutes at 4°C and for 30 minutes at room temperature before being unpacked. The frozen schizont antigen fixed in suspension and stored at -20°C is thawed at room temperature while the piroplasm antigen kept at 4°C is resuspended by agitation, passed through a 25 gauge needle to break the clumps (not necessary for schizont antigen) and diluted to previously standardised dilutions. The antigens are distributed onto the slides using a 100 μl pipette. By dispensing and immediately sucking up the schizont or piroplasm suspension, a monolayer of schizonts or piroplasms remains on each well. The slides are allowed to dry either at 37°C or at room temperature.

For initial screening 1:40 dilutions of test and control sera are made in lymphocyte lysate (195 μl lymphocyte lysate + 5 μl serum) and incubated for 30 minutes at room temperature. Further doubling or fivefold dilutions in 2% BSA in PBS can be made if endpoint antibody titre is desired. Twenty-five μl of the diluted sera are then transferred to the antigen slides. For each slide, the positive and negative control sera, diluted 1:40, are included. The slides are
incubated at room temperature in a moist chamber for 30 minutes, then washed twice in PBS for 15 minutes. Ten μl of antibovine immunoglobulin fluorescein isothiocyanate conjugate at recommended optimal dilution is applied to each well. Evans blue is incorporated in the conjugate at final dilution of 1:10,000 as a counterstain. The slides are incubated for 30 minutes at room temperature in a moist chamber, washed as before and mounted with cover-slip in 50% glycerol in PBS pH 8.0 and examined for fluorescence using a fluorescent microscope equipped with epi-Koem illumination (100 W mercury lamp), U.V. filter block, 6.3 x eyepieces and phaco FL 40/1.3 oil objective.

f) Characteristics of the IFA test

Using the test as described, the fluorescence is specific for the etiological agent only. The incorporation of Evans blue provides a good contrast, making reading easy. Mounting the slides in 50% glycerol at pH 8.0 reduces the rapid fading of fluorescein isothiocyanate and makes photography of the preparation possible. Slides once prepared are stable and can be read up to 72 h after preparation when kept at 4°C.

Using the schizont antigen, antibodies to *T. parva* and *T. annulata* following infection with sporozoites are first detected between days 10 and 14, while using the piroplasm antigen the presence of specific antibodies is first detected between days 15 and 21. Following recovery, antibodies may last for a variable period of time depending on several factors such as the establishment of a carrier state, chemotherapeutic intervention, and presence or absence of rechallenge. Following recovery from either East Coast fever or tropical theileriosis, some animals may have insignificant antibody titres in the IFA test after 4-6 months.

In *T. mutans* infections induced by sporozoite inoculation, the antibodies are first detected between days 10-15 after the appearance of piroplasms. In recovered animals low titres of antibody are most often detectable for at least 12-24 months.

In laboratories where the IFA test is used routinely for the detection of antibodies to one of the bovine *Theileria* spp. the test is sensitive and requires little standardisation. However, if the test is used to detect antibodies against different species of *Theileria*, specificity of the test needs to be carefully evaluated. For example, *T. annulata* and *T. parva* cross-react, although these cross-reactions are 4- to 6-fold lower than with the homologous sera. Such cross-reactivity does not seem to occur between *T. parva* and *T. mutans* or between *T. annulata* and *T. mutans*.

The failure to detect an antibody titre to *T. parva* or *T. annulata* is not necessarily confirmation that an animal is free of the parasite, as carriers of tick-transmissible infections have been shown to contain no antibodies by IFA. Xenodiagnosis can be applied but has shown that transmission may be intermittent.
g) Future tests for *Theileria* diagnosis

The IFA test is reasonably sensitive, specific and usually easy to perform. However, because of the problems of cross-reactivity between some *Theileria* species, the test has limitations for large-scale serological surveys in areas where these species overlap. There is a need for tests which are more specific, easy to interpret and robust enough to be used in field conditions. The enzyme-linked immunosorbent assay (ELISA) is now being used increasingly for the detection of parasite-specific antibodies, antigens and immune complexes. Such assays have been developed for African trypanosomiasis and have been shown to be far superior to the IFA test. Recently Katende *et al.* (9) have described an ELISA for *T. mutans*. The use of two monoclonal antibodies specific for *T. mutans* has been described in the ELISA system for the detection of antibodies and antigens in acute, subacute and chronic infections. The test is more specific and sensitive than the IFA test. This test needs further validation before it can be used on a wide scale.

Recently, DNA probes specific for *T. parva* (1, 3) and *T. mutans* (10) have been developed which are specific. The technology of polymerase chain reaction (PCR) is now available to amplify minute quantities of parasite DNA a millionfold, thus greatly increasing the sensitivity of the DNA probes (2). It is hoped that in the future, a combination of ELISA and DNA probes will greatly enhance our present capacity to identify antibodies and antigens at various stages of *Theileria* infections, thus making possible accurate serological surveys of *Theileria* species. Eventually, the aim would be to develop these technologies for the diagnosis of all the vector-borne diseases.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Cell culture vaccines for *Theileria annulata*

Vaccination against *T. parva* and *T. annulata* has been attempted since the causal organisms were first recognised early this century. However, reliable vaccines of known infectivity are a much more recent development. The most widely used is an attenuated schizont cell culture vaccine against *T. annulata*. The procedures for production and safety testing have been described (6, 8, 11, 12) and the vaccine is used widely in Israel, Iran, Turkey, India, Southern Russia and China.

1. **Seed management**

a) Characteristics of the seeds

Primary cultures of *T. annulata* infected cells may be established from trypsinised lymph nodes, liver or spleen taken aseptically from an infected animal after death or from the buffy coat of heparinised peripheral blood separated on a density gradient (Ficoll Hypaque) or using lymphocytes harvested using a simple 'plastic syringe' method (6).
Seed cultures are prepared from cryopreserved infected cell lines which have been isolated from cattle and attenuated as described below. In mass cultivation for vaccine production, cultivation is recommended from the seed culture following 20-30 passages because there is some uncertainty about the immunogenic stability of these cultures in long term passage.

b) Culture

The infected cells are cultured in Eagle’s minimum essential medium (MEM) supplemented with 20% calf serum and containing 100 µl/ml penicillin, 50 µl/ml streptomycin and 75 µl/ml mycostatin in 25 ml plastic screw cap tissue culture flasks. Medium is replenished every 3-4 days. The presence of bright refractile cells free in the medium (on examination using a phase contrast or inverted microscope) is indicative of cell growth. The cultures may establish as a monolayer or in suspension. Passage is effected by decanting the medium, adding 0.025% versene for 15 minutes to monolayer cultures, dispersing the cells, then counting and dispensing according to flask size. Approximately $10^6$ cells are introduced to a 25 ml flask and $16 \times 10^6$ to a Roux flask.

Serum is essential for maintenance of these cultures and is obtained from calves up to the age of 6 months and is tested for toxicity through three passages in an established cell line before use.

c) Safety

Attenuation of the virulence of *T. annulata* schizonts is achieved by prolonged growth and passage in culture. The mechanism of attenuation is unknown but the time a culture is maintained *in vitro* is thought to be more important than the number of passages. Attenuation is assessed by the inoculation of the culture into susceptible calves every 20-30 passages. A sample of culture should be cryopreserved every 10 passages in case of accidental loss or contamination. Complete attenuation is achieved when cultures do not cause fever or detectable schizonts and piroplasms in susceptible cattle. An attenuated culture will reliably infect cattle at $10^5$ cells and induce a serological reaction and will not produce disease at $10^9$ cells. Field isolates have required passage for periods of four to 30 months for complete attenuation. Cultures may be cryopreserved using either dimethyl sulphoxide or glycerol and two methods of storing and delivering the vaccine are described below.

2. Manufacture

The recommended vaccine preparation for field use varies.

a) Schizont-infected cells are mixed with dimethyl sulphoxide at a final concentration of 7% and dispensed into 2 ml plastic cups. These are frozen to -70°C in an ultra cold deep freezer and after 24 hours transferred to liquid nitrogen. Each 2 ml contains 10 doses of $10^7$ cells. Vaccine is transported to the field in liquid nitrogen and diluted in isotonic buffered saline in a screwcap
b) Cultured cells of two stocks (S11 and S15) are each concentrated in culture medium containing 9.5% glycerol to approximately $2 \times 10^6$ cells per ml and 12 ml of concentrated cells are dispensed into 20 ml vials. These are cooled at -20°C for 1 hour, then frozen to -70°C. The vaccine may be transferred to dry ice or liquid nitrogen storage. Vaccine is taken to the field in liquid nitrogen or dry ice. Twenty ml of one vaccine stock is thawed at 37°C and the animals are inoculated with $2 \times 10^6$ schizont-infected cells. The second stock is administered similarly within 30-60 days (8).

3. Batch control

Before release of cell culture vaccines in Israel, they are tested for purity by the procedures recommended by the US Code of Federal Regulation (11). These vaccines produce no adverse effects in healthy cattle. However, animals with existing infections, particularly viral infections, may not tolerate vaccination well. The administration of a viral vaccine, such as for foot and mouth disease, during the immunisation period (reaction period) is not recommended as the immune response may be compromised (8). In Iran it is not recommended to vaccinate cows that are more than five months pregnant, although studies with the vaccine stocks used in Israel have found no effect on pregnancy (12). The immunity is long lasting.

In general cattle should be immunised in the first few months of life and tick challenge under natural conditions re-inforces the immunity. Although antigenically different strains have been identified (12) it is generally considered that there is sufficient cross-immunity between strains to provide adequate protection against field challenge throughout countries, such as Israel. In the vast infected areas in the region of the Caucasus mountains in Southern Russia, a single stock has proved immunologically effective in 1.5 million cattle (4, 14). However, as described, two stocks are used routinely in Iran (8).

Infection and treatment vaccination for *Theileria parva*

Vaccination against *T. parva* is based upon a method of infection and treatment in which an aliquot of ground-up infected ticks is inoculated subcutaneously and the animals are treated with tetracyclines (13). A mild or inapparent reaction occurs and the immune response controls the infection. This method has also been applied effectively for *T. annulata* but the cell culture vaccine, which is not effective for immunisation against *T. parva*, is preferred.

1. Stabilate preparation

Infection is established by inoculation of healthy serologically negative cattle with a tick stabilate of *T. parva* or by application of infected ticks. During the parasitaemic phase of the ensuing disease reaction, clean laboratory raised nymphs of *Rhipicephalus appendiculatus* are fed on the animals and the engorged ticks
collected. The resultant adult ticks are applied to the ears of healthy rabbits in ear-bags within three weeks to four months after moult. About 600 ticks are applied to each ear and any unattached ticks are removed after 24 hours. After 4 days the ticks are removed and counted into batches of about 1,000. An estimate of the total number of ticks can be obtained by counting and weighing a given number of ticks and then weighing the total number of ticks. The ticks are washed in a sieve under fast flowing tap water and may be surface disinfected in 1% benzalkonium chloride or surface sterilised by washing in 70% alcohol, then washed again in water.

The ticks are placed (~1,000) in heavy glass specimen jars or plastic beakers and 50 ml Eagle's minimum essential medium (MEM) with 3.5% bovine plasma albumin (BPA) is added. The jars are kept on ice and the ticks are ground using a tissue grinder homogeniser (for instance Silverson LR2) for two minutes using a large aperture head and for three minutes using a small aperture head. The ground-up tick material is then centrifuged at 50 g for 5 minutes and the supernatant harvested. This supernatant is made up to 50 ml for every 1,000 ticks and an equal volume of 15% glycerol in MEM/BPA is added dropwise. The tick material is maintained chilled on ice and stirred by a magnetic stirrer. The final volume will contain sporozoites from the equivalent of 10 ticks per ml unless a different concentration is required because a tick batch was very heavily infected or had a very low infection rate. The infection rate is determined on dissected salivary glands from four-day fed ticks.

The ground-up tick material is then dispensed into glass vials by syringe or pipette for small total volumes or by automatic syringe for larger total volumes. Alternatively artificial insemination equipment for semen dispensation has been used with prelabelled plastic straws. This latter system is ideal for large volume stabilates and colour coding and labelling provide extra security by identifying each straw.

The final concentration of glycerol in the sporozoite stabilat e is 7.5% and the equilibration time should be 30-45 min before the stabilat e is placed in an ultra low deep freezer (-70°C). Once frozen it may be transferred to permanent storage in liquid nitrogen taking care not to allow any significant increase in temperature during transfer.

The infectivity of a standard dose of 1.0 ml is determined in susceptible cattle and the stabilat e is then titrated and the infectivity and lethality at different dilutions established. The sensitivity to tetracyclines is also determined, essentially to provide a dose of stabilat e that is controlled, preferably by a single dose of long-acting tetracycline administered at the same time as inoculation. The immunising dose should induce a very mild or inapparent infection and the animal should develop a serological titre and be immune to lethal homologous challenge. Should a single treatment with tetracycline fail to suppress the infection in all cattle, then either a lower dose of stabilat e is examined or two treatments of tetracycline (on days 0 and 4) may be used. An alternative method that has been used involves stabilat e infection and treatment with parvaquone at 20 mg/kg on day 8. This method can be applied where tetracyclines are not reliable but it requires that the animal be
handled more than once. A single treatment with buparvaquone at 2.5 mg/kg at the time of infection has also been shown to be effective with stabilate infections that were not controlled with a single treatment at 20 mg/kg of a long acting formulation of tetracycline.

Whichever method of immunisation or treatment is adopted, it must be adhered to strictly in the field or failure or breakdown will occur. It is also important to determine the stabilate and drug dose regimen in the most susceptible cattle in which it is likely to be used.

2. Safety precautions

At a recent meeting in Malawi the following recommendations on safety in the preparation, handling and delivery of *T. parva* infection and treatment vaccines were adopted (5).

a) Field collection of ticks

Consideration should be given to the possible hazard to man from pathogens present in field-collected ticks. The most important pathogen which has been recognised is Crimean Congo haemorrhagic fever virus, usually associated with ticks of the genus *Hyalomma* and widely prevalent within the geographic distribution of *Rhipicephalus appendiculatus*. Persons handling field tick collections should therefore be made aware of potential hazards. Ticks of *Hyalomma* species generally should not be removed from hosts; engorged or partially engorged ticks should not be crushed between the fingers. If removed, ticks should be handled with forceps.

b) Tick handling facilities

The handling of field-collected ticks in the laboratory must be controlled to avoid accidental attachment to personnel. Field-collected ticks should be fed on rabbits and cattle in isolation facilities. Animals on which ticks have fed should be destroyed following transmission and pick-up. Following engorgement of field-collected ticks on laboratory animals, aliquots should be homogenised and tested for extraneous human pathogens by inoculation in neonatal mice and BHK and VERO cells. The effects of these inoculations should be studied through three passages. Any unused ticks should be destroyed by chemical means or by incineration.

c) Stabilate preparation

Care should be taken in the preparation of sporozoite stabilates to avoid aerosol infection of personnel with extraneous pathogens during the grinding of ticks. Personnel grinding ticks should be educated in the potential hazards involved; access to areas where ticks are homogenised should be restricted to specified and informed personnel; personnel should wear protective clothing, including gloves and masks; and tick grinding should be carried out under a biohazard hood with a negative air pressure.
3. **Purity of stabilates**

Both ticks and experimental mammals are potential sources of contamination of stabilates with extraneous pathogens. In both cases, potential contaminants include *Ehrlichia bovis*, bovine *Borrelia* sp., Orbiviruses, Bunya viruses and others. Field-collected ticks should therefore not be used for the preparation of bulk stabilates. Well characterised and pathogen-free laboratory colonies of ticks should be used for this purpose. Only healthy cattle and rabbits, free of tick-borne parasites, should be used for tick feeding. Stabilates should be prepared in as sterile a manner as is possible. Under some conditions, the use of antibiotics at concentrations appropriate for tissue culture may be indicated. Prepared stabilates should be subjected to routine tests for safety by inoculation into neonatal mice and BHK and VERO cells, followed by three passages in these systems. Stabilates should be subjected to routine characterisation *in vivo*, which should involve infectivity testing in splenectomised and intact susceptible cattle, sensitivity to tetracyclines and other anti-theilerial drugs and cross-immunity *in vivo*.

Care must be taken to avoid contamination of the stock being used for preparation of stabilates in the laboratory with other *T. parva* stocks. A set of rules should therefore be established for the handling of infected ticks and the rules adhered to rigidly. Tick unit facilities should allow for strict separation of infected and uninfected ticks, and tick unit personnel should use separate overalls for each batch of ticks used in stabilate preparation and the overalls should be sterilised daily. Work should not be carried out on many different stocks simultaneously, and stabilate storage systems should incorporate clear labelling of each stabilate tube.

4. **Effect of an immunising stock**

The introduction of an immunising stock which does not originate from that area may result in that parasite, or a component parasite(s) of that stock, becoming established through a carrier state in cattle and ticks. The long-term effect of the introduction of new (and potentially lethal) parasites on the disease epidemiology should be monitored.

The characterisation of parasites in target populations should be carried out before immunisation and at intervals following immunisation.

5. **Vaccination strategy**

Unlike *T. annulata*, where a limited number of antigenically different strains exist in the field, a very much more complex situation exists for *T. parva*. Two strategies are used to try to overcome this antigenic complexity. A combination of three stocks which provides a broad spectrum protection has been tested in a number of countries. The combination is prepared under strict quality controlled conditions in Malawi by an FAO project for distribution within the *T. parva*-endemic region. If this combination fails to protect, the breakthrough strain will have to be isolated, characterised and added to the combination. The other strategy is to prepare national or local stocks for use within defined areas. This latter strategy is more costly in time and resources but it avoids, to some extent, the introduction of new
stocks into an area. The infection and treatment method of immunisation is effective but the attendant delivery problems and the risk of induction of carrier states, disease transmission and reduced productivity all emphasise the need for the identification of protective antigens for development of sub-unit vaccines.

REFERENCES


SUMMARY

Bovine venereal trichomoniasis is caused by Tritrichomonas foetus, a flagellate protozoan. It is worldwide in distribution and at one time was of major economic importance as a cause of abortion and infertility, especially in dairy cattle. With the widespread use of artificial insemination the prevalence is much reduced, though it may still be of importance in beef herds or where artificial insemination is not available.

Three serotypes of the organism occur: belfast, manley and brisbane, all being of equal pathogenicity.

Identification of the agent: Tritrichomonas foetus is a flagellate, pyriform eukaryotic protozoan with three anterior and one posterior flagellae and an undulating membrane, approximately 8-18 \( \mu \)m long and 4-9 \( \mu \)m wide. Organisms move with a jerky, rolling motion and are seen in preputial washings of infected bulls and vaginal washings or cervico-vaginal mucus of infected cows, or in aborted fetuses. Organisms can be cultured in vitro. They may be stained with the Romanowsky stains.

Transmission is primarily by coitus, but mechanical transmission by insemination instruments or gynaecological examination can occur. The organism can survive in whole or diluted semen at 5°C.

Serological tests: An agglutination test using mucus collected from the cervix and an antigen made from cultured organisms has been used as a herd test. Similarly, an intradermal test using a trichloracetic acid precipitate of the organism has been used in herds. Enzyme immunoassays are under development.

Requirements for biological products: There is no effective vaccine for trichomoniasis.

A. DIAGNOSTIC TECHNIQUES

Bovine venereal trichomoniasis is caused by the flagellate protozoan Tritrichomonas foetus. The natural hosts of T. foetus are cattle (Bos taurus, Bos indicus) and possibly the pig, horse and roe deer. Non-pathogenic species of trichomonads occur in the intestine of cattle; T. suis of pigs is indistinguishable morphologically and serologically from T. foetus.

* Nomenclature of parasitic diseases: see the note in Chapter 36 – Trypanosomiasis (B22).
Bovine trichomoniasis (B21)

*Tritrichomonas foetus* is pyriform, 8-18 μm long and 4-9 μm wide, with three anterior and one posterior flagellae and an undulating membrane. Living organisms move with a jerky, rolling motion. Detailed morphological descriptions, including electron microscopic studies, have been published by Warton and Honigberg (6).

The organism multiplies by longitudinal binary fission; there is no sexual reproduction and cysts have not been observed.

Three serotypes based on agglutination studies are recognised: *belfast*, *manley* and *brisbane*. The *belfast* strain predominates in Europe, Africa and the USA, *brisbane* in Australia, and only a few outbreaks are due to *manley*.

Organisms may be cultured *in vitro*, preferably in Diamond’s medium (1).

Transmission of infection under natural conditions is by coitus, by artificial insemination, or by gynaecological examination of cows. In the bull, infection is primarily of the preputial cavity, and little or no clinical manifestation occurs. Spontaneous recovery does not occur, and the bull becomes a permanent source of infection.

In the cow, the initial lesion is a vaginitis, followed by the invasion of the uterus by the organisms. Various sequelae result, including a placentitis leading to early abortion (8-16 weeks), uterine discharge and irregular oestrus periods. In some cases, despite infection, pregnancy is not terminated by abortion and a normal, full-term calf is born. On a herd basis there are irregular oestrus, uterine discharge, pyometra and early abortion. The cow usually recovers and is immune post-partum (5).

1. **Identification of the agent**

   A tentative diagnosis of trichomoniasis is based on the clinical history, signs of early abortion, repeated returns to service or irregular oestrus. Confirmation depends on the demonstration of organisms in placental fluid, stomach contents of the aborted fetus, uterine washings, pyometra discharge, vaginal mucus or, in the bull, preputial washings.

   The number of organisms varies in different situations. They are numerous in the aborted fetus, in the uterus several days after abortion and, in recently infected cows, they are plentiful in the vaginal mucus 12-20 days after infection. Thereafter the number of organisms varies according to the phase of the oestrus cycle, being highest 3-7 days after heat.

   Samples are collected by washing the vagina or irrigating the uterus with physiological saline with a plastic or glass pipette and a long rubber tube. Samples should be kept at 37°C. It is important to avoid faecal contamination as this may introduce intestinal protozoa which may be confused with *T. foetus*. Samples may be examined directly under a 100 x power microscope. Identification is based on
the size and shape of the organism as well as the jerky, aimless movements. Where specimens must be submitted to a laboratory and cannot be delivered within 24 hours a transport medium should be used (e.g. Winters' medium or buffered saline solution with 5% fetal bovine serum).

In grossly contaminated samples it may be necessary to sediment the material and to examine the sediment. Staining of preparations will allow more detailed examination of the material and more accurate diagnosis.

Where organisms are too few to allow accurate identification, cultures should be prepared. Several media can be used. The CPLM (cysteine-peptone-liver infusion-maltose) medium, BGPS (beef extract-glucose-peptone-serum) medium and Diamond's trichomonad medium are those of choice (4). It is important that sterile samples be obtained for inoculation into the media. Media should be cultured for 24 and 48 hours and 4 days, samples being examined at each period.

There are no specific macroscopic or microscopic lesions in the aborted fetus, and identification of the organisms is necessary for diagnosis.

2. Serological tests

A mucus agglutination test has been developed (3) and this detects about 60% of naturally infected cows, as antibody levels vary according to oestrus. Mucus samples are collected from the cervical region of the vagina, preferably a few days after oestrus. A sterile glass tube, 30 cm in length, 9 mm in diameter and bent at an angle of 150° about 9 cm from one end, should be used. Mucus is mixed with glucose saline and serially diluted in glucose saline. Alternatively, antibody in the mucus may be allowed to diffuse into saline, which is then diluted. The test organisms consist of a suspension of \textit{T. foetus} grown in culture containing 10,000 organisms/ml, and agglutination can be observed under the low power of a microscope after one hour's incubation at 37°C. Agglutination at a dilution of 1:10 is considered positive.

Antibodies appear in cervical mucus about six weeks after infection and persist for several months. The mucus agglutination test is most useful as a herd test, being capable of detecting latent infections. It is specific and does not cross-react with \textit{Campylobacter foetus} or \textit{Brucella abortus}. An intradermal test, using "Tricin", a trichloracetic acid precipitate of \textit{T. foetus} (2), has been used for herd diagnosis. Reactivity persists for some time after recovery from the infection.

Other serodiagnostic tests based on the antigen trapping ELISA technique are being developed (4).

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

There is no effective vaccine for trichomoniasis.
REFERENCES


SUMMARY

Tsetse-borne trypanosomiasis is a disease complex caused by several species of protozoan parasites of the genus Trypanosoma, transmitted cyclically by flies of the purely African genus Glossina (tsetse fly). The disease can affect all species of mammals but, from the economic point of view, tsetse-borne trypanosomiasis is particularly important in cattle. It is mainly caused by Trypanosoma congolense, T. vivax and T. brucei brucei.

Identification of the agent: Several parasite detection techniques can be used, including the microscopic examination of wet, thick or thin blood films or the microhaematocrit centrifugation technique, which is the most sensitive parasitological technique, particularly when combined with phase-contrast or dark-ground microscopic examination.

Serological tests: The indirect fluorescent antibody test is the most precise serological technique for detecting species-specific antibodies. A promising antigen detection enzyme-linked immunosorbent assay (antigen-ELISA) is under development for field use.

Requirements for biological products: No biological products are in use at the present time.

A. DIAGNOSTIC TECHNIQUES

Tsetse-borne trypanosomiasis is a disease complex caused by several species of protozoan parasites of the genus Trypanosoma, transmitted cyclically by flies of the purely African genus Glossina (tsetse fly). The disease can affect all species of mammals but, from the economic point of view, tsetse-borne trypanosomiasis is particularly important in cattle. It is mainly caused by Trypanosoma congolense, T. vivax and T. brucei brucei. T. uniforme, T. simiae and T. suis are other, less common tsetse-borne species. T. vivax is also transmitted by other biting flies, as exemplified by its presence in Latin America. Tsetse-borne trypanosomiasis also affects man, causing sleeping sickness through infection with either T.b. gambiense

*Note on nomenclature of parasitic diseases:

The World Association for Advances in Veterinary Parasitology has recommended a 'Standardised Nomenclature of Animal Parasitic Diseases' (Kassai T., Cordero del Campillo M., Euzeby J., Gaafar S., Hiepe Th. & Himonas C.A. (1988).- Vet. Parasitol. 29: 299-326). In principle, the disease name is constructed by adding the suffix 'osis' to the stem of the name of the parasite taxon. 'Trypanosomiasis' would therefore be 'trypanosomosis'. Because this nomenclature is not yet widely known, the Manual has retained the older names for the diseases.
Clinical and post-mortem signs of tsetse-borne trypanosomiasis are not pathognomonic but merely indicative of the disease. Therefore, positive diagnosis must rely upon parasitological techniques, which aim at microscopically confirming the presence of trypanosomes in animals, or serological diagnostic techniques, which involve various antibody or antigen detection tests.

1. **Identification of the agent**

Parasite detection techniques are specific, but their sensitivity is relatively low (i.e. a certain proportion of false negatives may be recorded), since parasitaemia is generally low and fluctuating. Therefore, a negative diagnosis does not always mean that the animal is not infected, and it is recommended that the tests be replicated before establishing a final diagnosis.

Several parasite detection techniques are available, all with varying sensitivity. The choice will depend upon the laboratory facilities available.

**Direct examination techniques**

The simplest techniques are wet and thick blood films. They are, unfortunately, also the least sensitive.

a) **Wet blood films**

These are made by placing a drop of blood on a microscope slide and covering with a coverslip. The blood is examined microscopically, using a x 40 objective with reduced condenser aperture. Trypanosomes can be recognised by their movement amongst the red blood cells.

b) **Thick blood films**

These are made by placing a drop of blood on a slide and spreading it over an area of about 2 cm in diameter, using the corner of another slide. It is dried by rapidly waving in the air. It is not fixed. The film is stained for 30 minutes with 10% diluted Giemsa stain in buffered water. The slide is then washed and examined using a x 100 oil-immersion objective. Trypanosomes are easily recognised by their general morphology, although they may be damaged during the staining process. This may make it difficult to identify species.

c) **Thin blood films**

These are useful for confirming the species of the trypanosomes. They are made by placing a small drop of blood on a clean microscope slide and spreading with the edge of another slide. This slide is placed at an angle of approximately 30° to the first slide, and drawn back to make contact with the blood droplet. The blood is allowed to run along the edge of the spreader, which is then pushed to the other end of the slide, drawing the blood out into a
thin film. All the blood must be evenly spread, with no surplus before the end of the slide is reached. The slide is dried quickly by waving in the air, and is fixed for 3 minutes in methanol. It is stained for 30 minutes in 20% Giemsa in buffered water. After staining, the slide is washed gently under tap water and allowed to dry. It is examined with a x100 oil-immersion objective. (A variation of this method is to fix for 2 minutes, then apply May-Grunwald stain for 2 minutes, then add an equal volume of buffered water and leave for a further 8 minutes, and drain off.) This allows *Trypanosoma* species to be identified by their characteristic morphological features.

The techniques described in a), b), and c) above can also be used for samples from puncture of lymph nodes.

**Parasite concentration techniques**

**a) Microhaematocrit centrifugation technique**

Microhaematocrit centrifugation techniques are the most sensitive for the detection of *T. congolense* and *T. vivax* (7) but they require specialised laboratory equipment which is not always available in the field.

The method described by Woo (10) is as follows:

i) Collect blood into a heparinised capillary tube. Fill to three quarters of its length.

ii) Seal one end of the tube either with plastic modelling clay or by heating, ensuring that the column of blood is not charred by the flame.

iii) Place capillary tubes in a microhaematocrit centrifuge with the sealed end pointing toward the outside. Load tubes symmetrically to ensure good balance.

iv) Screw on rotary cover and close the centrifuge lid.

v) Centrifuge at 12,000 rpm for 5 minutes.

vi) Place the tube on a slide where two pieces of glass 25 x 10 x 1.2 mm have been fixed on a slide, 1.5 mm apart, or use three tubes secured with adhesive tape.

vii) Place the tube in the groove, place a coverlid on top and flood the interphase with water.

viii) Examine the buffy coat-plasma junction. Trypanosome movement can first be detected using the x 10 objective with a reduced condenser aperture; the trypanosomes can be seen more clearly using the x 40, preferably long working distance, objective.
b) Dark-ground/phase-contrast buffy coat technique

This method (4) represents an improved technique for the detection of trypanosomes. It is carried out using steps i) to v) above, after which the tube is cut 1 mm below the buffy coat, to include the top layer of red cells, and 1 cm above to include the plasma. The contents of this piece of tube are expressed onto a clean slide, mixed together and covered with a coverslip. The preparation is examined using phase-contrast or dark-ground microscopy. Trypanosomes are easily recognised by their movements.

Trypanosome species can be identified by reference to the following criteria:

- **T. vivax:** Large, extremely active, traverses the whole field very quickly, pausing occasionally.
- **T. brucei:** Various sizes, rapid movement in confined areas.
- **T. congolense:** Small, sluggish, adheres to red blood cells by anterior end.
- **T. theileri:** More than twice the size of pathogenic trypanosomes.

If phase-contrast or dark-ground microscopes are not available, the phase-contrast effect can be achieved by using a normal field microscope with the condenser top out and/or the diaphragm closed.

If required, the preparation can be fixed and stained for species identification and for retention as a permanent record.

c) Packed cell volume determination

Measuring the packed cell volume (PCV) is useful for determining the degree of anaemia and therefore the stage of evolution of the disease. A sample may be prepared using steps i) to v) of the microhaematocrit centrifugation technique. The tube is then placed in the reader and the result expressed as a percentage of packed red blood cells to total volume of whole blood.

Animal inoculation

The mouse inoculation technique is the most sensitive for *T. brucei* infections. Two mice are injected intraperitoneally with 0.2 ml of the blood sample. They are bled three times a week and examined for the presence of trypanosomes by the wet film method.

2. Serological tests

a) Antibody detection

Several antibody detection techniques have been tried for the diagnosis of animal trypanosomosis, including the microplate enzyme-linked immuno-
sorbent assay (micro-ELISA) (3) and the card agglutination test (1). In general they have high sensitivity but often have poor specificity. Some give cross reactions with other protozoa and often fail to differentiate *Trypanosoma* species (8). They are only able to identify animals which have been previously exposed to trypanosome infection, but they do not reflect current infection, since the infection could have long been cured. At the present stage of development, the indirect fluorescent antibody (IFA) test is the procedure of choice, since it allows detection of species-specific antibodies.

**Indirect fluorescent antibody test**

The original method for this test (9) has been replaced by a new technique for the preparation of trypanosomal antigens (2), which involves fixation of live trypanosomes, using a mixture of 80% cold acetone and 0.25% formalin in saline. This new method is carried out by first preparing thin smears from heavily parasitaemic blood or from a trypanosome suspension. The smears are dried in air and fixed in acetone for 5 minutes. Glass slides are marked with 5 mm diameter circles, using nail varnish. A test serum, diluted 1:40, is placed in each circle, ensuring that each circle is completely covered. The slides are left for 30 minutes at room temperature in a moist chamber and then washed with 0.15 M phosphate buffered saline (PBS), pH 7.2. The slides are then immersed in PBS for 20-30 minutes, after which the conjugate is applied (goat anti-bovine Ig conjugated to FITC). The slides are mounted in PBS or buffered glycerol and examined for fluorescence.

**b) Antigen detection**

A promising recent test is the antigen detection enzyme-linked immunosorbent assay (antigen-ELISA) (6). This test appears to be considerably more sensitive than trypanosome identification procedures, and is specific to infections with *T. brucei*, *T. congolense* and *T. vivax*. In addition, it has potential as a test in kit form using a card ELISA technique which could be used under field conditions. It is presently under development for field use.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

No biological products are in use at the present time.

**REFERENCES**


Brucellosis in sheep is caused by Brucella melitensis and B. ovis, in goats by B. melitensis, and in swine by B. suis. Infections of these animal species by B. abortus have been reported but clinical disease is rare. Infections are manifested by one or more of the following: abortion, retained placenta, orchitis, epididymitis, and localised abscessation. B. melitensis and B. suis are highly pathogenic for humans. Control of brucellosis is based upon segregation of proven or suspected cases, slaughter of infected animals, and/or vaccination.

Identification of the agent: Brucella spp. may be isolated from uterine discharges, aborted fetuses, udder secretions or selected tissues such as lymph nodes, testes, or epididymides. Specimens are cultured using selective solid media. B. ovis and most biovars of B. abortus require incubation in a CO₂ atmosphere of approximately 10%. Suspected microorganisms are identified by various techniques such as sensitivity to dyes, agglutination with monospecific antisera, H₂S production, and lysis by phage. Species and biovar determinations are important for clinical and epidemiological purposes.

Serological tests: A large number of tests are available to assist in diagnosing brucellosis in all animal species. For pooled specimens from a herd or flock of sheep, goats and swine, there are no useful tests such as the milk ring test in cattle. For B. melitensis infections, the buffered Brucella antigen tests and complement fixation test (CFT) are usually recommended. The buffered Brucella antigen tests (rose bengal test or buffered plate agglutination test) are now most often used as the screening tests in sera where B. melitensis or B. suis infections are suspected. The serum agglutination test (SAT) is not considered satisfactory for use in small ruminants. However, it may be used in pigs. Sera which are positive to a buffered Brucella antigen test should be retested using the CFT or ELISA, especially if the animal has been vaccinated.

B. ovis infections may be detected by positive complement fixation or gel diffusion results especially if supported by physical examinations of sheep. The enzyme-linked immunosorbent assay (ELISA) has recently been found to be superior to other procedures in sensitivity.

Other procedures: The allergic or dermal hypersensitivity test is sometimes used in sheep and goats, usually as an intrapalpebral injection of Brucella allergen. Skin tests are also used in swine in some countries. They are useful screening tests for herds and flocks but must be interpreted in relation to the clinical signs and history and should be confirmed by serological or cultural examination.
Requirements for biological products: Seed cultures to be used for antigens for serological or intradermal tests and for vaccines should originate from reference centres (see B12). They must conform to minimum standards for viability, smoothness, pathogenicity, and immunogenicity, if applicable. Brucella allergen preparations must be free of lipopolysaccharide to prevent agglutinin production and local inflammatory reactions. Antigens for serological tests should be standardised against reference sera and the procedures and interpretations should conform with international recommendations.

A. DIAGNOSTIC TECHNIQUES

Brucellosis in sheep may be caused by *B. melitensis*, *B. ovis* and rarely *B. abortus*. With *B. melitensis*, abortions and retained placenta are the primary signs and sometimes orchitis and epididymitis occur. Breeds of sheep appear to differ in susceptibility to *B. melitensis* infections. *B. ovis* primarily causes epididymitis and rarely affects females. While *B. abortus* infections in sheep have been reported, clinical signs are rare.

*B. melitensis* is the primary pathogen of goats with similar signs to those in sheep. *Brucella*-induced arthritides are not uncommon in small ruminants. While goats are susceptible to *B. abortus*, natural infections appear to be rare. No natural cases of *B. ovis* or *B. suis* infections in goats have apparently been reported.

Swine brucellosis caused by *B. suis* results in abortions, orchitis and sometimes localised abscessation resulting in posterior paralysis. Cases of clinical disease in swine from *B. melitensis* or *B. abortus* are rare.

Brucellae may be excreted in uterine discharges, udder secretions and semen, and may be found in various tissues such as lymph nodes and those associated with reproduction. *Brucellae* may also be isolated from arthritic lesions. Abortions usually occur only once in infected animals. Excretion in goat milk may be prolonged and is of special public health concern with *B. melitensis*.

Special care must be taken by humans who work in environments where exposure to *B. melitensis* or *B. suis* may occur, as both organisms are highly pathogenic for man. An acute febrile illness may occur which may progress to more chronic forms and complications including abscessation. Those at occupational risk include those who work with susceptible animals, especially at times of parturition, and in laboratories where live cultures are used. Ingestion of unpasteurised dairy products is a common source of brucellosis in many countries. No confirmed cases of brucellosis in humans caused by *B. ovis* have been reported.

1. **Identification of the agent**

In countries where brucellosis exists in sheep, goats and swine, suspected animals should be investigated by serological procedures and culture methods if possible.
Brucellosis can only be confirmed by laboratory techniques. Stained smears of tissues or fluids may be useful using procedures such as the modified Ziehl-Neelsen method (6). *Brucella* spp. are intracellular bacteria and their presence is a presumptive diagnosis. Care must be taken in the interpretation of results as other infectious agents may have similar morphology. Serological tests offer a more dependable presumptive diagnosis, and culture is definitive.

Cultures are performed using solid media and several are available. For contaminated material Farrell’s selective medium (1) is suitable for isolation of *B. abortus*, *B. melitensis* or *B. suis*. For *B. ovis* the less inhibitory medium containing vancomycin (3 μg/ml), colistin methane sulphate (7.5 μg/ml), nystatin (12.5 units/ml), and nitrofurantoin (10 μg/ml) may be used. *B. ovis* and most *B. abortus* biovars require incubation in an atmosphere of approximately 10% CO₂ and addition of 5-10% serum to the basal medium.

Direct inoculation of the specimen (e.g. vaginal discharge, placental cotyledon, fetal tissues or abomasal contents) on solid medium is generally satisfactory. Milk may be centrifuged to concentrate the bacteria in the sediment or cream. If the specimen is heavily contaminated, guinea pigs or mice may be inoculated. However, inoculation of multiple plates of media containing a combination of inhibitors (such as Farrell’s) is usually superior to the use of guinea pigs or mice.

Experienced laboratory workers can usually identify *Brucella* colonies on the basis of morphology, slide agglutination tests using monospecific antisera, and growth characteristics. Pure cultures may be submitted to reference laboratories for additional procedures such as oxidative metabolism tests, H₂S production, sensitivities to dyes, phage lysis, and surface antigen determination, in order to unequivocally identify the species and biovar.

Identification of the vaccine strain *B. melitensis* Rev. 1 requires special techniques used only in reference laboratories. *B. melitensis* strain Rev. 1 has the normal properties of a biovar 1 strain of *B. melitensis* but grows much more slowly on ordinary media, does not grow in the presence of fuchsin or thionin at 20 μg/ml or benzylpenicillin at 3 μg/ml but grows in the presence of streptomycin at 5 μg/ml, has very weak urease activity and shows a reduced virulence for guinea pigs and mice. (For further details see refs. 1, 3, and 4.)

2. **Serological tests**

**Sheep and goats (*B. melitensis*)**

Serological tests on individual animals are less accurate in sheep and goats than in cattle. Therefore detection on a flock or herd basis is the most reliable means of serological diagnosis. This involves the collection of blood from several animals for serological tests. The milk ring test, which is widely used in dairy cattle, is not effective in sheep and goats. The serum agglutination test (SAT) is not considered satisfactory for use in small ruminants.
a) Buffered Brucella antigen tests [rose bengal test (RBT) or buffered plate agglutination test (BPAT)]

The difficulties of interpreting the results of the SAT have led to the use of the buffered Brucella antigen tests (the RBT and BPAT), which are rapid and simple. Most workers agree that these tests are reliable in screening serum samples on a flock or herd basis (see B12). Positive sera may be further tested with the complement fixation test or ELISA. Acidified plate test antigens are prepared with *B. abortus* Strains 99 or 1119-3 (see B12 or ref. 8).

b) Complement fixation test (CFT)

Most workers agree that the CFT is the most accurate test for use with sheep and goat sera. *B. abortus* antigen is used. Negative results may sometimes be obtained with infected animals. The test is complex and may be difficult to perform in some countries. See B12 for a description of the procedure. Titres of less than 20 IU are considered negative.

c) Enzyme-linked immunosorbent assay (ELISA)

Recent work has indicated that the ELISA performs at least as well as the CFT while being a simpler test to perform.

Sheep (*B. ovis* infection)

An accurate diagnosis of infection in individual sheep depends upon clinical examinations, cultures and serological tests. No single test will detect all infected sheep and combinations of clinical examinations and multiple tests are superior. The recommended serological test, considered by many workers to be the most specific method, is the CFT. *B. ovis* Strains 10/2, 424/2, or 63/290 are usually used as antigen. The antigen is extracted by autoclaving or boiling cells of *B. ovis* in saline. A microtitre CFT using *B. ovis* as the source of antigen has been described. The sheep red blood cells, haemolysin, complement and antigen must be standardised using control sera (1). In the evaluation of the test, 50% inhibition of haemolysis at a serum dilution of 1/40 should be regarded as positive and at a dilution of 1/20 as doubtful.

The agar gel immunodiffusion test (AGID) has been used by many workers to replace the CFT since it is simpler. Some studies have found the AGID to be less sensitive than the CFT. The ELISA is more sensitive than other tests for detecting *B. ovis* antibodies (9). For satisfactory use of ELISA and other procedures, the reagents and technique should be standardised for the conditions of each laboratory using control sera.

There is an international standard anti-*B. ovis* serum (Int. Standard 1985) and all *B. ovis* tests should be standardised against this.

Positive serological reactions may be obtained in vaccinated animals when the tests mentioned above are used.
Swine (B. suis infection)

As with sheep and goats, the serological diagnosis of individual swine with brucellosis may be difficult. Some infected swine fail to react with any test and heterospecific antibodies may be present. Cross-reactions are especially common with the SAT and sometimes result from infection with Yersinia enterocolitica O:9.

The buffered Brucella antigen tests (buffered plate or RBT) (10) are considered to be among the most practical and accurate screening tests if carried out on a herd basis.

3. Dermal hypersensitivity tests

Allergens (brucellins) may be used to diagnose B. melitensis infections in sheep and goats and B. suis infections in swine. Dermal hypersensitivity results cannot be relied upon to detect all infected animals and should be used only as herd or flock tests. In sheep, a solution of 50 μg of brucellin in buffered saline may be injected into the lower eyelid either subcutaneously (0.5 ml) or intradermally (0.1 ml) and observed at 48 hours. In goats, a solution of 50 μg may be injected intradermally (0.1 ml) in the neck, lower eyelid or dorsum of the ear. Swine may be injected in the ear or vulva.

The absence of allergic reactions in a flock or herd should be interpreted as signifying the absence of infection, while the presence of a reaction indicates that further investigation is necessary using more sensitive and specific diagnostic techniques such as culture and serology. Positive reactions may occur in vaccinated animals.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

The Brucella antigens which are used in serological tests for antibodies to B. melitensis and B. suis are made from smooth B. abortus Strains 99 or 1119-3 (5). These strains may also infect humans. Original seed should be obtained from the Central Veterinary Laboratory, Weybridge KT15 3NB, Surrey, UK, or the USDA, National Veterinary Services Laboratories, 1800 Dayton Avenue, Ames, Iowa 50010, USA. Dyes are used to stain the cells for the acidified plate tests. Further information on B. abortus antigens is given in the chapter on bovine brucellosis (B12).

B. melitensis Rev. 1 vaccine is used for the immunisation of sheep (7) and goats. It has been shown to be of low virulence and to produce good immunity (5). Vaccination is usually performed at the age of 4-6 months with a recommended dose of 10^9 cells. The duration of protection is several years. A reduced dose of Rev. 1 vaccine (10^5-10^7) given to adult sheep and goats has been shown to be effective in experimental and field studies.

Two additional vaccines are available in some countries for immunisation of sheep and goats against B. melitensis. B. melitensis Strain H38 vaccine is a killed virulent
strain in an adjuvant. It can be given to animals of all ages. However, it has the disadvantage of causing severe local lesions and prolonged serological reactions.

*B. suis* Strain 2 was developed in China. It is an attenuated smooth strain of *B. suis* biovar 1 and the growth characteristics are similar. It reportedly does not cause abortions nor persist in tissues (12). It also confers significant protection against infection with virulent *B. suis* in swine (6). The vaccine is normally administered by the oral route.

Rev. 1 vaccine has been shown to provide good protection against *B. ovis* infections in sheep. Commercial bacterins are also used in some countries.

1. **Seed management**

   a) **Characteristics**

   For *B. melitensis* Rev. 1 vaccine production original seed should be obtained from the Institut National de la Recherche Agronomique, Tours-Nouzilly, France. It should be propagated to produce a seed-lot which must be a pure, smooth culture and conform to the characteristics for *B. melitensis* biovar 1. Additionally it should be resistant to streptomycin and sensitive to basic fuchsin, thionin (1/50,000 w/v), and penicillin when cultured in air for 48 hours. Seed-lots should be produced in sufficient amount to be no more than three passages from the original seed culture (1, 11).

   b) **Culture**

   The sources and growth characteristics of seed strains used to produce smooth cells as antigens for serological tests for antibodies to *B. melitensis* and *B. suis* are given in Chapter B12.

   There are published techniques for growth of *B. ovis* antigen (1). In the United States, *B. ovis* Strain 3572 is grown on tryptose agar enriched with 5% bovine serum. After 72 hours of incubation, the growth is harvested in distilled water and filtered through gauze. The suspension is autoclaved and phenol added to a final concentration of 0.5%. The antigen is titrated prior to use in a checkerboard fashion with standardised antiserum.

   c) **Validation as a vaccine**

   Rev. 1 vaccine has been shown in numerous experimental and field studies to be a superior immunising product for protection against *B. melitensis*. Its virulence is unchanged after passage through pregnant sheep. If the $10^9$ dose is used, abortions may result when it is injected into pregnant sheep, and Rev. 1 can be found in the milk and the vaginal discharge. Prolonged agglutinins are produced in adult sheep and goats. To overcome these disadvantages, it has been recommended that vaccination be restricted to sexually immature animals. However, reducing the dose by 2 or more logarithms greatly reduces these problems and makes field usage of Rev. 1 vaccine much more practical,
although a $10^9$ dose is still mandatory in some countries. It has been reported (7) that conjunctival inoculation of Rev. 1 vaccine confers good immunity and reduces postvaccinal antibodies.

2. Manufacture

Serum dextrose and trypticase soy agars are satisfactory for propagating the Rev. 1 strain. Liquid medium may also be used. It is recommended that harvest of a vaccine lot should not be more than 3 passages from a seed lot (9). It is essential that the Rev. 1 strain be maintained at established levels of biological activity, immunogenicity and residual infectivity. The original seed culture or local seed-lot must be examined for dissociation before use by visual examination and by low power microscopy after staining with a 1:2,000 solution of crystal violet. In the culture the S/R ratio must be greater than 99.9%.

The techniques for growth of cells used in the manufacture of brucellin are given in part B of Chapter B12. The techniques for growth and maintenance of quality control for *B. suis* Strain 2 have not yet been established.

3. In-process control

Following growth of Rev. 1 strain in Roux bottles or in liquid medium, a pooled suspension is inoculated onto agar, incubated for 3 days, and the characteristics of the growth and possible contamination are examined. The number of viable organisms is calculated and percent of dissociation determined. The suspension is diluted so that final vaccine will contain $1 \times 10^9$ cells per dose of vaccine following loss of viability during lyophilisation.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

b) Safety

See part 4b of Chapter B12. Guinea pigs or mice are used to determine if there has been any change in the virulence of attenuated strains of brucella. Tests for virulence of brucella strains in laboratory animals have also been described in detail (1).

c) Potency

The potency of Rev. 1 vaccine can be determined in guinea pigs or mice using a procedure similar to that described in part 4c of Chapter B12. *B. melitensis* H38 is used for the challenge instead of *B. abortus* strains (2). There is, however, some dispute as to the validity of mouse or guinea pig assays for determining the potency of brucella vaccines.
REFERENCES


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CAPRINE ARTHRITIS-ENCEPHALITIS
(B25)

SUMMARY

Caprine arthritis-encephalitis (CAE) is an infection of goats caused by a lentivirus that occurs worldwide, although indigenous flocks of some countries are free of the disease. The virus is related to the maedi-visna virus (MVV) and the human immunodeficiency virus 1 (HIV 1). All breeds of goat are susceptible. The characteristic signs include arthritis, principally affecting the carpal joints, which may be accompanied by bursitis and synovitis. Pneumonia, induration of the mammary gland, and signs of central nervous system disorder may also occur.

Identification of the agent: In cases showing the classical signs of arthritis with or without bursitis, it should be possible to aspirate the joint fluid with a syringe. In dead animals, the joints should be opened aseptically and samples taken of the articular cartilages and synovial cells. Both aspirates and samples can then be used to inoculate tissue cultures of goat synovial membrane or cartilage cells, or other suitable cell cultures, such as those made from the choroid plexus, fetal testis or the cornea. A number of subcultures should be made every 2-4 weeks, until multinucleated cells appear. After concentration, the culture fluids are used in tests for the detection of virus or viral antigen. These tests include agar gel immunodiffusion, enzyme-linked immunosorbent assay and electron microscopy. Direct immunofluorescence tests can be performed on cell cultures.

Serological tests: The most widely used test is agar gel immunodiffusion, but the enzyme-linked immunosorbent assay technique is now also being employed. Serological tests can be conducted on sera or on milk that has been concentrated. Colostrum contains high levels of antibody. It is possible to use MVV antigen in these tests since the viruses are related.

The immune (Western) blot technique yields more information, as it will identify the group-specific lentivirus antigen p28.

Requirements for biological products: There are no biological products available.

A. DIAGNOSTIC TECHNIQUES

Occurring throughout the world, caprine arthritis-encephalitis (CAE) is a slow virus infection of goats. The causative agent is a lentivirus which is closely related to the maedi-visna virus (MVV) of sheep and to the human immunodeficiency virus I (HIV I). It should be noted that the indigenous flocks of some countries are free of the disease, so there should be no laxity in the control of the international movement of goats (1, 2, 6, 7, 8).
The laboratory diagnosis of CAE is in general not as easy as for diseases caused by viruses of other groups. Two approaches are possible, namely, attempts to isolate the causative virus as well as to detect the presence of specific viral antigen; and the alternative is to detect the presence of specific antibodies. The choice of method will depend on the disease situation.

Although all breeds of goat appear to be equally susceptible to infection, some countries have still not yet conducted tests on their national flocks to determine whether the disease is present or not. This is probably understandable when it is remembered that the presence of infection does not necessarily mean that signs of the disease are invariably present. A high percentage of animals is slaughtered for human consumption at an age when typical lesions have not yet become detectable (except in those countries where leukoencephalomyelitis occurs in kids aged 1 to 4 months old) (7).

The classical form of the disease consists of arthritis, which may or may not be complicated by the presence of bursitis and synovitis. The changes occur predominantly in the carpal joints, although other joints may be involved. The leukoencephalomyelitis observed in young goats, as well as the granulomatous encephalomyelitis seen in older goats, are not as widely distributed as is arthritis. It is still debatable whether or not an accompanying progressive pneumonia that may be seen in cases of viral arthritis is specifically caused by the CAE virus. This is because this aspect of the disease has not yet been reproduced experimentally. The same is true for the induration of the mammary gland that can be found in goat herds affected with CAE (2, 3, 4).

1. Identification of the agent

Whenever the presence of the CAE agent is suspected, it is advisable to try to isolate the virus itself. This may take as long as six months. At the same time, suitable serological tests should be carried out.

If an animal shows the characteristic signs of arthritis, with or without the presence of bursitis, which predominantly affects the carpal joints, material should be aspirated by syringe from the affected joint cavities. If it is possible to sacrifice the animal, small samples of the cartilage or the synovial membrane of the affected joints, together with synovial cells, can be used to establish tissue culture explants and to inoculate into synovial tissue or cartilage cell culture monolayers. Cultures of other cell types can also be employed, such as those of the choroid plexus, fetal testis or from the cornea. All cultures should be passaged a number of times every 2 to 4 weeks until polymenuclear cells begin to appear, or until tests carried out on the supernatant culture fluids prove to be positive for the presence of virus (6, 7, 8).

The CAE virus can be identified on cell culture by direct immunofluorescence. In tests on culture media that are carried out for the detection of virus, the fluids should first be concentrated at least 100-fold. If other tests are conducted on culture media subsequently, any of the available serological tests can be applied. These may be supplemented by electron microscopy using a negative staining
technique, or else by embedding cells in glutaraldehyde or another fixative suitable for the examination of ultrasections.

2. Serological tests

The most widely used test is the agar gel immunodiffusion test. Details for this procedure may be found in the chapter on maedi-visna (B33, Chapter 47). Enzyme immunoassay is also acceptable, e.g., the ELISA (1, 7, 8).

When the disease is endemic, the investigation of new outbreaks should start with the demonstration of antibodies either in sera or in milk, preferably colostrum. Antibody levels are frequently low and in terminal cases may even be absent. However, they are very high in colostrum and are therefore easily detectable. Since these levels drop rapidly, the ELISA is the preferred test to be applied to milk – after prior concentration, if necessary. The close relationship of the CAE virus with MVV makes it possible to use MVV antigen for the identification of the CAE agent.

The serological tests described above will not distinguish between CAE virus and other lentiviruses. In most cases, however, these will be sufficient, particularly as it is still uncertain whether viruses of the maedi-visna group occur in goats. If required, a more precise diagnosis can be made by means of the immune (Western) blot technique, which will distinguish between CAE virus and MVV.

Although antibody levels to the p28 core protein of CAE virus may be low or absent in terminal and some other cases, these animals may still have significant levels of antibodies directed against the glycoprotein gp135 antigen; such animals may give positive results with this antigen in the immunodiffusion test and in ELISA.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

At present no biological products are available.

REFERENCES


SUMMARY

Contagious agalactia, a disease of sheep and goats that includes mastitis, arthritis and keratoconjunctivitis, was originally associated only with Mycoplasma agalactiae. Now three other mycoplasmas, M. capricolum, M. mycoides subsp. mycoides (LC) and M. putrefaciens, have also been shown to cause similar diseases, at times accompanied by pneumonia.

Identification of the agent: Differentiation of the above-mentioned aetiological agents depends on the use of suitable media for isolation and on carrying out biochemical and serological tests on the isolates.

Serological tests: Detection of antibodies in serum by complement fixation, growth inhibition or ELISA is useful in diagnosis and in epidemiological studies, but does not replace identification of the causal organism for specific diagnosis.

Requirements for biological products: Killed vaccines are not considered efficacious for prevention of M. agalactiae infection, but show promise for that caused by M. mycoides subsp. mycoides (LC). Live vaccines made from attenuated cultures have shown better results than dead vaccines with M. agalactiae infection, but are not acceptable in all countries.

A. DIAGNOSTIC TECHNIQUES

Contagious agalactia is a disease syndrome of goats and sheep manifesting as mastitis, arthritis and keratoconjunctivitis, that has been known for over 170 years. It occurs mainly in Europe, Western Asia and North Africa, and is caused by Mycoplasma agalactiae (4, 6). In recent years M. capricolum and M. mycoides subsp. mycoides (LC) have also been isolated from mastitis and arthritis in goats and sheep in many countries. The clinical signs in these infections were sufficiently similar to those of contagious agalactia to lead Perreau (28) to suggest that these infections could also be considered as contagious agalactia. For this reason all three organisms will be included in the present discussion. In addition, very recent cases of mastitis and arthritis in goats associated with M. putrefaciens (10) have brought to notice a further condition with characteristics similar to those of contagious agalactia. Whether regulatory authorities will prefer to preserve the name for the disease caused by M. agalactiae or whether the conditions caused by these other mycoplasmas will be included in "contagious agalactia" remains to be determined.

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, lameness and, in some
animals, keratoconjunctivitis (8). Pregnant does may abort. *M. agalactiae* may also be found in lesions of vulvo-vaginitis in goats (36) and in lung lesions, but pneumonia is not a consistent finding. Bacteraemia is common 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 *M. mycoides* subsp. *mycoides* (LC), which is widely distributed, occurring on all the continents, including some countries to which *M. agalactiae* has not spread or where it occurs rarely (2, 9, 28, 33). With few exceptions (27) it is confined to goats. Usually cases occur sporadically but the disease may smoulder and slowly spread within a herd. After parturition the opportunity for spread in does being milked increases and kids ingesting infected colostrum and milk become infected. The resulting septicaemia with arthritis and pneumonia gives rise to high mortality in the kids (9, 11).

*M. capricolum* has occurred in geographically diverse situations (Africa, Australia, Europe and USA) but the frequency of occurrence is low (6, 28). Goats are more commonly affected than sheep and clinical signs of fever, septicaemia, mastitis and severe arthritis may be followed rapidly by death (6, 8). Pneumonia may be seen at necropsy. The severe joint lesions (8) seen in experimental infections with this organism are accompanied by intense periarticular subcutaneous oedema affecting tissues to some distance from the joint. The genital lesions seen in sheep in the United Kingdom (19) are unusual.

There are few records of natural infection in goats with *M. putrefaciens*, and none so far in sheep. Perreau (28, 29) commented that *M. putrefaciens* infection in France was associated with mastitis and agalactia, but not usually with arthritis or ocular lesions. Quite recently in California, a large outbreak of mastitis and agalactia in goats was apparently caused by a pure infection with *M. putrefaciens* (10). There was a high rate of abortion in does, followed by death. Severe arthritis later became a prominent sign in does and kids although at no time was pyrexia seen in infected goats (10).

1. **Identification of the agents**

The usual techniques used in the isolation of mycoplasmas apply to all four organisms under discussion. Media containing heart infusion broth, yeast extract (1-2%), and horse or pig serum (15-20%), with bacterial inhibitors, ampicillin (150 mg/litre) and thallous acetate (250 mg/litre) will be adequate to isolate and grow *M. agalactiae*, *M. capricolum*, *M. mycoides* subsp. *mycoides* (LC) and *M. putrefaciens* (7, 24). However, thallous acetate has not been used in some countries because it inhibits the growth of certain mycoplasmas. Sites of choice for culture are, in the living animal, milk, blood, aspirated joint fluid and nasal exudate, and at necropsy, udder and associated lymph nodes, joint fluid and lung lesions. The organisms may be isolated from liver, kidney and spleen when bacteraemia is present. Material obtained aseptically is sown to liquid, and plated on solid media. The solid medium is incubated in a moist atmosphere at 37°C in 5% CO₂ or in a candle jar (3% CO₂). Liquid medium is incubated at 37°C and plated on solid medium when growth is evident, or at 5-7 days even if growth cannot be seen. All
four mycoplasmas grow well with typical fried-egg colonies, those of *M. capricolum* and *M. mycoides* subsp. *mycoides* (LC) becoming large (up to 2 mm diameter) in 3-4 days, depending on growth conditions.

Before proceeding further with the cultures they should be purified. From well separated colonies single representatives are picked off into liquid medium and incubated. When grown, the culture may be filtered through membranes of 0.22-0.45 μm pore size and the filtrate plated to solid medium and incubated. This process is repeated twice more (37).

The colony appearance is useful only to the experienced eye, but selected biochemical tests (7, 12) can help with initial screening.

a) Biochemical tests

Growth in liquid medium containing 1% glucose, 0.2% arginine, 0.01% phenolphthalein diphosphate, on solid medium containing horse serum or egg yolk for demonstration of film and spots, and on casein agar or coagulated serum agar to test for proteolysis, are amongst the most useful tests for differentiating among the four mycoplasmas (7).

The characteristics of the four mycoplasmas are:

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<td>G*</td>
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<tr>
<td><em>M. agalactiae</em></td>
<td></td>
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<tr>
<td><em>M. capricolum</em></td>
<td>+</td>
</tr>
<tr>
<td><em>M. mycoides</em> subsp. <em>mycoides</em> (LC)</td>
<td>+</td>
</tr>
<tr>
<td><em>M. putrefaciens</em></td>
<td>+</td>
</tr>
</tbody>
</table>

*G = catabolism of glucose, A = arginine hydrolysis, F = production of surface film and spots, P = presence of phosphatase, C = digestion of casein, S = liquefaction of coagulated serum, O = odour of putrefaction in cultures.*

With the testing of more strains of each species, variations in some of these results have been found. Some *M. agalactiae* (34) and *M. putrefaciens* strains (Cottew, unpublished data) have failed to produce film and spots. Some strains, otherwise resembling *M. mycoides* subsp. *mycoides* (LC), have not liquefied coagulated serum (18). *M. capricolum* strains may be slow to hydrolyse arginine, requiring prolonged incubation (30; Cottew, unpublished data) or passages in the medium to effect this. Amongst the four species, the odour of putrefaction in cultures is peculiar to *M. putrefaciens*. Despite these limitations the biochemical characteristics can narrow the range of possibilities, but serological tests are required for precise identification.

b) Serological identification

Identification of isolates using specific antisera is usually carried out with the growth inhibition (GI) test (5) and the indirect fluorescent antibody (IFA) test (12, 24).
In the GI test, diluted liquid cultures are seeded to solid medium and allowed to be absorbed. For this, a streak culture using the "running drop" method gives good results. Filter paper discs, each saturated with a specific antiserum and allowed to dry, are placed on the agar surface. Stronger reactions are obtained if the antisera are added to wells (4 mm diameter) made in the agar. Plates are incubated and examined. With suitable homologous antiserum, a zone of up to 7 or 8 mm in width around the disc, or well, will be free of growth (complete inhibition). Partial inhibition can occur with weak antisera.

In the IFA test specific antisera are applied to colonies on growth medium. Homologous antiserum remains attached after washing and is demonstrated by adding fluorescein-conjugated antiglobulin, washing and viewing the colonies in an epi-fluorescence microscope (12, 24).

The growth precipitin test (13) is also used. This reaction detects antigens diffusing from growing culture and is regarded as group specific rather than species specific (12).

Antiserum for these serological tests has traditionally been prepared against the type strains of the various *Mycoplasma* species, and with this most field isolates have been readily identified. However, as more strains have been examined, some have been found to react poorly with these antisera, while reacting well with antisera to other representative strains of the species. Intraspecific variation in antigenic composition has not been reported for *M. putrefaciens* but occurs to some degree with *M. agalactiae* (34) and, in the author's experience, not uncommonly with *M. capricolum* strains. Thus, diagnosticians may need to have available more than one antiserum to enable all strains of a species to be identified.

Sharing of antigens between species or subspecies can make the allocation of an isolate to a species difficult, as is found in differentiation among strains of *M. capricolum*, F 38 and the bovine Group 7 mycoplasma (20). The difficulty of placing field isolates into a subspecies of *M. mycoides* is now well known (18). In the diagnostic laboratory, should the suggested use of several representative antisera fail to place an isolate in a species (or subspecies), help from a specialised laboratory could be sought. In such laboratories, work towards speciation of problem strains has made use of non-immunological techniques, such as the determination of the iso-enzyme composition (14) and more complex methods of examining the protein and DNA content of the mycoplasmas, and the use of DNA probes (12), but these techniques are not ready for routine use in a diagnostic laboratory.

2. **Serological tests**

a) **Complement fixation**

Perreau *et al.* (31) reported a standard complement fixation (CF) test that has also been applied to other mycoplasmas involved in the contagious agalactia
syndrome (25). 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 one hour, and the test is carried out in microtitre plates with overnight fixation in the cold, followed by addition of the haemolytic system and reading of the test 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*, *M. capricolum*, and *M. mycoides* subsp. *mycoides* (LC). It is regarded as a herd test and at least 10 sera are tested from each herd, preferably from acute and convalescent cases.

Some sera from healthy flocks react in the CF test with *M. agalactiae* up to a serum dilution of 1/20 but rarely react with the other two antigens (25). 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.

b) Growth inhibition

Sera from infected animals, when mixed with cultures of *M. agalactiae* may inhibit the growth of the organism, and this finding was used to develop a diagnostic growth inhibition test in Turkey (1). Le Goff and Perreau (25) also used a growth inhibition test and found that the most reliable serological diagnostic method was the combined use of the CF test (31) and the growth inhibition test.

c) ELISA

An enzyme-linked immunosorbent assay (ELISA) for contagious agalactia was described some years ago (35). The version of this test described by Lambert and Cabasse (23) uses only one dilution of each test serum (1/100) and a reference serum is included on each plate. The optical density reading of each sample is converted by a programmed computer to units of antibody by taking into account the optical density readings of the reference serum. The ELISA results correlate well with those of the CF test in current use (31) but the ELISA is more sensitive. However, the ELISA test is not yet sufficiently developed to recommend to non-specialised laboratories.

Serological testing for *M. putrefaciens* has not been carried out to the same extent as that for the other mycoplasmas under discussion, but the tests mentioned are equally applicable.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Vaccines for the prevention of contagious agalactia due to *M. agalactiae* have been used in countries in Europe and western Asia. No single vaccine has been universally adopted and no standard methods of preparation and evaluation would apply to all that have been used.
1. **Inactivated vaccines for *M. agalactiae* infection**

In those countries where live vaccines for *M. agalactiae* are not acceptable, research has focussed on the use of killed organisms, usually with adjuvant. While some of these have given a degree of protection against clinical disease resulting from experimental challenge (15, 16), this type of vaccine is considered to lack efficacy in the field (26). It is, of course, possible that in some instances, the apparent lack of protection given by such a vaccine could have resulted from the animals concerned being infected with one of the other three mycoplasmas involved with the syndrome of contagious agalactia.

2. **Live attenuated vaccines for *M. agalactiae* infection**

Live attenuated vaccines prepared from *M. agalactiae* in Turkey produced better protection than dead vaccines in controlled experiments (15, 16); generalised infection and clinical signs were prevented but transient infection of the udder could occur after challenge. Lambs born to vaccinated ewes were protected. This vaccine has been used successfully in the field in Turkey (Arisoy, personal communication) but it has not been adopted in Europe. However, Lambert (22) in France, reported recently that studies on a live attenuated vaccine gave "considerable but not total, protection" against challenge and also showed no reversion to virulence during or after 10 passages in sheep.

The recent thorough review of contagious agalactia by Lambert (21) has summarised important aspects of the application of dead and live vaccines. 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 make contact are vaccinated at the same time.

3. ***M. mycoides* subsp. *mycoides* (LC)**

In Israel, an inactivated vaccine protected kids against infection with *M. mycoides* subsp. *mycoides* (LC) resulting from ingestion of infected milk, and from subcutaneous challenge (3).

4. ***M. capricolum* and *M. putrefaciens***

Although infections with *M. capricolum* 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.

**General comment**

Eventual prevention of contagious agalactia by vaccination may be possible, but is complex. In sheep the condition is almost always due to the single mycoplasma, *M. agalactiae*, but in goats any of four mycoplasmas may be involved. Further, should the antigenic variation that occurs with two of these be associated with the protective antigens, then the vaccine may have to contain several representative strains of each species. As has been discussed, effective vaccines have already been
described for *M. agalactiae* and *M. mycoides* subsp. *mycoides* (LC) but the former is a live attenuated culture which may not be acceptable in some countries. The prevalence of these conditions varies among countries, and these differences would affect decisions on whether large scale, regional or local vaccination should take place. There is much to be done before vaccination will be widely used in prevention of contagious agalactia. Therefore, it is not possible at this stage to establish the standard requirements which would be universally acceptable to all concerned.

**Control**

While a discussion on control of contagious agalactia is not called for in this paper, it is of value to draw attention to the informative accounts by Gaillard-Perrin and Lenfant (17) and Picavet (32) on control measures and their application in the field in the absence of specific prophylaxis by vaccination.

**REFERENCES**


SUMMARY

The causal agent of classical, acute contagious caprine pleuropneumonia (CCPP) is a mycoplasma known at present as the F38 biotype. The F38 biotype is closely related to three other caprine mycoplasmas: M. mycoides subsp. mycoides, M. mycoides subsp. capri and M. capricolum. All three mycoplasmas may confuse the diagnosis of CCPP, both because the diseases they produce can resemble the disease caused by the F38 biotype and because they share several serological and biochemical characteristics with the F38 biotype.

Respiratory disease in goats may also involve Pasteurella haemolytica, Pasteurella multocida, Mycoplasma ovipneumoniae and Mycoplasma agalactiae.

Identification of the agent: Diagnosis of the disease is assisted by microscopical examination of lung material for evidence of the presence of F38 biotype, but definitive diagnosis requires culture of the causative organism from lung tissue samples taken at post-mortem. After purification, isolates can be identified by the use of several biochemical and immunological tests.

Serological tests: Serological tests have not been widely applied for identifying the cause of pleuropneumonia in goats and 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 in the diagnosis of CCPP. Indirect haemagglutination is also used, and other serological methods, including ELISA, are being developed.

Requirements for biological products: No vaccines against CCPP are commercially available at present.

A. DIAGNOSTIC TECHNIQUES

Contagious caprine pleuropneumonia (CCPP) is a major cause of loss in at least thirty countries in Africa and Asia containing a total goat population of more than 250 million. Classical, acute CCPP is now considered to be caused by a mycoplasma known as the F38 biotype (15). This organism was first isolated and shown to cause CCPP in Kenya (17), and subsequently it has been isolated in Sudan, Tunisia, Oman, Turkey and Chad. Disease indistinguishable from naturally-occurring CCPP has been experimentally reproduced with F38 biotype organisms by several groups of workers.
The diagnosis of outbreaks of respiratory disease in goats, and of CCPP in particular, is complicated by two aspects, one clinico-pathological and the other taxonomic. Pleuropneumonic disease resembling F38 biotype-associated CCPP can also be produced by Mycoplasma mycoides subsp. capri (Mmc) and caprine variants of M. mycoides subsp. mycoides (Mmm). Mmc was originally considered to be the cause of CCPP, but its full importance as a pathogen of goats has now become doubtful, both because of the discovery of the F38 biotype and because many isolates previously classified as Mmc have subsequently been found to be caprine variants of Mmm. Mmc has been recorded from several countries in Africa and Asia, and from Australia. The disease reproduced experimentally with Mmc is largely restricted to the thoracic cavity, with or without a septicaemic phase and death. In contrast, caprine variants of Mmm generally cause a syndrome which may include not only pleuropneumonia but also mastitis, polyarthritis, keratoconjunctivitis, acute septicaemic death, sometimes with symptoms of the central nervous system, and abortion. Mmm is a major cause of disease in goats in the USA, France, Israel and India. Experimentally, the disease caused by the F38 biotype differs from that produced by Mmc and Mmm in: being readily contagious to susceptible goats; not affecting sheep or cattle; not producing local oedematous reactions when injected subcutaneously; and being characterised histopathologically by an interstitial, intralobular oedema of the lung, compared with the thickening of the interlobular septa which is seen with Mmc and Mmm (12).

Pasteurella haemolytica (both biotypes A and T) and P. multocida have also been associated with pleuropneumonia in goats, although experimental evidence of their pathogenicity in this host is meagre.

The taxonomy and identification of caprine mycoplasmas are also particularly difficult and confusing. The F38 biotype, Mmm (both caprine and bovine variants) and Mmc all share serological and/or genetic relationships with each other and with two other mycoplasmas, M. capricolum and an unnamed group of isolates from cattle known as Leach's bovine group 7. These six species, serotypes or biotypes have become known as the "M. mycoides cluster" or "group" (3, 5). The F38 biotype and M. capricolum are particularly closely related genetically, and it has been suggested that they form two subspecies of the same species or taxon (3). However, in most other respects M. capricolum differs considerably from the F38 biotype in being a widely distributed pathogen of goats that causes principally sporadic arthritis and mastitis. M. capricolum may also produce a pneumonia in kids which does not resemble CCPP.

The caprine and bovine variants of Mmm are serologically indistinguishable from each other, but they differ in a variety of other respects. One notable difference is that caprine variants generally, but not invariably, produce larger colonies on agar; they have thus become known as "large colony" (LC) and "small colony" (SC) forms, respectively. More fundamentally, the protein composition of MmmLC more closely resembles that of Mmc than that of MmmSC (3). It has therefore been proposed that MmmLC and Mmc should together form a taxon or species separate from MmmSC.
1. Identification of the agent

a) Microscopy of lung exudates, impression smears or sections

The F38 biotype shows a branching filamentous morphology in vivo which can be observed by dark-field microscopy in exudates or tissue suspensions from lesions, or pleuritic fluid. Alternatively, smears made from cut lung lesions can be stained by the method of May-Grünwald-Giemsa and examined by light microscopy. The other caprine mycoplasmas show a short filamentous or coccobacillary morphology. Neither of these techniques provides definitive diagnosis, but application of the fluorescent antibody test to lung tissue sections (21) should provide specific identification of the causative agent.

b) Gel precipitin tests to detect antigen in tissue specimens

The gel precipitin tests are based on the precipitation in agar by specific hyperimmune serum of soluble antigens produced by the mycoplasma and present in affected lung tissue. Various forms of the test have been used in the diagnosis of contagious bovine pleuropneumonia (CBPP), which have also proven effective in the diagnosis of MmmLC infection in sheep (23). The predominant antigen detected in Mmm infection is a polysaccharide, galactan.

The strong cross-reactions demonstrated between MmmSC, MmmLC, Mmc and the F38 biotype using this test (16) indicate that it would not identify the causative agent in cases of CCCP. F38 also releases an antigenic polysaccharide. Nevertheless, this test can be valuable for identifying the presence of the mycoides cluster, particularly when specimens are no longer suitable for culture owing to delay in transit.

c) Isolation of mycoplasmas

i) Selection of samples

The best necropsy samples are lung lesions, particularly from the interface between consolidated and unconsolidated areas, and pleuritic fluid.

If microbiological examination cannot be performed immediately, samples or whole lungs can be stored deep-frozen at -20°C for considerable periods (months) with little apparent loss in mycoplasma viability. The transport of samples should always aim at keeping them as cool as possible, since mycoplasma viability diminishes rapidly with increasing temperature. Lung samples can be dispatched to other laboratories in freeze-dried form, but freeze drying reduces the titre of many mycoplasma species including biotype F38.

Many mycoplasma species, including MmmLC, Mmc and M. capricolum, but not biotype F38, may commonly be harboured in the ear canal by healthy goats and in the mites occurring therein. Though of no diagnostic value, the finding is of interest epidemiologically. Infection with MmmLC
can be detected by the culture of blood samples taken during the acute, pyrexic phase of the disease (25).

ii) Treatment of samples

Swabs are suspended in 2-3 ml of culture medium. Tissue samples are best chopped with scissors, then shaken vigorously, or pulverised in medium (e.g. with the Stomacher 80, A.J. Seward, London) 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 desired a high quality bacteriological medium such as nutrient broth may be used to provide a suspension suitable for both forms of examination.

Pleuritic 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 media. Dilutions should also be plated on solid medium.

iii) Mycoplasma media

The medium used to culture F38 biotype organisms by MacOwan and Minette (17), termed "viande foie goat" (VFG), contained inter alia goat meat-liver broth and goat serum. Alternative suitable media are WJ (11), AC and TPM (10), modified Hayflick’s and Newing’s tryptose (13). WJ was found in one study to be superior to VFG and TPM (11). Its formulation is as follows: Autoclaved portion – Bacto PPLO broth without crystal violet (Difco) 1.5 g; Bacto-Tryptose (Difco) 1.5 g; Bacto-Peptone (Difco) 0.3 g; Bacto-Yeast Extract (Difco) 0.1 g; deionised water 50 ml (agar medium 55 ml); agarose (agar medium only) 0.9 g. Membrane-filtered portion – newborn calf serum (inactivated at 56°C for 30 min) 45 ml (agar medium 40 ml); 10x Medium 199 without NaHCO₃, with glutamine (Gibco) 5 ml; fresh yeast extract 5 ml; calf thymus DNA (highly polymerised; 0.2% w/v) 1 ml; glucose (50% w/v) 0.5 ml; NADH (10% w/v) 0.1 ml; ampicillin (100 mg/ml) 0.25 ml; thallium acetate (10% w/v) 0.25 ml; phenol red (0.4% w/v) 1.5 ml. The medium is adjusted to pH 7.6-7.8 with 1 M NaOH.

MrmmLC, Mmc and M. capricolum readily grow in WJ medium, but M. ovipneumoniae grows better in less highly enriched media such as OB (11) or modified Friis (7). A mycoplasma diagnostic system should also include a broth medium adapted for the culture of arginine-hydrolysing mycoplasmas, of which two recognised species occur in goats and sheep, namely, M. capricolum and M. arginini. Adjustment of any of the media referred to above to pH 7.0, and supplementation with arginine (generally L-arginine hydrochloride to a final concentration of 1% w/v) specifically enhances the growth of arginine-hydrolysing mycoplasmas in a culture, which is visualised by an alkaline shift of the phenol red indicator.
iv) Media 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 in 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 screwcapped 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 used as fresh as possible and stored for no more than 2 weeks at 4°C before use.

v) Cultivation

Cultures are incubated at 37°C. Plates are best incubated in a humidified 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, namely, 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 to 3 days using a stereo microscope (5 to 50 x magnification) and transmitted and incident light sources. 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 culture.

In early passage, many mycoplasma species – including the F38 biotype – produce colonies of bizarre morphology, often small, centreless and of irregular shape. This effect is often associated with the use of marginally suitable medium and it is interesting that it has never been observed with VFG medium (16). With passage, such isolates demonstrate conventional "fried egg" colony morphology, except M. ovipneumoniae, which retains centreless colonies. Colonies of MmmLC and M. capricolum may be up to 3 mm in diameter.
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 3-5 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. F38 biotype organisms in early passage often do not produce marked change in the pH of WJ broth.

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, being expressed as colour changing units (CCU) per transfer volume. Growth on plates is expressed as colony-forming units (CFU) per ml.

d) Identification of mycoplasmas

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

i) Biochemical tests

Biochemical tests cannot unequivocally identify an isolate, which at present can only be done by serological or genetic means. Intraspecific variation in some biochemical reactions is often considerable, but some tests do 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 tetrazolium chloride (aerobically and anaerobically), phosphatase activity, serum digestion and digitonin sensitivity. The reactions in these tests of the twelve recognised mycoplasma species (excluding ureaplasmas) which occur in goats and sheep are shown in Table 1.

The first three of these tests are performed routinely in isolation and cultivation procedures. Glucose breakdown is indicated by acid (yellow) change and arginine hydrolysis by alkaline (red) change in broth media, using phenol red as indicator. "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 aging 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 tetrazolium reduction provides corroborative evidence of the mycoplasmal nature of *M. agalactiae* isolates, since this organism is neither glycolytic nor arginine-hydrolysing. Serum digestion (6) distinguishes members of the "M. mycoides cluster" from other small ruminant mycoplasmas, and phosphatase production (1) separates *M. capricolum* from other members of this cluster. Digitonin sensitivity distinguishes members of the order *Mycoplasmatales* from those of the order *Acholeplasmatales* (8).

ii) Serological identification

Mycoplasmal antigens used in hyperimmune serum production are invariably 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 with homologous animal components, e.g. growth in VFG medium to immunise a goat.

### TABLE 1

**Biochemical reactions of the mycoplasmas of goats and sheep**

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<thead>
<tr>
<th>Test</th>
<th>F38</th>
<th>MmmLC</th>
<th>Mmc</th>
<th>Mc-c</th>
<th>M.oovi</th>
<th>M.arg</th>
<th>M.agal</th>
<th>M.conj</th>
<th>M.putr</th>
<th>A.laid</th>
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<td>Glucose fermentation</td>
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<td>Digitonin sensitivity</td>
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**Key:**

- MmmLC: *M. mycoides subsp. mycoides* (large colony type)
- Mmc: *M. mycoides subsp. capri*
- Mc-c: *M. capricolum*
- M.oovi: *M. ovipneumoniae*
- M.arg: *M. arginini*
- M.agal: *M. agalactiae*
- M.conj: *M. conjunctivae*
- M.putr: *M. putrefaciens*
- A.laid: *Acholeplasma laidlawii*
- A.gran: *A. granularum*
- A. ocul: *A. oculi*
- W: Weak reaction
- ND: Not done
- OTC: Ornithine transcarbamylase presence
- *: Small colony strains of Mmm (both bovine and caprine origin) and bovine group 7 (PG 50) are negative in the OTC test
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.

*a) Growth inhibition test (GIT)*

The GIT is the simplest and most specific but least sensitive of the tests available. It depends on the direct inhibition of growth on solid media by specific hyperimmune serum, and detects primarily surface (membrane) antigens (4).

The GIT is particularly useful in identifying F38 biotype and *M. ovipneumoniae* isolates. The former 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 (11). F38 biotype cross-reacts with Leach's bovine group 7 (PG50), *M. equigenitalium* and *M. primatum* in the GIT when polyclonal antisera are used, but a monoclonal antibody specific for F38 biotype in the GIT has been produced (30). A small proportion of F38 biotype isolates also cross-react in the GIT with antiserum to *M. capricolum*.

*M. ovipneumoniae, M. capricolum* and the *M. mycoides* subspecies are serologically heterogeneous to a greater or lesser degree, and pooled antisera against several strains of each organism should be used for the identification of field isolates by the GIT. Because *M. ovipneumoniae* produces centreless colonies that are readily washed off agar, the fluorescent antibody test cannot be used, and the GIT is thus the identification method of choice for this organism.

**Procedures for the GIT:**

1. 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.

2. Agar plates are dried for 30 min at 37°C.

3. Sterile paper disks of 6-7 mm diameter are impregnated with a drop (10 to 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 (4), which allows storage at 4°C.

4. Using a separate plate for each dilution of culture, 1 ml or 2.5 ml volumes are pipetted onto 5 cm or 10 cm diameter plates, respectively. The inoculum is dispersed evenly over the plate, then the excess removed.

5. The plates are dried at 20-25°C for 15-20 min, 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.

6. 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 F38 biotype, *MmnLC*, *Mmc*, *M. capricolum* and *M. ovipneumoniae*. A disk containing 1.5% digitonin should also be included on the plates.

7. 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.

b) *Growth precipitation test (GPT)*

The GPT detects soluble cytoplasmic and extramembranous antigens released by growing cultures and allowed to diffuse through solid mycoplasma growth medium towards mycoplasma antiserum during growth (14). As with the gel precipitin test, there are strong cross-reactions within the mycoides cluster (16).

c) *Indirect fluorescent antibody test (IFAT)*

The direct and indirect FATs are the most effective of the various serological methods of identification for most mycoplasmas (26). They are simple, rapid, sensitive, yet economical in the use of antiserum. Several forms have been described, the most commonly used and perhaps best being the indirect FAT (IFAT) 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.

The IFAT is almost identical to the GIT in its specificity for the different members of the "M. mycoides cluster".

Procedures for the IFAT:

1. Two agar plates, pre-dried at 37°C for 30 min, are each flooded with a different dilution of test broth culture, the dilutions being selected
according to the vigour of growth of the strain on agar medium. Alternatively, a drop of undiluted culture is spread over a single 5 cm plate using an L-shaped glass rod.

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

3. Several blocks of approximately 0.5 to 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 placed colony-side up on different slides, each slide being used for a different mycoplasma antiserum. The colony side is identified for future reference by undercutting one corner.

4. Rabbit anti-mycoplasma serum (ra-m) or normal rabbit serum (NRS) at a suitable dilution in normal saline or phosphate buffered saline pH 7.2 (PBS) is gently pipetted onto each agar block until the surface area is totally covered. The optimal dilution of ra-m is determined by checkerboard titration against the fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin serum (a-r Ig-FITC) used.

5. The flooded blocks on their slides are incubated at room temperature for 30 min in a humid box.

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

7. The plugged tubes are rotated at 18-30 rpm for 10 min, then the PBS is decanted and replaced with fresh PBS and the tubes are again rotated for 10 min.

8. The PBS is decanted and the blocks placed colony side up on their respective slides. Excess moisture is blotted off.

9. All blocks are flooded with a-r Ig-FITC at optimal dilution.

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

11. The blocks, replaced colony-side upward on their respective slides, are examined by epi-immunofluorescent microscope using the settings recommended by the manufacturer for FITC.
Notes on the IFAT

1. Working dilutions of ra-m and a-r Ig-FITC should be kept at 4°C, which limits useful life to about one week.

2. Isolates from CCPP should be examined using antisera against F38 biotype, MmmLC, Mcm and M. capricolum; positive control cultures should comprise their type strains, namely, F38, Y goat, PG3 and California kid, respectively.

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

4. Interpretation of the IFAT 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 M. capricolum. Otherwise, poor results are usually ascribable to the use of agar cultures that have been allowed to grow for too long, or to the use of antiserum that has deteriorated with dilution and age.

iii) Other identification tests

Metabolism inhibition (16, 32) and tetrazolium reduction inhibition (31) are other tests sometimes used in the identification of caprine mycoplasmas.

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 Mcm can produce a background of positive titres to these organisms in a proportion of apparently healthy animals (11), and under experimental conditions seroconversion to M. mycoides can occur in goats without disease expression. Acute cases caused by the F38 biotype rarely show positive titres to the organism before death (19, 22), perhaps because antibodies are "eclipsed" by circulating mycoplasma antigens (22). Seroconversion to the F38 biotype in experimentally infected animals is observed by CFT and IHAT to start 7-9 days after the appearance of clinical signs, to peak between days 22 to 30, and to decline rapidly thereafter (22). 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 to 8 weeks apart should be examined.

a) Complement fixation test (CFT)

The CFT in various forms remains the most widely used serological test for the diagnosis of contagious bovine pleuropneumonia (9, 24). In CCPP, the CFT was used for F38 infection (16, 18) and it has been found to be more specific, though less sensitive, than the IHAT (22). Its main disadvantage is the high
### Table 2

**Suggested mycoplasmological procedures for the diagnosis of CCPP**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>i) Phase-contrast or dark-field microscopy of exudates</td>
<td>LUNG TISSUE (or PLEURITIC FLUID) (Precipitin test)</td>
</tr>
<tr>
<td>ii) Giemsa stain of lung impression smear</td>
<td>Homogenisation/suspension in nutrient broth</td>
</tr>
<tr>
<td>iii) Fluorescent antibody test on lung sections</td>
<td>Bacteriological (and virological) examination</td>
</tr>
</tbody>
</table>

**Broth media**

At least 3 tenfold dilutions in broth media

- i) If no colour change by 7 days, make blind subculture
- ii) Filter (0.45 μm) if bacterial contamination present.

**Cloned culture**

- Biochemical tests
  - Glucose breakdown
  - Arginine hydrolysis
  - "Film and spots"
  - Phosphatase activity
  - Proteolytic activity
  - Digitonin sensitivity
  - (Tetrazolium reduction)
  - (OTC presence)

**Typical Mycoplasma colonies**

- Serological tests
  - GIT
  - IFAT
  - (GPT)
  - (GIT/GPT)
  - (MIT)

**Atypical colonies**

- Agar medium without inhibitors

Parentheses indicate a less commonly used sample or technique.

* Either WJ, VFG, AC or TPM; a broth medium supplemented with arginine should also be used.
level of technical expertise required to perform the test (9).

One method for performing the test is as follows. To prepare the antigen, 2 litres of culture of titre higher than $10^9$ CFU/ml is centrifuged at 40,000 g for one hour at 5°C. The deposit is resuspended and washed 3 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 second control antigen is freeze dried broth reconstituted at 200 mg/ml. Prior to testing the antigen is diluted 1:60 and ultrasonicated for 3 min at low power in a container of iced water. The sonicate is centrifuged at 3,000 rpm for 30 min to remove any debris, and stored at -20°C. If stored for more than 2 or 3 weeks the antigen should be recentrifuged.

Microtitre plate tests are performed using 0.025 ml volumes, 2 volumes containing 3 mean haemolytic doses of complement and a 1.5% (v/v) final concentration of sheep red blood cells.

b) Indirect haemagglutination test (IHAT)

The IHAT (2) has been used for diagnosis of CCPP (22).

The IHAT is most commonly performed with erythrocytes which are either fresh and tanned, or treated with glutaraldehyde. The former is more sensitive but shows greater variability between tests, and requires sensitization of cells with antigen each time the test is performed. Glutaraldehyde treatment of erythrocytes reduces sensitivity but produces a much more useful diagnostic test, since sensitised erythrocytes remain effective for a year or more if kept refrigerated, and require little further manipulation before use in the test.

The specificity of the IHAT for the "M. mycoides cluster" has been evaluated using rabbit hyperimmune sera and glutaraldehyde treated erythrocytes sensitised with lightly sonicated mycoplasma cell suspensions (11). MmmLC and strain F38 sensitised cells cross-reacted with antisera to the other three species, but Mmc and M. capricolum-sensitised cells each cross-reacted only with the antiserum to strain F38.

The polysaccharide produced by the F38 biotype has been found to bind to untreated goat erythrocytes, and as such was successfully used in an IHAT to identify animals with experimental and natural CCPP (29).

Cells sensitised with the four principal caprine mycoplasmas have been used in field studies in Oman and Sudan. Conclusions from the two surveys differed. The Oman survey revealed widespread seropositivity to Mmc, whereas strain F38 reactors were largely confined to herds in which the F38 biotype had been shown by cultural means to occur (11). In contrast, more animals with signs of CCPP in Sudan were seropositive to MmmLC than to any of the other mycoplasmas tested, only 7% reacting with strain F38 antigen. Seropositivity to two or more antigens was noted in a proportion of animals in both surveys.
c) **Latex agglutination test (LAT)**

Latex beads sensitised with the polysaccharide produced by F38 biotype and present in culture supernatant have been used in a slide agglutination test (27). The test is simple, rapid and can be performed with undiluted serum or whole blood. The LAT proved to be more sensitive than the CFT in one study (27).

Both CFT and IHAT findings emphasize the difficulties inherent in the serological diagnosis of CCPP when using whole cell or membrane preparations as antigen. The use of more defined antigens – for example, the different polysaccharides elaborated by *MmmyLC*, *Mmnc* and the F38 biotype – may provide greater specificity, but serological diagnosis of CCPP should always use antigens of all four of the principal caprine mycoplasmas.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

The first experimental vaccine against F38 biotype comprised live strain F38 in high passage (20). 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 strain F38 vaccines have been in use for several years) contains lyophilised strain F38 suspended in saponin; this formulation gives a shelf-life of at least 14 months. The optimal dose of 0.15 mg of mycoplasma provides protection for more than one year (28).

**REFERENCES**


ENZOOTIC ABORTION OF EWES
(OVINE CHLAMYDIOSIS)
(B28)

SUMMARY

The diagnosis of enzootic abortion, also known as ovine chlamydiosis or chlamydial abortion of ewes, depends on the demonstration or, possibly, isolation of Chlamydia psittaci in the products of abortion or vaginal excretions of freshly aborted ewes. A humoral antibody response may be detected after abortion. Goats as well as sheep can be affected. C. psittaci of small ruminants is a zoonotic organism which must be handled with care. Pregnant women are particularly susceptible.

Identification of the agent: The basis for a positive diagnosis of infection with C. psittaci 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 ewes 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. psittaci and Coxiella burnetii, the agent of Q fever.

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

Serological tests: A rise in antibody titre to C. psittaci, detected by complement fixation (CF), is common after abortion or stillbirth but this does not occur in every case. C. psittaci shares common antigens with some Gram-negative bacteria, so that the CF test is not wholly specific, nor does it distinguish between responses to vaccination or 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 biological products: Inactivated vaccines are available that reduce the incidence of abortion, but do not prevent infection. They assist in disease control but will not eradicate it. Serological screening during the period after lambing helps to identify infected flocks, to which control measures can then be applied.
A. DIAGNOSTIC TECHNIQUES

*Chlamydia psittaci* causes enzootic abortion in ewes and has a zoonotic potential, and must therefore be handled with adequate microbiological containment.

Infected ewes shed vast numbers of infective chlamydiae at the time of abortion or parturition, particularly in the placenta and uterine discharges. Human infection may be acquired from these sources or from carelessly handled laboratory cultures of the organism, with effects that range from subclinical infection to acute influenza-like illness. Recent authenticated cases of human placentitis and abortion caused by *C. psittaci* of ovine origin indicate that pregnant women are at special risk and should not be exposed to sources of infection (3).

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 (11). In positive cases stained by the latter method and examined under high power microscopy, 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 ewes that have aborted within the previous 24 hours or from the moist fleece of a freshly aborted or stillborn lamb. In general, such preparations contain fewer organisms than placental smears.

In morphology and staining characteristics, *C. psittaci* resembles the rickettsia *Coxiella burnetii* which causes Q fever of animals and may provoke abortion. 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. psittaci* and *C. burnetii* can be detected serologically. Immunofluorescent tests using specific antiserum or monoclonal antibody may be used for identification of *C. psittaci* in smears.

b) 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 (4) is rapid and simple.
c) Isolation

*C. psittaci* can be isolated in embryonated chicken eggs or in cell cultures, the latter being the method of choice for isolation of new strains.

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 are commenced, should be maintained in a suitable transport medium in the interim. The most satisfactory medium is sucrose-phosphate-glutamate medium supplemented with 10% fetal calf serum, antibiotic (streptomycin and gentamycin are suitable, but not penicillin) and a fungal inhibitor. 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.

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

**Cell cultures:** *C. psittaci* of ovine origin can be isolated in a variety of cell types but McCoy, BEM or 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 coverslip. Confluent coverslip 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 2,500 g for 30 minutes onto the coverslip monolayer to promote infection. After further incubation for 2-3 days the coverslip monolayers are fixed in methanol and stained with Giemsa or after Giménez (2, 7). After methanol fixation, infected cultures contain basophilic (Giemsa) or eosinophilic (Giménez) intracytoplasmic inclusions. Similar procedures are used in culturing *C. psittaci* for antigen preparation. Immunofluorescence 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 coverslip monolayers are exposed include cycloheximide at 1 µg/ml in the maintenance medium (8), emetine at 1 µg/ml for 5 minutes before infection (9) and 5-iodo-2-deoxyuridine (1) at 80 µg/ml for 3 days prior to infection. Unless preconditioned cells are available, the latter isolation procedure requires increased time for successful isolation.
2. Serological tests

Complement fixation (CF) is the most widely used procedure. On a flock basis, the CF test will detect evidence of vaccination or infection. Infection is evident principally during active placental infection in the last month of gestation and following the chlamydiaemia that often accompanies abortion. Consequently, paired sera collected at the time of abortion and again three weeks later may reveal a rising CF antibody titre which will provide a basis for a retrospective diagnosis. Antigenic cross-reactivity between C. psittaci and 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 non-specific.

Antigen is prepared from heavily infected yolk sac membranes obtained from chick embryos which have been inoculated in the same manner as those used to isolate the organism from field material. 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 resuspended in a small volume of saline and a smear of this examined to ensure a high yield of chlamydiae. The suspension is held in a boiling water bath for 20 minutes or autoclaved, and sodium azide (to 3%) added as a preservative. Antigen may also be prepared from cell cultures infected with C. psittaci. Infected monolayers are suspended in phosphate buffer, pH 7.6, and the cells 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 standard complement and antisera will establish the optimal working dilution for each batch of antigen.

Other types of test applied to chlamydial serology include latex agglutination, passive haemagglutination and enzyme-linked immunosorbent assay (ELISA). As yet, none has been adequately standardised for universal application.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Inactivated vaccines can be prepared from infected yolk sacs (5) or cell cultures (12). A chemically-induced, temperature sensitive mutant derived from an ovine abortion strain of C. psittaci has been tested as live vaccine (10). No adverse effects have been observed in inoculated sheep or goats, and protection against experimental infection is good. These trials have not been completed, but there will be the need to exclude any risk of reversion to virulence or any potential risk of infection to man. Both humoral and cellular immunities are important.

1. Seed management

a) Characteristics

One or more ovine abortion isolates which 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 chick embryo
by multiple passage (>100) can be used. This permits more of the embryo to be used for vaccine production (6).

b) 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 that case the infectivity of an aliquot of each lot should be separately titrated to ensure that each matches the requirements (see 2, below). Store under refrigeration conditions.

2. Manufacture

The inactivated harvest is centrifuged and resuspended in phosphate buffered saline containing 0.2% formalin to a volume representing a pre-inactivation infectivity titre of approximately \(10^8\) infectious units/ml (12). Usually, the aqueous suspension is blended with an oil adjuvant, either directly (12) or after precipitation by potassium alum (5) \((\text{Al}_2\text{K}_2\text{SO}_4\cdot 12\text{H}_2\text{O})\). A preservative, such as 0.01% thiomersal, may be added also.

3. In-process control

The main requirements are to ensure adequate growth of \(C.\ psittaci\), 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 the chapter on General Information.

b) Safety

Subcutaneous inoculation into two or more sheep of a standard dose (usually 1.0 ml) of manufactured vaccine should elicit no systemic reaction, but
oil-adjuvant vaccines can cause a non-harmful 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 again 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 to 3 years, according to the exposure risk.

e) **Stability**

Vaccine stored under refrigeration (5±3°C) should remain stable for at least one year. Before use it should be held at room temperature for 24 hours and the container shaken vigorously immediately before vaccine is withdrawn.

**REFERENCES**


SUMMARY

Sheep pulmonary adenomatosis (SPA) is a contagious tumour of sheep and, exceptionally, of goats. Adult animals are usually affected. It causes an inevitably fatal progressive respiratory disease. 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 SPA is distinct from the non-oncogenic ovine lentivirus but related to type B and D retroviruses.

Identification of the agent: As the retrovirus of SPA cannot yet be propagated in vitro, no routine diagnostic method, such as virus isolation, is available for diagnosis. Diagnosis, therefore, has to rely on clinical history and examination, as well as on the findings at necropsy and by histopathology.

Regular inspections and prompt removal of suspected cases may help to limit the spread of infection within affected flocks. There is no available vaccine.

Serological tests: As the virus involved has yet to be cultured, there are no serological tests available for diagnosis.

Requirements for biological products: There are no biological products available.

A. DIAGNOSTIC TECHNIQUES

Sheep pulmonary adenomatosis (SPA), also known as jaagsiekte, is a contagious lung tumour of sheep and, possibly, of goats. It is the most common pulmonary tumour of sheep and occurs in many countries of the world, although it is absent from Australia and New Zealand.

Both a herpesvirus and a retrovirus are closely linked with SPA, but the former does not have an aetiological role in the disease. At present, diagnosis of SPA relies upon clinical and pathological investigations, and there is no vaccine against the disease. The present understanding of the possible viral aetiology of SPA is summarised below.

1. Identification of the agent

Herpesvirus: The consistent association between a herpesvirus and the SPA tumour tissue from which it is isolated probably stems from reactivation of a latent infection coupled with replication of the virus in the numerous alveolar macrophages that characterise cases of SPA. To date, ovine herpesviruses have been recovered only from SPA tumour tissues, and all isolates are antigenically
distinct from other members of the *Herpesviridae*, including herpesviruses of bovine origin. In accordance with the recommendations of the International Committee for the Taxonomy of Viruses, these viruses have been provisionally designated caprine herpesvirus 1 (2).

Experimental infection of young lambs with this virus results in a subclinical interstitial pneumonia, the pulmonary histiocyte being the main, and perhaps exclusive, site of the virus replication (3). These findings imply that the respiratory tract is a predilection site for the virus and account for the readiness with which it can be isolated from cases of SPA. On serological evidence, infection of sheep with the virus is widespread, even in countries where SPA does not occur, which implies that any relationship between the herpesvirus and SPA is coincidental and not causal (4).

**Retroviruses:** Implication of retroviruses with SPA has been recognised for several years. In some instances retroviruses isolated from SPA tumours have been propagated *in vitro* and shown to have properties that identify them as members of the non-oncogenic subfamily of ovine lentiviruses. Almost certainly, these isolates have no aetiological role in SPA. On the other hand, cumulative morphological, biochemical and immunological evidence now strongly indicates that the particular virus involved in SPA is related to retroviruses of types B and D (1, 4, 10, 11).

Tumour homogenates containing the SPA retrovirus and, more significantly, concentrated cell-free lung fluid from natural cases of SPA transmit the tumour. Following the experimental inoculation of adult sheep, clinical disease develops only after several months or years. Similar inoculation in newborn lambs results in a reduced incubation period of 3-6 weeks, and many of the transformed epithelial cells contain intracytoplasmic retrovirus particles (7, 9, 11). Furthermore, cell cultures prepared from the tumours occurring in young lambs can support virus replication (8). Following intratracheal inoculation of concentrated supernatant fluids of such cultures into three lambs within 24 hours of birth, clear histological evidence of SPA was detected in one of them six months later (5).

Future progress, as well as the development of routine laboratory tests for the early detection of infected animals, now depends on overcoming the present difficulties in culturing the SPA retrovirus. Alternative diagnostic approaches, using techniques of molecular biology to exploit the relationships between the SPA retrovirus and prototypes of types B and D retrovirus, are under active investigation.

**Clinical signs:** Since SPA 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 under one year old. 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 unique feature of SPA, giving rise to moist rales 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. This is a useful diagnostic aid in differentiating from maedi. Coughing and inappetance 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 one due to *Pasteurella haemolytica*.

Necropsy: In typical cases the main features are pulmonary enlargement, with areas of grey solid tumour in the ventral portion of the lungs being clearly demarcated from adjacent normal tissue. An associated increase in lung weight is common. Frothy fluid exudes from the trachea and also from bronchioles when grossly affected areas are incised. In the early stages of SPA only small individual tumour nodules may be present, often in the dorsal aspects of the diaphragmatic lobes, whilst advanced cases are often complicated by pleurisy and secondary bacterial pneumonia.

**Histopathology:** Three main features can be recognised: (i) continuous lining of alveoli by cuboidal or columnar cells, which often project into the alveoli as adenomatosis-like nodules, (ii) polyp-like epithelial cell ingrowths in the terminal bronchioles, and (iii) nodular or diffuse masses of myxomatous tissue in the tumour lesions. In addition, numerous large macrophages occur in the non-affected lung surrounding the tumour. The cells involved in the alveolar lesions are type II alveolar cells while the bronchiolar polyps are formed by the non-ciliated cells of Clara.

For more detailed accounts of the clinical, post-mortem and histopathological aspects of SPA, the reader is referred elsewhere (6, 11).

2. **Serological tests**

There are no serological tests available in the absence of the ability to grow the agent as a separate entity.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

There are no vaccines available at the present time.

In the absence of specific diagnostic tests, disease control relies upon regular flock inspections and prompt culling of suspected cases and their offspring.

**REFERENCES**


NAIROBI SHEEP DISEASE  
(B30)

SUMMARY

Nairobi sheep disease (NSD) is caused by a Nairovirus of the family Bunyaviridae. It should be suspected when a mortality rate which may reach 90% occurs in a sheep or goat population, especially when this follows movement into enzootic areas. The disease is characterised by pyrexia (41.5°C), collapse, and diarrhoea. 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.

Identification of the agent: Plasma from febrile animals, mesenteric lymph nodes or spleen are best for virus isolation. Laboratory-reared sheep, suckling mice aged 2-4 days inoculated intracerebrally, or cell cultures may be used for primary isolation. Sheep are the most sensitive animals for this, whereas a BHK cell line, and lamb or hamster kidney cell cultures are the most sensitive cells. Sub-inoculation 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. Infected tissue cultures or mouse brain suspensions may be used as sources of complement-fixing antigens. Infected spleen may also be used as a source of antigen in immunodiffusion tests.

Serological tests: Complement fixation, indirect fluorescent antibody and indirect haemagglutination tests have been used to conform NSD outbreaks in the field. Virus neutralisation tests give equivocal results, a feature which also occurs with other members of the Nairovirus group.

Requirements for biological products: An experimental attenuated live virus vaccine has been investigated, and a killed tissue culture vaccine has been shown to be immunogenic.

A. DIAGNOSTIC TECHNIQUES

Nairobi sheep disease (NSD) is a disease of sheep and goats caused by a Nairovirus of the family Bunyaviridae. It is characterised by a mortality rate which may range between 40-90%. It should always be suspected when animals have recently been moved from a area free of the disease into one where it is endemic. The virus is transmitted by the tick Rhipicephalus appendiculatus, and any infestation with such parasites should arouse suspicion of the presence of the disease.
1. **Identification of the agent**

NSD virus may be isolated from material collected from field cases by the use of laboratory animals or cell cultures. 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 as inoculum directly, 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 containing penicillin (500 IU/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. 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 10 times more sensitive than mice to NSD virus infection.

Infant mice, 2-4 days of age, can be inoculated intracerebrally with 0.01 ml of a 1:10 dilution of plasma or of the tissue suspension. Two litters should be used for each sample and 1 blind passage is made routinely. The mice become debilitated and die within 5-9 days after inoculation. Their brains should be harvested aseptically, pooled and diluted 1:100 for passage into further mice.

Cell cultures may be used for the primary isolation of NSD virus since they have shown levels of sensitivity similar to that of the intracerebral inoculation of suckling mice. The BHK21-C13 cell line is especially valuable, 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 sub-inoculation. 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. The cultures should be used both with and without flying coverslips. 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 coverslip cultures by immunofluorescence or by staining with haematoxylin and eosin. The latter method reveals an unusual intracytoplasmic 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 after 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 antisera 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 mouse brain suspensions or infective tissue culture fluids can be used as antigens for complement fixation tests for virus identification. Both have proved satisfactory after partial purification with fluorocarbon; or, the mouse brain can be used in the form of a suspension in a borate buffer solution.

2. **Serological tests**

Immunodiffusion, complement fixation, indirect fluorescent antibody tests, haemagglutination and enzyme-linked immunosorbent assay (ELISA) have all been used with NSD virus. Results of virus neutralisation tests are unreliable, as are those of the same tests on other members of the *Nairovirus* genus.

Indirect fluorescent antibody tests have been widely used. Antigen in the form of infected cells is prepared on flying coverslips, or in multiwell slides. Cultures of BHK or VERO cells are convenient for this purpose when a high passage strain of NSD virus is used as the inoculum. The cultures are fixed in acetone at 4°C when 10-20% of the cells show fluorescence: these may be stored at 4°C or -20°C for 6 months until required. Staining procedures follow standard methods for the indirect test using antispecies conjugates. Titres of 1:10,000 have been found in sheep sera in the immediate post-infection period.

The indirect haemagglutination test has been used in field survey work, but great care is necessary to produce a pure antigen from cell cultures with which to coat red blood cells. After 24 hours, the infected cultures are washed and serum-free medium added. The viral antigen is precipitated by the addition of 6% polyethyleneglycol and used at a dilution of 1:20 to coat a suspension of sheep red cells which have been previously treated with tannic acid (tanned). The test sera are absorbed before use with a suspension of 2% tanned sheep red cells which have not been coated with the antigen.

Complement fixation tests are complicated by the marked anti-complementary 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. This can be a useful test when care is taken to employ a pure antigen preparation.

The ELISA has not been used other than under experimental conditions and problems have been encountered with non-specific reactions given by test sera.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

Epidemiological investigations have shown that in a state of enzootic stability, no problems are encountered with NSD. The disease arises from animal movements into endemic areas and can be avoided when such areas have been defined. Ecological changes which 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 usage.

A tissue culture adapted strain of NSD virus has been grown to high titre in rolled culture. When precipitated with methanol, inactivated, and administered with an adjuvant, this was found to afford good protection following 2 inoculations given at an interval of 14 days.

REFERENCES


SUMMARY

The genus *Salmonella* contains only two species, *Salmonella enterica* and *S. bongori* (see the note on the next page, concerning the nomenclature of *Salmonella*). *S. enterica* is divided into six subspecies that include over 2,000 serovars. The salmonellae of all subspecies are pathogenic for man and many animal species, but a few serovars are adapted to a host species. In the case of those of subspecies I, which are the most frequent, the clinical signs, post-mortem findings and epidemiological patterns may vary according to the serovar involved. Most salmonellae are ubiquitous.

Some serovars are host-specific. *S. abortus ovis*, a sheep-adapted serovar, causes a contagious disease with abortion as the main symptom, sometimes accompanied by death of lambs. *S. abortus equi* has seldom been isolated from animals other than the Equidae. In addition to abortion, it has been associated with various other clinical conditions in horses, including death of the newborn, polyarthritis in foals, and orchitis. This chapter covers the salmonellae in general, but some details specific to *S. abortus ovis* and *S. abortus equi* (which appear on the OIE List B of diseases) are included.

Many serovars of salmonella have been shown to cause human food poisoning, although they may not be pathogenic in the animal from which the food was derived. (*S. typhimurium* and *S. enteritidis* are dealt with in the following chapter.)

Identification of the agent: Samples should be collected as aseptically as possible. The salmonellae are cultured selectively on a series of different types of media. These may be liquid or solid and permit differential growth of bacteria. Non-selective media often yield more rapidly growing colonies and can be used for obtaining pure cultures prior to further characterisation tests. Various biochemical and serological tests can be applied to the pure cultures, to provide a more definitive characterisation of the isolated strain.

The salmonellae possess antigens designated O, Vi and H which may be identified by the use of specific antisera. Reference laboratories are able to confirm the serological identity of an isolate.

Serological tests: Serological tests are preferably conducted on a representative sample of a population and not merely on one animal. However, serological tests for the diagnosis of salmonellosis are of little value, except in endemic infections. Paired samples may be of greater value.

Although there are various tests, those such as precipitation or complement
fixation do not present real advantages over agglutination reactions as in rapid slide or tube agglutination tests. ELISA tests are now being developed for the serological diagnosis of a number of serovars.

Requirements for biological products: Many inactivated vaccines are used against salmonellosis and some live vaccines have been developed. Live vaccines contain attenuated salmonella strains. Inactivated vaccines are produced from virulent strains. The efficacy of these inactivated vaccines is often low, so that oil adjuvants are recommended. Tests for efficacy are usually not performed except for the expected properties of strains used for the production of these products. Innocuity tests are performed in laboratory animals and, in the case of inactivated vaccines, sterility tests using bacteriological enrichment media are done.

Live and inactivated vaccines are available for S. abortus ovis and S. abortus equi.

SPECIAL NOTE ON NOMENCLATURE OF GENUS SALMONELLA

The classification of Salmonella species has been controversial for several years. The Manual was prepared following a recent taxonomy of this genus and corresponding nomenclature proposed by the WHO Collaborating Centre for Reference and Research on Salmonella (8). Earlier proposals for the nomenclature of Salmonella species are described in refs. 7 and 11.

According to the latest nomenclature, which reflects recent advances in Salmonella taxonomy, the genus Salmonella consists of only two species, S. enterica and S. bongori. S. enterica is divided into six subspecies (listed below). These six subspecies are distinguishable by certain biochemical characteristics, and some of them correspond to what Kauffmann called sub-genera.

Subspecies I = Subspecies enterica (choleraesuis)
  " II = Subspecies salamae
  " III = Subspecies arizonae IIIa & IIIb
  " IV = Subspecies diarizonae
  " V = Subspecies houtenae
  " VI = Subspecies indica

In the Manual we have followed the example of WHO (11) in using the familiar 'old' terms such as S. typhimurium, rather than the more complete expressions such as S. enterica subsp. enterica ser. typhimurium. However, because these organisms are treated as serovars rather than species in the nomenclature of Le Minor et al. (8), their names are not italicised.
A. DIAGNOSTIC TECHNIQUES

The genus *Salmonella* includes two species, *S. enterica* and *S. bongori*. *S. enterica* is divided into six subspecies that include 2,252 serovars (as of 1989 - ref. 8). The species is sub-divided as follows: I = 1,333, II = 452, III = 92, IV = 302, V = 64, and VI = 9. *Salmonellae* of sub-genera II and IIIa, IIIb and IV are frequently isolated from the intestine of cold-blooded animals (2) and from the environment. *Salmonellae* of subspecies I cause transmissible infection both in man and many domesticated and wild animal species. Some serovars only affect certain hosts. Salmonella infections are often inapparent.

The course of infection, the clinical signs, the post-mortem findings and epidemiological patterns vary according to the serovar and animal species involved (2). The most common form comprises an enteritis. Septicaemia, genital infections with sporadic or endemic abortions and affections of joints are often other signs of a salmonella infection. The signs and lesions are not pathognomonic.

Many serovars of salmonella have been shown to cause human food poisoning, although they may not be pathogenic in the animal from which the food was derived. *S. enteritidis* and *S. typhimurium* are particularly important causes of food poisoning and are dealt with in the following chapter.

The isolation and subsequent identification of the pathogen involved will enable epidemiological markers including phage type and antibiotic sensitivity to be determined. The isolation and identification of salmonellae depend not only on the quality of the samples but also on the culture medium and growth characteristics of the serovars, particularly those adapted to a host species (1, 3, 4, 5).

1. **Identification of the agent**

The frequency of sampling and the type of samples obtained will depend largely on the clinical signs, epidemiological data, laboratory facilities available and the objectives.

Individual samples for bacteriological tests are taken as aseptically as possible and before any antibiotic treatment has commenced. Samples are collected preferably during the acute phase of the disease or as soon as possible after death. Precautions should be taken to avoid contamination between samples before they are received at the laboratory. Packages should be kept cool and be accompanied by adequate information. For small animal species, it may be preferable to transport 1-2 sick or recently dead animals to the laboratory if it is feasible to do so.

**Isolation media for salmonellae**

Salmonellae are isolated by using four different types of medium, as follows:

a) **Pre-enrichment media**

These are liquid and non-selective. They assist in revitalising bacteria in
samples that have been frozen, heated, or have become dried. They are mainly employed in food and feed-stuff bacteriology, but also in environmental sampling of animal housing.

b) Enrichment media

These are liquid and contain additives that selectively permit salmonellae to be cultured whilst inhibiting the growth of other bacteria. However, some are also inhibitory to certain serovars of *Salmonella*, e.g. brilliant green to *S. dublin* and *S. abortus ovis*. The formulation of the medium, temperature and duration of incubation, and the volume of the sample used to seed the medium, all may serve to improve the isolation rate. These variables should be taken into account to obtain optimal adaptation to specific situations, particularly for the detection of serovars adapted to a host species, although these are not characteristic of all host-adapted species. After shaking, an aliquot is seeded into isolation medium. Examples of selective enrichment media are sodium tetrathionate as in Muller-Kauffman broth, selenite F, brilliant green, and Rappaport-Vasiliadis broths.

c) Selective plating-out media

These are solid, selective and permit differential growth to varying degrees. They inhibit growth of other bacteria and give information on some of the principal differential biochemical characteristics of salmonellae. Results are read after 24 and 48 hours of culture at 37°C. Salmonellae form characteristic colonies on such media and are usually distinguishable from the colonies of other bacteria that may not have been inhibited. However, it may not be easy to distinguish *Proteus* and *Citrobacter*. It should be noted that lactose fermentation by salmonellae may occasionally be seen. Examples of these isolation or separation media include brilliant green, deoxycholate-citrate, *Salmonella-Shigella* and bismuth sulphite agars.

*S. abortus ovis* is a slow-growing serovar (10). In differential and selective media such as *Salmonella-Shigella* or Hektoen, colonies observed after 36 or 48 hours may remain white, without black centres indicating H$_2$S production. Direct plating on blood agar of material containing *S. abortus ovis* yields a higher recovery rate than preliminary enrichment in selenite or tetrathionate.

As with *S. abortus ovis* infection, diagnosis of *S. abortus equi* infection rests on laboratory findings, the identification of the agent, and serological tests. *S. abortus equi* has only one form of flagellar antigens, i.e. it is a monophasic serovar.

d) Ordinary agar media

These are non-selective and often yield more rapidly growing colonies. They are used for obtaining pure cultures prior to further characterisation tests.
Identification of suspect colonies

Sub-culturing of suspect colonies on selective media will inhibit some bacteria. Impure cultures can lead to errors in identification. The use of a magnifying glass and sub-culturing on non-selective ordinary agar media to check the specificity of isolated colonies for further examination is recommended, as well as a slide agglutination of suspected colonies with polyvalent salmonella antiserum. If necessary, additional culture can be performed, for example on blood agar and MacConkey agar. This is appropriate for clinical samples such as blood after incubation in non-selective broth.

The interval between sampling and testing, the nature of any treatment carried out prior to sampling, the presumed numbers of viable salmonellae in relation to contaminants, and a knowledge of the characteristics of strains previously isolated in an area are all factors which require a variety of techniques. Samples that may be contaminated with other bacteria, such as faeces, mucus or genital lochia, are treated separately and are seeded in both enrichment and isolation media. With samples rich in salmonellae and only lightly contaminated by organisms resembling them, isolation will often be possible by direct seeding of selective media without prior enrichment. The samples of normally sterile fluids or tissues are seeded directly into isolation and non-selective media.

The homogeneity of a bacterial population, its morphology and Gram-staining character can be examined microscopically. Biochemical tests on pure bacterial cultures and then serotyping are used for identification purposes. Screening for motility now usually employs the inoculation of a soft agar medium.

Biochemical tests will confirm that the organisms are salmonellae. Any undetermined suspect colonies should be studied further. Different complex media, in tubes or plates, will give a rapid identification by a combination of characteristics. In the case of slow-growing strains such as S. abortus ovis it is sometimes necessary to read reactions after 36-48 hours.

The determination of the O factor, and in special cases also of the Vi antigen as well as the H antigen, are performed by direct slide agglutination tests using specific O, Vi or H antisera. This will account for the more common serovars in an area. Screening is facilitated by the availability of antisera directed against several factors, and this can be pursued further by the use of monovalent antisera. All strains do not always express all their antigenic characteristics when first isolated. Reference laboratories are often required to confirm the identity of an isolate and give information as to its exact serovar, biotype, phage type, antibiotype and plasmid profile.

2. Serological tests

Blood samples should not contain anti-coagulant and should be collected from several clinically affected and convalescent animals. Samples should be kept cool and sent for examination as soon as possible. Second samples may be taken if an evaluation of the antibody response is required.
Serological tests for the diagnosis of salmonellosis are of little value, except in endemic infections. Paired samples may be of greater value. Natural agglutinins have been reported in the sera of normal sheep. In sporadic cases caused by ubiquitous serovars, screening for the causal serovar by agglutination tests can only be done by specialised laboratories which have an assortment of reference antigens. In practice, a preliminary bacteriological diagnosis is recommended, otherwise the identity of the probable causal serovar can be sought by using a restricted number of antigens selected according to the results of bacteriological tests conducted previously.

Precipitating antibody or complement fixation tests do not present real advantages compared with the detection of agglutinating antibodies. Agglutinating antigens may be dense suspensions of either live or killed bacteria. The use of live antigens cannot be recommended, however, because they present a hazard to those performing the tests, and in fact it is preferable to use separate O and H antigens, involving treatments which inactivate the strain.

Both somatic and flagellar antigens are available. Bacterial suspensions which contain only O or H antigen (sometimes Vi antigen) factors will allow separate screening for O and H agglutinins. The O antigen is a suspension standardised to Browne's tube 2 of smooth phase salmonellae that has been treated for 24 hours at 37°C with an equal volume of 96% ethanol. The H antigen consists of a dense suspension of highly motile salmonellae which have been cultured in a nutrient broth and then treated with 0.5% formaldehyde (v/v final concentration) for over 24 hours at 37°C. To select the most motile bacteria, the strain is first passaged several times in U tubes (Craigie's tubes) or on agar plates containing semi-solid agar. If the strain used is bi-phasic, H antiserum corresponding to the phase to be suppressed is added to the agar. These O or H antigens may be stained (9). Slow microplate agglutination with stained antigens facilitates the test and reading of results. Monovalent antisera can be used to determine any O or H agglutination of antigen suspensions diluted in isotonic saline. A reference serum is titrated simultaneously as a control.

Two techniques are available to associate a sample under examination with the two antigen types. Rapid slide agglutination is rarely used. The specificity of this reaction is reflected by the rapidity of agglutination. The specificity of slow agglutination depends on the dilution of the sample. The antigen is dispensed into a tube containing diluted serum which is then incubated at 37°C, or at 50°C (for 24 h for the O antigens and 4 h for the H antigens), or which is centrifuged to give a more rapid result.

Seronological examinations are generally conducted on a representative sample of a population and not on a single animal. An interpretation of the results will depend on a knowledge of the humoral antibody response to infection of the animal species involved. Whatever test is used, the results obtained should be evaluated after taking into account the techniques used and the clinical and epidemiological findings.

Unfortunately, results of serological tests often fail to correlate with shedding of
salmonellae by infected animals. A further disadvantage is that agglutinating antibodies may not be demonstrable until about three weeks after infection.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Many inactivated vaccines are used against salmonellosis due to different serovars in different animal species, most of them being prepared by local laboratories, some being part of plurivalent preparations. Some live vaccines have been developed more recently, a few being approved by some national control authorities. This diversity of vaccines means that only general recommendations can be made in this chapter.

1. Seed management

a) Characteristics

For dead or living vaccines, the bacterial strain (or each bacterial 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 and attenuation of virulence should be stable, and preferably obtained by two independent mutations.

A primary seed lot for each bacterial strain used in a vaccine preparation should be used during the entire period of use of the vaccine.

b) Culture

The seed culture is propagated and maintained using suitable media, of which many have been described (in textbooks) suitable 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.

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 non-selective media
- Metabolic requirements as indicated by biochemical tests
- Detection of markers, phage type
- Agglutination with specific antiserum.

ii) Safety

The LD$_{50}$ or ID$_{50}$ may be determined in mice. Ten times the field dose of
vaccine must be given to the target species at the recommended age and route. The animals are observed for absence of adverse reactions. Stability and non-reversion to virulence after serial passages in susceptible species should be shown.

iii) Efficacy
Laboratory experiments and field trials must 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.

2. Manufacture

Vaccine must be made in suitable clean rooms with access restricted to approved persons. Care must be taken to avoid cross-contamination between infected and uninfected areas and contamination from operators and/or the environment. Operators must be without illness. Personnel must be provided with protective clothing in production areas and in animal rooms.

Culture seed-lots are prepared from the primary seed-lot, and the vaccine must be less than four passages removed from the seed-lot, i.e. five passages from the primary seed-lot. The vaccine may be prepared by inoculation of a suitable medium, such as nutrient broth, with a fresh culture 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 phosphate buffered saline (pH 7.0) and may be freeze dried.

The time of inactivation of dead vaccines should be at least three times that taken to reduce the viable number to an undetectable level. The inactivation process must be applied to the whole volume of vaccine.

Preservatives, excipient for lyophilisation, or 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. A standard freeze-drying process should be developed for a particular apparatus, loss of viability of about 50% being considered as a starting point; the bacterial concentration shall be adjusted to allow for loss of viability during the freeze-drying process.

3. In-process control

The following points require attention:
- Visual control of the suspension, homogeneity by Gram stain, culture on non-selective medium
- Slide agglutination with specific antisera
- Titration of bacteria by turbidimetry and/or plate count
- Test of effective inactivation (dead vaccine) by plating on non-selective medium
- 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 the chapter on General Information.

   b) **Safety**

   A laboratory test in mice, to determine the \( \text{LD}_{50} \) and/or \( \text{ID}_{50} \) may be used, having previously shown a correlation with safety in the target species. Each batch should be tested in the target species at the recommended age and route, using at least twice the field dose.

   c) **Potency**

   Potency is tested for using vaccination-challenge assay in mice and immunological response in target species.

**Vaccines for S. abortus ovis and S. abortus equi**

Autogenous or commercial adjuvanted bacterins composed of killed S. abortus ovis or S. abortus equi are injected into all females on endemic farms or areas on two or three occasions at weekly to monthly intervals, starting 1-3 months before the breeding season. Living vaccines have been said to give good results against S. abortus equi infection (e.g. in China) and against S. abortus ovis infection (e.g. in France). The results of vaccination need to take into account the flock immunity developing after a natural infection.

**REFERENCES**


SALMONELLOSIS: S. TYPHIMURIUM AND S. ENTERITIDIS
(Not on OIE Lists A or B)

SUMMARY

The genus Salmonella contains only two species, Salmonella enterica and S. bongori (see the note below, concerning the nomenclature of Salmonella). S. enterica is divided into six subspecies that include over 2,000 serovars. The salmonellae of all sub-species are pathogenic for man and many animal species, but a few serovars are adapted to a host species. In the case of those of sub-species I, which are the most frequent, the clinical signs, post-mortem findings and epidemiological patterns may vary according to the serovar involved. Most salmonellae are ubiquitous.

Many serovars of salmonella have been shown to cause human food poisoning, although they may not be pathogenic in the animal from which the food was derived. This chapter has been included in the Manual because S. enteritidis and S. typhimurium are particularly important causes of food poisoning.

Identification of the agent: The considerations applying to salmonella in general (see the previous chapter) apply. At present the preferred method for monitoring poultry breeding flocks and hatcheries for salmonella is by means of bacteriological examination of samples obtained from these establishments. Samples should be taken at specified regular intervals and examined in a laboratory authorised for that purpose by the veterinary authorities. Rappaport-Vasiliadis medium is widely used for the detection of salmonella in samples from poultry flocks and hatcheries.

Serological tests: The considerations applying to salmonella in general (see the previous chapter) apply.

Requirements for biological products: The use of vaccines for the control of S. typhimurium or S. enteritidis may assist control but may interfere with serological testing.

SPECIAL NOTE ON NOMENCLATURE OF GENUS SALMONELLA

The classification of Salmonella species has been controversial for several years. The Manual was prepared following a recent taxonomy of this genus and corresponding nomenclature proposed by the WHO Collaborating Centre for Reference and Research on Salmonella (6). Earlier proposals for the nomenclature of Salmonella species are described in refs. 4 and 5.

According to the latest nomenclature, which reflects recent advances in Salmonella taxonomy, the genus Salmonella consists of only two species, S. enterica and S. bongori. S. enterica is divided into six subspecies (listed below). These six subspecies
are distinguishable by certain biochemical characteristics, and some of them correspond to what Kauffmann called sub-genera.

Subspecies I = Subspecies *enterica (choleraeuis)*

" II = Subspecies *salamae*

" III = Subspecies *arizonae IIIa & IIIb*

" IV = Subspecies *diarizonae*

" V = Subspecies *houtenae*

" VI = Subspecies *indica*

In the Manual we have followed the example of WHO (7) in using the familiar 'old' terms such as *S. typhimurium*, rather than the more complete expressions such as *S. enterica subsp. enterica ser. typhimurium*. However, because these organisms are treated as serovars rather than species in the nomenclature of Le Minor *et al.* (6), their names are not italicised.

**A. DIAGNOSTIC TECHNIQUES**

For an introduction covering salmonella in general, see the previous chapter (Chapter 44 - Salmonellosis).

Many serovars of salmonella have been shown to cause human food poisoning, although they may not be pathogenic in the animal from which the food was derived. *S. enteritidis* and *S. typhimurium* are particularly important causes of food poisoning. Recently this has been associated with poultry meat and eggs, although these organisms are also isolated from other livestock.

1. **Sampling for salmonella**

   The frequency of sampling and the type of samples obtained will depend largely on the clinical signs, epidemiological data, laboratory facilities available and the objectives.

   Individual samples for bacteriological tests are taken as aseptically as possible and before any antibiotic treatment has commenced. Samples are collected preferably during the acute phase of the disease or as soon as possible after death. Precautions should be taken to avoid contamination between samples before they are received at the laboratory. Packages should be kept cool and be accompanied by adequate information.

   **Monitoring of poultry breeding flocks and hatcheries for salmonella: general considerations**

   At present the preferred method for monitoring poultry breeding flocks and hatcheries for salmonella is by means of bacteriological examination of samples obtained from these establishments. ELISA tests are being developed which may be widely used in future for mass screening.
For the purposes of monitoring, a 'flock' is defined as any group of birds continuously housed within a building or part of a building with a common air space. Samples for bacteriological monitoring are obtained in the case of rearing flocks from the premises in which the birds are housed, or in the case of adult laying birds either from the premises in which the birds are housed or from the hatchery to which the hatching eggs from that flock are consigned.

The samples to be taken are:

a) On the premises in which birds are housed – fresh faeces (each sample at least one gram), dead or culled birds or in the case of day old birds the chick box liners.

b) At the hatchery – meconium, dead in shell and culled chicks.

Additionally it is recommended that environmental samples are also taken in both the premises and the hatchery at a similar frequency. Where the laying flock is sampled only on the premises, environmental sampling of the hatchery is required.

The total number of samples to be taken on each occasion is shown below and is based on the random statistical sample required to give a probability of 95% to detect one positive sample given that infection is present in the population at a level of 5% or greater.

<table>
<thead>
<tr>
<th>Number of birds in the flock</th>
<th>Number of samples to be taken on each occasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-29</td>
<td>20</td>
</tr>
<tr>
<td>30-39</td>
<td>25</td>
</tr>
<tr>
<td>40-49</td>
<td>30</td>
</tr>
<tr>
<td>50-59</td>
<td>35</td>
</tr>
<tr>
<td>60-89</td>
<td>40</td>
</tr>
<tr>
<td>90-199</td>
<td>50</td>
</tr>
<tr>
<td>200-499</td>
<td>55</td>
</tr>
<tr>
<td>500 or more</td>
<td>60</td>
</tr>
</tbody>
</table>

Up to five samples may be pooled together for bacteriological culture.

All samples should be selected at random to represent the house or in the case of samples taken at the hatchery to represent the hatching eggs from that poultry flock.

The following minimum frequency of sampling is recommended:

a) **Rearing flocks:** At day old and three weeks prior to moving to laying accommodation. Where birds are moved from the rearing premises other than direct to laying accommodation a further sample should be taken three weeks
prior to such movement.

b) Breeding flocks in lay: The laying flocks should be sampled at least at monthly intervals during the laying period.

All samples should be fully marked and identified as to the date of sampling and the flock to which the samples relate. Samples should be stored in a refrigerator at between 1°C and 4°C until they are dispatched to the laboratory (for not more than five days).

All samples should be examined in a laboratory authorised for that purpose by the Veterinary Authorities.

2. Bacteriological methods for culture of salmonella from poultry flocks and hatcheries

As described in the previous chapter (Chapter 44 – Salmonellosis: Section 1: Identification of the agent), many methods are available for the culture of salmonella. A practical method which is in wide use, using Rappaport-Vassiliadis medium for the detection of salmonella in chick box liners, cloacal swabs, composite faeces samples, carcasses and environmental samples, is described below.

Samples submitted for testing for the presence of salmonella should be examined on consecutive days. Samples should be stored in a refrigerator between 1°C and 4°C until required for examination.

Day 1

a) Chick box liners: a one gram portion shall be taken from a soiled area on each liner and the portions from separate liners shall be bulked together and placed in buffered peptone water (BPW) (3) at the rate of 1 gram of liner in 10 ml of BPW up to a maximum of 10 grams in 100 ml of BPW.

b) Faeces samples: the composite faeces sample shall be thoroughly mixed and a sub-sample weighing not more than 10 grams shall be placed in BPW at the rate of 1 gram faeces to 10 ml BPW up to a maximum of 10 grams in 100 ml BPW.

c) Cloacal swabs: cloacal swabs shall be bulked together in batches and placed in BPW at the rate of 1 swab to 4 ml BPW up to a maximum of 30 swabs in 120 ml BPW.

d) Carcasses of birds: the following organs shall be removed from the carcasses of birds:
   - from chicks – samples of the yolk sac, liver and terminal intestines (to include portions of small intestines, large intestines and caecal tonsil).
   - from birds of four weeks of age – samples of liver and terminal intestines (to include portions of small intestines, large intestines and caecal tonsil).
   - from mature birds – samples of gonads should be included.
The samples of organs taken from the carcasses of birds submitted shall then be bulked together and placed in BPW at the rate of 1 gram of bulked tissue in 10 ml BPW up to a maximum of 10 grams of tissue in 100 ml BPW.

e) Environmental samples:
- an individual surgical swab moistened with sterile BPW from each fan housing;
- an individual surgical swab moistened with sterile BPW from each of the four corners of the house at floor level, from the centre of each of the four walls of the house at floor level, and from two crevices in the house;
- each food-weighing hopper or each food-dispensing hopper within each house; and
- in the case of any nest boxes which have been removed from a house, before they are put back in the house, swabs moistened with sterile BPW should be taken from the interior of the boxes at the rate of one in 20.

The inoculated BPW shall then be incubated at 37°C for 18-24 hours.

Day 2

0.1 ml from the incubated BPW shall be inoculated into 10 ml of Rappaport-Vassiliadis (RV) broth (2) and incubated at 41.5 ± 0.5°C for 18-24 hours.

Day 3

The RV broth should be plated out on to one or two plates of brilliant green agar (BGA) (1) using a 2.5 mm diameter loop. The plates should be incubated at 37°C for 18-24 hours, and the RV broth reincubated at 41.5 ± 0.5°C for a further 18-24 hours.

Day 4

a) The plates of BGA shall be examined and a minimum of three colonies from the plates showing suspicion of salmonella growth shall be sub-cultured on to DCA or BGA and MacConkey agar plates and into biochemical composite media or equivalent. These media shall be incubated at 37°C for for 18-24 hours.

b) The reincubated RV broth shall be plated out, and the plates incubated, as described for Day 3.

Day 5

a) The incubated plates and composite media or equivalent shall be examined and the findings recorded, discarding cultures which are obviously not salmonella. Slide serological tests shall be performed using salmonella polyvalent O (Groups A-S) and polyvalent H (phase 1 and 2) agglutinating sera on selected suspect colonies collected from the blood agar or MacConkey plates. If
reactions occur with one or both sera, the colonies shall be typed to Group level by slide serology.

b) The plates of BGA prepared at Day 4 (a) shall be examined and further action taken as described in Day 4 (a) and Day 5 (a).

3. **Serological tests**

See Section 2: Serological tests, in the previous chapter (Chapter 44 – Salmonellosis).

### B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

The use of vaccines for the control of S. typhimurium or S. enteritidis is not recommended when serological testing may be used unless the vaccine contains a specific marker which can be serologically identified.

### REFERENCES


SCRAPIE
(B32)

SUMMARY

Scrapie occurs naturally in sheep and goats. The disease is caused by one of the unconventional slow agents, the biochemical nature of which remains unknown. The natural infection is commonly passed from ewe to lamb before parturition and during the period to weaning. Infection can also pass to unrelated animals, especially when lambing occurs in confined areas. The disease only develops if the infection enters the central nervous system. The incubation time between primary infection and clinical disease is nearly always longer than one year and may sometimes exceed the commercial life-span of the sheep. Animals that never develop clinical signs can still be a source of infection to others.

Identification of the agent: No definitive diagnostic test for scrapie infection is available. Scrapie can however 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 routine diagnostic procedure. Scrapie infection can only be diagnosed if it leads to neuropathological disease. The disease is recognised by clinical signs of pruritus and incoordination. Clinical diagnosis is supported by pathological diagnosis of spongiform encephalopathy. This is characterised by the histological demonstration of bilateral and usually symmetrical spongiform change of neuropil and neuronal vacuolation in the grey matter of the brain stem and, often, spinal cord. These are not found in the white matter in this disease. The detection of specific scrapie-associated fibrils in extracts of diseased brain may become a useful, additional criterion for scrapie diagnosis.

Sero logical 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.

Requirements for biological products: There are no biological products available.

A. DIAGNOSTIC TECHNIQUES

The clinical signs of scrapie start insidiously with the appearance of non-specific behavioural changes. For example, an affected sheep may either lead or trail behind a flock that is being driven (1, 7, 10). These changes progress to more definite signs that are of 2 kinds:

a) Pruritus: There is often an extensive loss of wool from the flanks and hindquarters as the result of scraping or rubbing against objects and by
nibbling. The compulsive nature of this behaviour can often lead to the development of skin lesions. Evidence of self-inflicted lesions may also be found on the head, ears, forelegs and base of the tail. Affected sheep often tremble and exhibit a nibbling reflex when rubbed over the rump, or even when rubbing themselves.

b) **Incoordination:** Affected sheep usually have an exaggerated and incoordinated gait. Some sheep exhibit a high-stepping or trotting action of the forelegs, but more generally there is ataxia of the hind limbs, with much stumbling if the animal is obliged to move. When at rest, an affected sheep may stand awkwardly while supporting its hindquarters against a fence, or it may be seen to sway drowsily and even lose its balance.

Characteristically, clinical signs are progressive and can lead to incoordination and an inability to stand. The clinical disease has a very variable course and may last for a week or up to several months, but invariably ends in death. The general bodily condition often remains good for most of the period of clinical disease, although a significant loss of weight may occur just before death in many but not all cases.

There is a great variation in the clinical signs seen in individual animals. This variation is probably due to differences between breeds of sheep, between strains of the agent and from environmental factors. It is not uncommon for affected animals with pruritus to show no incoordination, and vice-versa. The diagnosis of isolated cases can be very difficult. Many cases of scrapie probably go unrecognised, especially in poorly supervised flocks in which an affected sheep may rapidly become incoordinated and die from exposure. In differential diagnosis, it should be remembered that an intense pruritus may arise from the presence of ectoparasites, and posterior incoordination has been associated with infections by other microbial agents that invade the central nervous system, and a variety of other environmental and metabolic factors.

1. **Identification of the agent**

As there is no definitive diagnostic test for scrapie yet available, such as a tissue culture technique for the isolation of the agent, it is essential to confirm any clinical diagnosis by histological means.

The histological lesions of scrapie are confined to the central nervous system (2). Characteristically, there are no inflammatory changes and no demyelination. The most striking lesion is the vacuolisation of neurones in the medulla, pons, mid-brain and spinal cord. Single or multiple vacuoles may be present. These push the neuronal perikaryon contents to one side. Eosinophilic globular, or finely granular material can be seen within some of the intracytoplasmic vacuoles in neurones of affected sheep and goats. The surrounding cytoplasm may show signs of degeneration, although vacuolation may occur in cells which in other respects appear normal. Occasionally there may be a loss of neurones. Spongiform changes of grey matter neuropil are often found in the same areas as neuronal vacuolation. Astrocytosis, as demonstrated by gold impregnation techniques, is often seen but is
a non-specific lesion of scrapie. Recently, a mild cerebrovascular amyloidosis has been recognised in some field cases of scrapie (4), but its occurrence varies between animals.

The whole brain and, ideally, the spinal cord with dorsal root ganglia should be removed and preserved with formalin. Autolysis or freezing of the specimens invariably makes the tissue sections unsuitable for diagnostic histopathological examination. Paraffin-embedded, haematoxylin and eosin stained sections should be prepared from standard representative areas to establish the pathological degrees of spongiform encephalopathy and differential or concurrent diagnosis.

Neuronal vacuolation of the type found in scrapie is not strictly pathognomonic of scrapie because vacuoles may occasionally be found in the brains of normal sheep. However, in the majority of cases with clinical signs of scrapie the number of neuronal vacuoles is so great as to support the diagnosis (5, 11, 12). Examination of the medulla alone is inadequate. It is important that the whole brain should be removed immediately after death and an examination made of many different areas (6).

There are experimental situations where clinical disease occurs with virtually no neuronal vacuolation (1) and it is possible that scrapie cases that show no lesions might occur naturally. Without independent diagnostic criteria it is impossible to assess how serious this problem might be. Therefore, even a complete absence of vacuolation in whole brain sections cannot be taken as proof that scrapie was not the cause of the clinical disease and it is certainly not evidence of the absence of scrapie infection, which can occur in the absence of clinical signs and of lesions.

The need for additional diagnostic criteria for scrapie becomes most apparent on those rare occasions when histological examination of brain material yields inconclusive results. Two new approaches to scrapie diagnosis have been evaluated. In one approach, histochemical techniques have been applied to brain sections to detect certain types of enzymic inclusions in neurones (8). However, this method has not been adopted outside the laboratory of origin and the specificity of the inclusions for scrapie is unknown. The second approach has been tested more widely. It depends on making biochemical extracts of unfixed, frozen brain from suspected cases and demonstrating the presence of scrapie-associated fibrils (SAF) which can be observed by electron microscopy (3, 9). Immunological techniques can also be used to detect the protein forming SAF but only under conditions that distinguish the SAF from the precursor protein which is present in normal, uninfected sheep brain. Suitable methods are currently being developed to enable a thorough investigation of the diagnostic potential of SAF. Detecting SAF has yet to be established as a routine procedure for scrapie diagnosis.

2. Serological tests

To date it has not been possible to detect an immune response to infection with the scrapie agent.
B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

There are no biological products available.

REFERENCES


SUMMARY

Maedi-visna of sheep (ovine progressive pneumonia or OPP) is one of the so-called "slow virus" diseases. The causative agent is a lentivirus. A closely related agent causes infectious caprine arthritis-encephalitis. The disease is associated with a progressive interstitial pneumonitis, or a meningo-encephalitis. Arthritis and indurative mastitis may also occur. Most infected sheep exhibit few clinical signs, but such individuals remain virus carriers and may transmit infection. Clinical disease can occur in antibody-negative sheep.

Diagnosis is confirmed by histological examination of the appropriate tissues collected at necropsy, and is assisted by serological examination of a proportion of the flock.

Identification of the agent: Virus isolation can be attempted from live clinical or subclinical cases by cocultivating peripheral blood or milk leucocytes with appropriate ovine cell cultures, such as fibroblastic choroid plexus cells. But 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. The cytopathic effects are characteristic, consisting of the appearance of refractile stellate cells and syncytia. The presence of maedi-visna virus can be confirmed by immunolabelling methods and electron microscopy.

Serological tests: The majority of infected sheep possess specific humoral antibodies which can be detected by a number of different serological assays. The two most commonly used are the agar gel immunodiffusion test (AGID) and the indirect enzyme-linked immunosorbent assay (ELISA). Following infection, the time required for seroconversion is relatively prolonged and unpredictable, being measured in months rather than in weeks. A small proportion of infected individuals may remain continuously antibody-negative. However, after seroconversion the antibody response usually persists and antibody-positive sheep are identified as virus carriers.

Requirements for biological products: There are no biological products available.

A. DIAGNOSTIC TECHNIQUES

Maedi-visna of sheep (ovine progressive pneumonia) is one of the so-called "slow virus" diseases. A closely related agent causes infectious caprine arthritis-encephalitis. The Icelandic name describes two of the possible clinical syndromes occurring in sheep that are persistently infected with the causative agent, a lentivirus. "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. Arthritis and indurative mastitis may also occur. The majority of infected sheep exhibit few signs of disease; however, such individuals remain virus carriers and are capable of transmitting infection. Clinical disease can occur in antibody-negative sheep.

Where clinical disease caused by maedi-visna virus is suspected, confirmation of the diagnosis can only be achieved by histological examination of the appropriate tissues collected at necropsy; lung in the case of maedi (progressive interstitial pneumonitis), brain and spinal cord for visna (meningoencephalitis), udder for indurative mastitis, and the affected joints for arthritis. To assist confirmation of the disease, a proportion of the flock should be tested for virus antibody.

1. Identification of the agent

Isolation and characterisation of maedi-visna (MV) virus [ovine progressive pneumonia (OPP) virus] would not normally be attempted for routine diagnostic purposes. Due to the persistent nature of the infection, the establishment of a positive antibody status is sufficient for the identification of virus carriers.

There are two basic approaches to the isolation of MV virus, one in the case of the live animal and the other for the collection of necropsy tissues.

a) From the live animal

The MV DNA provirus is carried in circulating monocytes and tissue macrophages. Virus isolation from the live animal therefore requires the establishment of leucocyte preparations with aseptic precautions from peripheral blood, or milk during lactation, and culturing them together with indicator cells. Sheep choroid plexus (SCP) cells are commonly used for this purpose. These can be prepared as primary explant cultures from fetal or new-born virus-free lambs and their number multiplied over 3-4 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 MV virus becomes reduced.

Leucocyte preparations can be made from peripheral blood as buffy coats by the centrifugation at 1,000 g of heparinised EDTA or citrated samples for 15 minutes. The cells are aspirated off, suspended in Hanks' balanced salt solution (HBSS), and further purified by centrifugation at 400 g onto a suitable cushion 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 resuspended in medium to a concentration of approximately $10^6$ cells per ml. Ten ml of this suspension is then added to a washed monolayer of slightly sub-confluent SCP cells in a flask with an area of 25 cm$^2$.

Leucocytes 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% carbon dioxide 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, coverslip cultures should be prepared. These are fixed, and evidence of viral antigen is sought by immunolabelling, usually by means of indirect fluorescent antibody or indirect immunoperoxidase methods. In addition, the cells of any suspect monolayers are deposited by centrifugation, and preparations made for the identification of any characteristic lentivirus particles by transmission electron microscopy.

b) From necropsy tissues

Samples of suspect tissues 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 placed carefully on each. Each flask is then incubated at 37°C in a humidified 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 outgrowth, 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 cocultivations.

Adherent macrophage cultures are easy to establish from lung-rinse material 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 cocultivation of macrophages and SCP cells as described for leucocytes above.

2. Serological tests

Ovine lentivirus infections (maedi-visna/ovine progressive pneumonia) are persistent, so that antibody detection is a valuable serological tool for identifying virus carriers. MV and ovine progressive pneumonia viruses are antigenically indistinguishable by methods that employ polyclonal antisera. The assays now commonly used are agar gel immunodiffusion (AGID) (1, 2, 5) and the indirect ELISA (3). AGID is specific, reproducible and simple to perform, but experience is required for reading the results. The indirect ELISA is economical and stages of the process can be automated, thus making it useful for screening large numbers of sera. However, the specificity of the indirect ELISA depends on the quality of the antigen. In the case of MV/OPP viruses the production of satisfactory antigen preparations has limited its routine application. Modifications to the MV/OPP ELISA are emerging, such as employing double antibody sandwich
methods and monoclonal antibodies (4), which may ultimately result in its wider application. But in general the AGID remains the most frequently used test.

a) **Agar gel immunodiffusion**

There are 2 MV/OPP 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 is commonly used; this has been distributed by Dr. R.C. Cutlip, USDA, Ames, Iowa, USA.

The predominant precipitating antibody response detected routinely is directed against the gp135 antigen. An anti-p28 response, although occasionally detected, is rarely encountered in the absence of an anti-gp135 response. It is therefore essential that the reference serum produce a distinct anti-gp135 precipitin line.

The gel medium is 0.7-1% agarose in 0.05 M Tris buffer, pH 7.2, with 0.8% NaCl. The test is conveniently performed in plastic petri dishes, or in 10 cm square 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 diameter) and small (3 mm diameter) peripheral wells 2 mm apart and 2 mm from a central 3 mm diameter antigen well. The large peripheral wells are used for test sera and the small ones for reference sera. The plates are incubated overnight at ambient room temperature in a moist chamber and then examined for precipitin lines.

b) **ELISA**

Operating protocols for available ELISA methods are described in refs. 3 and 4.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

There are no biological products available.

REFERENCES


Summary

Contagious equine metritis is a bacterial venereal disease of the mare caused by Taylorella equigenitalis (Haemophilus equigenitalis). It is a true venereal pathogen transmitted at coitus but accidental transmission can also occur if strict hygiene is not observed when animals are being handled or examined. T. equigenitalis infections cause endometritis characterised by a vaginal discharge which may vary both in amount and purulence. It usually leads to a loss of fertility. Infected mares risk becoming carriers of the organism.

The disease can only be controlled by the regular swabbing of all breeding mares and stallions and the identification of carrier animals before they go to stud. A code of practice recommends the sites and frequency of swabbing to detect the infected mare and carrier animal. The mare that has been infected but still carries the organism asymptotically is the most likely reservoir of infection but colts and filly foals may become carriers at birth or as foals at foot from an infected dam and remain asymptomatic carriers until of breeding age.

Identification of the agent: T. equigenitalis is a Gram-negative, non-acid-fast, non-motile short rod or coccobacillus with occasional filaments 5-6 μm in length. It grows well on a rich peptone-based chocolate agar medium. Cultures must be incubated in an atmosphere enriched with 5-10% v/v carbon dioxide. Optimal temperature for growth is 35-37°C. T. equigenitalis can be identified by slide or tube agglutination tests, or by direct or indirect immunofluorescence tests.

Serological tests: Techniques used to detect specific antibodies in horses include the serum agglutination test (SAT), complement fixation test (CFT), passive haemagglutination test (PHAT), enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID). Serology can only be used in the identification of mares during the acute stage of an infection. It is of no value for detecting chronic or carrier infections.

Requirements for biological products: Vaccination is not effective and no vaccine is available commercially.

A. Diagnostic Techniques

Confirmation of contagious equine metritis is based on the isolation of Taylorella equigenitalis by bacteriological culture from swabs. A voluntary code of practice operates in many countries where the horse, particularly the thoroughbred, is of economic importance. Such codes provide guidelines for veterinary surgeons and breeders for control of the disease. Most codes are based on the Code of Practice
published by the Horserace Betting Levy Board of Great Britain in 1977. This Code is reviewed annually and revised and modified as required to take into account the incidence of infection during the previous breeding season (3). It recommends the sites to be swabbed and the frequency of swabbing for maximum effective isolation of the organism and control of the disease. These recommendations relate to the situation in the specific countries operating the Code, i.e. France, Ireland and Great Britain, and should not be considered universal. However, the principle that each and every breeding horse be tested for the presence of the causative organism before going to stud is applicable in all countries.

The most likely reservoir of infection is a carrier mare. A mare can be designated as being at "high risk" of carrying the organism on the basis of her previous breeding history. The high risk mare is one from which the organism was isolated during the previous season, was covered by a stallion known to be transmitting the organism during the previous season, or has been resident on stud premises where there was no control of the disease or the standard of control was unsatisfactory. Carriage of the organism is asymptomatic and gives no serological indication of its presence. Carriage can therefore only be identified by bacteriological examination of swabs and it is recommended that high-risk mares be swabbed at least once before going to the stallion stud farm. Ideally swabs should be taken on at least three occasions from the clitoral fossa and the clitoral sinuses and on at least one occasion from the endometrium during early oestrus. Although carrier mares have been identified by the isolation of the organism from swabs taken from the clitoral sinuses there is no evidence that this is the only site where the organism is retained. It is the recommended site for swabbing high-risk mares and a small swab suitable for penetrating the clitoral sinuses should be used.

An infected mare may occasionally conceive. The organism does not interfere with pregnancy but is retained with the normal bacterial flora and can be transmitted to the foal at birth. It has been recovered soon after birth from the external genitalia of foals whose dams were known to have been infected at covering. A mare infected at covering may also transmit the organism to her foal at foot and the organism has been recovered from the external genitalia of colts and filly foals for which the only known source of contact was the infected dam. Infected foals can carry the organism in the normal flora until sexually mature and, if undetected, constitute a potential reservoir of infection.

A mare that is not considered to be at "high risk" should be swabbed from the clitoral sinuses and fossa before going to stud.

In the stallion, T. equigenitalis can establish itself in the normal genital flora of the penile sheath and the urethral fossa, where it may survive for many years. Bacterial identification is the only method of detecting carrier stallions, as serological tests are universally negative. For this reason, and for maximum control of the disease, it is recommended that all stallions be swabbed before the covering season and special attention should be paid to new stallions; the first mare covered by a first season stallion should be thoroughly screened for evidence of the organism as an additional precaution. All stallions should have swabs taken for bacteriological
examination from the penile sheath, urethra and the urethral fossa, on two or three occasions at not less than seven days’ interval before starting covering duties.

Swabs should be sent in transport medium to a laboratory which has the staff expertise and equipment for the culture and identification of *T. equigenitalis*.

The carrier state can be eliminated by treatment with antibiotics. Several courses of treatment may occasionally be required (4).

1. **Identification of the agent**

The causative agent of contagious equine metritis is *T. equigenitalis* (1). It is a Gram-negative, non-acid-fast, non-motile cocccobacillus, 0.8 μm in diameter, with occasional filaments 5-6 μm in length. It grows well on agar medium containing a rich peptone base with 2-5% chocolate horse or sheep blood. The medium should be supplemented with L-cystine (300 mg/litre) or L-cysteine hydrochloride (100 mg/litre), and sodium sulphite (200 mg/litre). The plates should be incubated in an atmosphere enriched with 5-10% v/v carbon dioxide or in an anaerobic environment (10% v/v hydrogen, 10% v/v carbon dioxide, 80% v/v nitrogen). Optimal temperature for growth is 35-37°C. Visible colony formation requires at least 48 hours. Chocolate blood agar is best for supporting the growth of the organism. The X and V factors do not stimulate growth. Although initial observations suggested that the organism would not grow on fresh blood agar plates, it will do so if the plates are incubated in the correct environment. The organism will not grow in aerobic conditions. Acid is not produced from carbohydrates. Oxidase, catalase, phosphatase and phosphoamidase are produced. Nitrates and nitrites are not reduced. Lysine and ornithine decarboxylase, gelatinase, lipase, urease and deoxyribonuclease are not produced; indole and H₂S are not produced. Citrate is not utilised (2).

The G + C content of the DNA is about 36.5 mol % (Tm). Two strain variants of *T. equigenitalis* have been identified on the basis of their sensitivity to streptomycin. One is resistant to streptomycin and will grow in concentrations of 256 to 512 μg/ml. The other is unable to grow at concentrations of 1 μg/ml and is considered to be streptomycin-sensitive.

Swabs taken from animals should be placed immediately into transport medium, such as Stuart’s medium (6), and dispatched to the laboratory. A loss of viability occurs during transport which increases with time and temperature. Samples should be kept cool and reach the laboratory within a maximum of 36 hours. In the laboratory, the swabs should be plated onto two sets of chocolate blood agar, supplemented with cysteine and sodium sulphite and a fungicide such as amphotericin B (5 μg/ml). One set of plates should in addition contain 200 μg/ml of streptomycin sulphate. A control positive culture should be run to test the medium. The plates should be incubated in a humidified CO₂-enriched environment for 48 hours before examination and for up to 6 days. On chocolate blood agar, colonies are small, smooth, and grey to beige in colour. They may exhibit considerable variation in colony size. On chocolate blood agar, colonies
are more easily distinguished from those of other bacteria which are likely to be present on the swabs. Dextrose should not be present in the medium as it has been shown to inhibit *T. equigenitalis* in the presence of other bacteria, especially *E. coli*. Suspected colonies should be Gram stained and tested for oxidase and catalase production. The Gram stain, showing very small Gram-negative cocci or short rods, is very characteristic.

Any colony which gives a fast oxidase positive reaction and is of the correct morphology should be checked by serum agglutination (slide or tube) or by direct or indirect immunofluorescence for a positive diagnosis. Certain strains of *T. equigenitalis* exhibit autoagglutination when emulsified in saline. Such strains can be positively identified using direct or, preferably, indirect immunofluorescence. In the latter technique, a purified washed culture is allowed to react on a slide with rabbit *T. equigenitalis*-specific antiserum. The slide is washed and fluorescein conjugated anti-rabbit antiserum, which is commercially available, is added. After further washing, the slide is examined under a fluorescent microscope.

Reference cultures of both streptomycin-sensitive and streptomycin-resistant strains have been deposited with national culture collections. In Great Britain, three cultures have been deposited with the National Collection of Type Cultures:

- NCTC 11184 Streptomycin-resistant
- NCTC 11225 Streptomycin-sensitive isolated in the U.S.A.
- NCTC 11226 Streptomycin-sensitive isolated in Europe

Specific rabbit antisera to the National Type Cultures have been raised by Dr. P. Høgh, State Veterinary Serum Laboratory, Bulowsvej 27, DK-1870, Copenhagen V, Denmark.

2. **Serological tests**

Various serological tests are available to investigate the disease (5). These include a serum agglutination test (SAT), a complement fixation test (CFT), a passive haemagglutination test (PHAT), an enzyme-linked immunosorbent assay (ELISA), and an agar gel immunodiffusion (AGID) test. A serological response can only be demonstrated in the mare following clinical signs of infection with *T. equigenitalis*. Serum antibodies can be detected from around seven days after infection, peaking at around 21 days and declining from between 6-10 weeks, depending on the test used. No antibody response has been demonstrated in carrier mares (i.e. those from which the organism can be isolated without clinical signs of infection or a history of clinical signs of infection) or from stallions.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

Current vaccines are not effective and no commercial vaccine is available.
REFERENCES


DOURINE
(B35)

SUMMARY

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

Dourine is the only trypanosomiasis which is not transmitted by an invertebrate vector. T. equiperdum differs from other trypanosomes in that it is primarily a tissue parasite which rarely invades the blood. There is no known natural reservoir of the parasite other than infected equine animals. 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 tolerant than horses. Infection is not invariably transmitted by an infected animal at every copulation. Rats can be infected experimentally, and can be used to maintain strains of the parasite indefinitely.

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: Specific humoral antibodies are present whether clinical signs exist or not. CF tests are used and these can detect latent infections. Non-infected animals, especially donkeys, often yield inconsistent results. The indirect fluorescent antibody (IFA) test can be used to confirm infection or resolve inconclusive CF test results.

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

A. DIAGNOSTIC TECHNIQUES

Dourine is a chronic or acute contagious disease of breeding solipeds which 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, Beschälseuche, slapsiekte, sluchnaya bolyezn, covering disease (1, 5).

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 trypanosomiasis which is not transmitted by an invertebrate vector. T. equiperdum differs from other trypanosomes in that it is primarily a tissue parasite which rarely invades the blood. There is no known natural reservoir of the parasite other than infected equine animals.

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, and then parasites 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 (3). 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, a week only. Although dourine is a fatal disease with an average mortality of 50% (especially in stallions), spontaneous recovery occurs. Subclinical infections are recognised. Donkeys and mules are more tolerant than horses.

Trypanosomes are not continually present in the genital tract throughout the course of the disease; thus 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 3-7 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 semi-transparent 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.

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:

- 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.

- The trypanosomes are only sparsely present and extremely difficult to find, even in oedematous areas.

- The trypanosomes are only fleetingly present in the blood, and in small numbers that defy detection.

In practice, diagnosis is based on clinical evidence supported by serology.

In infected animals, trypanosomes are present only in low numbers 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 only for a few days, 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.

Since 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 are present, stained preparations are necessary to identify the Trypanosoma species. T. equiperdum is indistinguishable from T. evansi. Both are monomorphic, slender trypomastigotes with a free flagellum, although pleomorphic, stumpy, proteonuclear forms are recognized.

Typical Indian strains are from 15.6 μm to 31.3 μm in length. Russian strains range from 22.3 μm to 29 μm.

2. Serological tests

Humoral antibodies are present in infected animals, whether they display clinical signs or not. The complement fixation (CF) test (7) is used to confirm clinical evidence and to detect latent infections. Uninfected equine animals, particularly donkeys and mules, often give inconsistent or non-specific 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.

a) Complement fixation test

Standard or microtitre techniques may be used (4).

Antigens: Antigens for the CF test are prepared as follows:

i) Inoculate a rat with T. equiperdum cryopreserved stock. At maximum parasitaemia, blood is collected into an anticoagulant such as heparin.

ii) Inoculate 20 large rats 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. Blood is previously taken from the tail for thick smears and examined microscopically. When parasitaemia is maximal, the rats are bled out 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) Filter the blood through muslin gauze and centrifuge at 800 g for 4 minutes. The red cells are mostly deposited while the trypanosomes remain in suspension.
iv) Transfer the supernatant fluid to a fresh tube, the upper layer of red cells mixed with trypanosomes to a second tube, and the next layer to a third. Add citrate saline to tubes 2 and 3 to prevent clotting of cells, mix and centrifuge all tubes at 1,500 g for 5 minutes.

v) Discard the supernatant fluid and transfer the upper white layer of trypanosomes 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) Add physiological saline, mix and centrifuge again at 1,500 g for 5 minutes to separate the trypanosomes. Repeat washing until all the trypanosomes are collected as a pure white mass. Ten rats should produce 3-5 g of antigen. This procedure of purification can also be carried out using a column of DE52 diethylaminoethyl cellulose in phosphate buffered saline (pH 8.0) (6).

vii) Dilute the concentrated trypanosomes 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 Griffiths tube (8). This antigen may be divided into aliquots 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. Dilutions of sera positive in the screening test are titrated against 2 units of antigen. Test sera are screened at a dilution of 1:5. Sera showing more than 50% 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 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 which is at least 3 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 1 hour may result in degradation of the anticomplementary effect.

b) Indirect fluorescent antibody (IFA) test

An IFA test for dourine can also be used (7) as a confirmatory test or to resolve inconclusive results obtained by the CF test. The test is performed as for equine piroplasmosis (see B40 – Chapter 54).
c) Other serological tests

Other serological tests have been used, including enzyme-linked immunosorbent assay (ELISA), radio-immunoassay, and counter immunoelectrophoresis (2).

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

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


EPIZOOTIC LYMPHANGITIS
(B36)

SUMMARY

Epizootic lymphangitis is a contagious, chronic disease of solipeds characterised by suppurative inflammation of the cutaneous lymphatic vessels and adjacent skin tissues, with extension to the regional draining lymph nodes. It is characterised by suppurative lymphangitis, lymphadenitis, ulcers of the skin, regional lymph nodes and by keratitis or pneumonia. It is caused by a host-specific, diamorphic fungal organism, Histoplasma farciminosum. Isolation and microscopic identification of H. farciminosum are the acceptable laboratory criteria for disease confirmation.

Identification of the agent: The organism grows slowly and aerobically at 25-30°C in a variety of media, which include Sabouraud's glucose agar, brain-heart infusion agar as well as broth preparation of the same media. The purulent contents of closed nodular lesions are aspirated aseptically and streaked on plates of media containing chloramphenicol and cycloheximide. Swabs and samples of the nodular and ulceral discharge are also collected and directly immersed in broth media. Smears are also made from the affected tissues of the animals and stained with Giemsa and/or a mixture of basic fuchsin and methylene blue. The specimens for culture should be collected into a solution containing 500 units of penicillin per ml. They should be refrigerated, transported rapidly to the laboratory and cultured promptly.

Stained smears from closed nodular lesions may reveal relatively large, single-celled round structures, 1-5 μm in diameter, mostly phagocytosed in macrophages. This organism is difficult to culture on artificial media, and growth is very slow and uncertain. After 3-4 weeks' incubation of culture, a brown deposit may be visible at the bottom of the Sabouraud's broth tube. Upon direct microscopic examination of preparations made from such deposits, chlamydospores and aleuriospore-like structures are seen. They are found in the short branches arising from irregular, relatively thick hyphae. On solid media, colonies are not noticed before 2-3 weeks of incubation. The colonies are dull grey in colour, dry, and 2-3 mm in diameter.

Serological tests: Specific antibodies develop before clinical skin disease signs are visible and may be detected by serological tests. Fluorescent antibody, ELISA, agglutination, haemagglutination inhibition and skin hypersensitivity tests have been developed and are promising tools for disease diagnosis and identification of H. farciminosum organisms. Serological tests are useful as an aid in disease diagnosis and are awaiting field evaluation in disease surveillance.

Requirements for biological products: No vaccines are available for the disease.
A. DIAGNOSTIC TECHNIQUES

Epizootic lymphangitis is a contagious, chronic disease of equidae characterised by inflammation and thickening, or "cording", of the cutaneous lymph vessels. Along the lymph vessels, suppurative nodules are formed and there is inflammation of the regional lymph nodes. It has been known by several names such as pseudo-glanders, equine blastomycosis, equine histoplasmosis, African farcy, Japanese glands and culebrilla. The infection is caused by a fungus, *Histoplasma farciminosum*. In most outbreaks, the disease occurs in the cutaneous form, although the infection may be confined to the eyes, producing conjunctivitis, keratitis, and granulomatous lesions (10). Pulmonary and systemic infection with *H. farciminosum* has also been reported in equidae. Therefore, the form of the disease may vary with the type of exposure.

The disease is endemic in countries bordering the Mediterranean Sea, particularly in North Africa. It is also found in parts of Asia, Russia and the Far East, as well as in certain countries of central and southern Africa. The last occurrence in North America was in 1923. The disease is enzootic in Colombia, Venezuela, and Uruguay. Horses under six years of age are highly susceptible. Disease incidence is increased when large numbers of animals are assembled. With the exception of rabbits and guinea pigs, most laboratory animals are refractory to infection. The infectious *Histoplasma* is usually introduced through wounds and abrasions, especially along the lower parts of the legs. From there, it may be transmitted by contaminated harnesses and grooming tools. Camels are also affected by the disease.

1. Identification of the agent

Field diagnosis

The clinical signs (2, 7) of lymphangitis and lymphadenitis may enable a presumptive diagnosis to be made. However, laboratory diagnosis is mandatory as the disease signs are similar to those for glanders, bacterial lymphangitis and sporotrichosis. The incubation period is approximately two months. The lymphatic vessels in the affected skin areas are thickened and described as "corded". Nodular lesions are observed at intervals along the thickened vessels. The nodules suppurate and develop into abscesses containing thick, purulent, oily, yellow or greenish-yellow pus. The abscesses rupture and form chronic indurated ulcers with an inverted border and a central crater. In the early disease stages, the ulcers are seen on the legs, but as the disease progresses, they are found on other areas of the body. The disease course is long. Lung lesions, if present, are a mass of coalescing granulomas. Frequently, abscesses are present in the pleura, spleen, liver, testis, and on bone marrow. However, visceral involvement is most unusual.

Laboratory diagnosis

Aseptically aspirated pus from unopened nodules is the diagnostic specimen of choice. This material should be placed in a suitable liquid nutrient medium containing 500 units of penicillin per ml. The specimens should be refrigerated
during transportation to the laboratory, and culture attempts should be made immediately.

Smears made from swabs of fresh ulcers or aspirated pus from nodules should be dried and wrapped separately.

For histopathology, biopsies from representative nodules and ulcers should be preserved in 10% neutral formalin.

Confirmation of the disease is dependent upon demonstration of *H. farciminosum*.

a) **Direct microscopic examination**

i) **Unstained wet preparations:** The tissue phase of the fungal organism appears as small, yeast-like cells which are often lemon-shaped in appearance. The cells may be seen singly or in groups, either free or phagocytosed by macrophages, polymorphonuclear cells or giant cells. The organisms are 3-4 μm in length and 2.5-3 μm in width.

ii) **Gram-stained smear:** The cytoplasm of most cells remains unstained. The organisms may occur singly or in groups, either free in the lymph or in leukocytes.

iii) **Biopsy:** The lesions contain histiocytic cells with a predominance of large macrophages. The organisms are seen in aggregates, either free in the tissue spaces or engulfed by macrophages which appear distended. Mycelial forms are rarely observed. In a fully developed nodule, there are many giant cells with large numbers of double-walled organisms of *H. farciminosum*.

b) **Cultural properties**

Growth of *H. farciminosum* is slow on laboratory media. Primary cultures on agar containing horse blood take 4-6 weeks to develop at 37°C, but the growth period may be shortened to 48-72 hours by using Littman's medium (1). Growth occurs also on plain agar, broth-coagulated egg medium and coagulated serum medium. Among the media giving comparatively successful primary isolation is a pleuropneumonia-like organism medium (Difco) composed of 50 g brain-heart infusion (BHI) agar, 10 g Bactopeptone, 5 g sodium chloride, and 14 g agar. The medium is enriched with 2% dextrose and 2.5% glycerol and adjusted to pH 7.8. Addition of antibiotics to media used for primary isolation is practiced (0.5 g/ml cycloheximide and 0.05 g/ml chloramphenicol). However, it has been claimed that in media containing antibiotics, growth of the fungus was delayed with slight inhibition in comparison to media without antibiotics. On solid media, colonies appear as white, aerial mycelia. The hyphae are hyaline, septate,
branched, and 2.5-3.0 μm in diameter.

To obtain the yeast form, it is necessary to subculture on brain heart infusion agar with 5% horse blood and incubate in an atmosphere of less than 15% CO₂. The yeast colonies are flat, raised, wrinkled, and white to grayish brown. Incubation at 25°C hastens conversion to mycelial forms. Microscopically, the colonies appear to contain short septate mycelia. To isolate *H. farciminosum* from nodular lesions, it is necessary to inoculate several cultures of nutrient media, as growth may not occur in all of them. Whenever the solid media show signs of dryness because of the long time needed to obtain satisfactory growth, liquid nutrient medium is added to the culture. Once the fungus has been isolated in pure culture, it can be maintained on Sabouraud's dextrose agar with 2.5% glycerol.

*H. farciminosum* will remain viable at -15°C for 18 weeks and at 5°C for 16 weeks in both soil and water. It will be viable for only six weeks in non sterile water and eight weeks in soil at 37°C. The organism has survived ten weeks in water at 26°C (8).

c) **Direct fluorescent antibody test**

This test is used to identify the antigen of *H. farciminosum*. Blood is collected aseptically from the jugular veins of horses in which the disease is confirmed microscopically and by culture. Serum is obtained aseptically, stored at -20°C and thawed immediately before use. The globulin fraction is purified by salting out with 35% saturated ammonium sulphate and gel filtration. The protein concentration is determined, quantified and then the preparation is labeled with fluorescein isothiocyanate at a ratio of 1:60. Excessive fluorescein is removed by gel filtration. The labeled globulin is stored at -70°C and thawed shortly before use. An antigen is prepared by making thin films of the mycelial and tissue phase of *Histoplasma* grown on Sabouraud's dextrose agar at 26°C or brain-heart infusion agar at 32°C. Films of the purulent nodular pus fixed with heat can also be used. Dilutions (1:5, 1:10 and 1:20) of labeled antibody are applied to fixed antigen preparations and incubated at 37°C for 60 minutes. They are then treated by standard methods and examined with a high quality fluorescent microscope. For controls, sera from the tested horses and from noninfected horses are treated in the same manner. Three replicates are made for each serum dilution. The intensity of fluorescence for test and control specimens under identical conditions is reported subjectively (1+ to 4+). Conjugated sera diluted 1:5 usually yield the highest intensity of fluorescence. In a valid test, none of the test controls shows fluorescence.

2. **Serological tests**

During the incubation period and the disease course, specific antibodies are produced, allowing the use of serological diagnostic tests. The following diagnostic techniques, which are helpful in disease detection and surveillance, were largely developed by Fawi (3) and Gabal and co-workers (5, 6). These include the tube agglutination, haemagglutination, skin hypersensitivity, ELISA and fluorescent
antibody tests. Blood samples are collected aseptically from the jugular veins of infected horses after the organism has been demonstrated in Giemsa stained smears. Second samples are taken one week later. Harvested sera are stored at -20°C, and thawed immediately before use.

The same antigen (histoplasmin) is used for tube agglutination, passive haemagglutination and skin hypersensitivity testing. It consists of eight-week-old mycelial forms of *H. farciminosum* isolates from clinical cases. The mycelial/spore growth is scraped from the medium aseptically, ground in a porcelain mortar and resuspended in sterile saline. The suspension is then sonicated for 20 minutes. For standardisation, the sonicated suspension is filtered through four layers of sterile gauze and diluted in sterile saline. Various dilutions of antigen, corresponding to known wet weight of cells, are tested in chessboard titrations with known antisera to determine the optimum antigen dilution. Usually, an antigen dilution of 1:160 is satisfactory for agglutination and passive haemagglutination tests. In the tube agglutination test, the test sera are diluted 1:8, followed by serial doubling dilutions to 1:64. The tubes are incubated at 37°C in a water bath for two hours before recording the final titres. Each serum dilution is tested in replicates and the average titer is recorded.

In the passive haemagglutination test, tannic-acid-treated sheep red-blood cells are used as the antigen carrier. Sheep blood is aseptically obtained from a single healthy sheep. The blood is collected in an equal volume of Alsever's solution and refrigerated at 4°C for 3-7 days. The cells are sedimented at 1,250 rpm for 10 minutes and washed three times in saline. The cells are then suspended in buffered saline (10 ml physiological saline, 23 ml 0.15 M KH₂PO₄, 76 ml 0.15 M Na₂HPO₄; pH 7.2) to form a 2.5% suspension.

Tannic acid solution (1:20,000) is freshly prepared, mixed with an equal volume of the red blood cell suspension and incubated at 37°C for 15-30 minutes. The cells are sedimented by centrifugation, washed in buffered saline and resuspended in saline to the original volume of the red blood cell suspension. To determine the optimum antigen dilution, varying dilutions of the antigen preparation are mixed with equal volumes of tanned red-blood cells and incubated in a 37°C water bath for 60 minutes. The red cells are collected by centrifugation, washed three times in buffered saline and resuspended in saline to make a 1% cell suspension. Sera to be tested are inactivated by heating at 56°C for 30 minutes and absorbed with an equal volume of washed, normal, sheep red blood cells. Serial twofold dilutions of serum in 1:100 normal rabbit serum are prepared, starting at 1:8 dilution. A 0.5 ml volume of each serum dilution is placed in a 13 x 75 mm tube. Antigen coated tanned red blood cells (0.05 ml) are added and the mixture is gently shaken. The tubes are placed on a mirror reading rack and observed at 2 and 12 hours.

Haemagglutination is detected when the red cells form a uniform layer on the bottom of the tube. A negative test is indicated by the presence of a "button" of red cells at the bottom of the tube. The titre is expressed as the reciprocal of the final dilution in which complete agglutination occurs. The test is duplicated on each serum sample and the average of the reactions is reported. The normal controls for this procedure, including sera from noninfected horses, are performed.
3. **Skin hypersensitivity test**

The same antigen is diluted 1:100 for use in the hypersensitivity test. The antigen is tested for sterility on Sabouraud's dextrose agar at 26°C for four weeks. A volume of 0.2 ml is injected intradermally in the caudal cervical area. A positive reaction is a local elevated induration at the injection site 48-72 hours post injection. The skin reaction subsides gradually and disappears by approximately seven days.

### B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

No vaccines are available for this disease.

### REFERENCES


EQUINE ENCEPHALOMYELITIS (EASTERN and WESTERN) (B37)

SUMMARY

Eastern and western equine encephalomyelitis viruses cycle between birds and mosquitoes; the disease occurs sporadically in horses and humans from mid summer to late fall. 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 an often-fatal disease, while western equine encephalomyelitis (WEE) virus can cause a subclinical or mild disease with less than 30% mortality.

EEE and WEE viruses cause disease in humans, and 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 in a biocontainment facility.

Identification of the agent: A presumptive diagnosis of EEE or WEE can be made when susceptible equines display the characteristic somnolence and other signs of neurologic disease when hematophagous insects are active. There are no characteristic gross lesions. Histological 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.

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

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

A. DIAGNOSTIC TECHNIQUES

Eastern equine encephalomyelitis (EEE) and western equine encephalomyelitis (WEE) viruses are members of the genus Alphavirus (arbovirus serogroup A) of the family Togaviridae. The viruses cycle between birds and mosquitoes; from mid summer to late fall, clinical disease may be observed in humans and horses, which are tangential dead-end hosts. EEE is usually a fatal disease of horses and has been diagnosed in Quebec and Ontario in Canada, the states east of the Mississippi River in the United States, 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 United States and Canada, Mexico, and Central and South America. Highlands J, a virus antigenically related to WEE virus, has been isolated in the eastern United States. Although it is generally believed not to cause disease in domestic animals (6, 7, 10), it has been isolated from the brain of a horse dying of encephalitis in Florida (3).

Even though the mortality is lower for WEE, the clinical signs of EEE and WEE can be identical. Following an incubation period of one to three weeks, clinical signs include fever, anorexia, and depression. In severe cases, the disease 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. WEE virus infection in horses is often observed over a wide geographical area, e.g. sporadic cases over 1,000 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 domestic fowl, primarily pheasants, chukars, and quail. Most encephalomyelitis infections in domestic fowl are caused by EEE virus and occur in the East Coast states of the United States. The virus is introduced by mosquitoes, but transmission within the flocks is primarily by feather picking and cannibalism.

EEE virus causes severe disease in humans, with a mortality rate of approximately 65% 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 done in a Biosafety Level 2 laboratory using biological safety cabinets, and personnel must be immunised against EEE and WEE (9). Precautions should also be taken to prevent human infection when performing necropsy examinations on horses suspected of being infected with the equine encephalomyelitis viruses.

1. Identification of the agent

The most definitive method for the diagnosis of WEE or EEE is the isolation of the virus (10). EEE virus can usually be isolated from the brains of horses unless more than five 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 the 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 tissues be collected and split, one set for virus isolation and the other set in formalin for histopathological examination. Specimens for virus isolation should be shipped 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 solution (pH 7.8) containing 0.75% bovine serum albumin (fraction V), 100 units/ml penicillin, and 100 µg/ml streptomycin. The suspension is clarified by centrifugation at 1,500 g for 30 minutes.

A newborn mouse is considered to be the most sensitive host system. One- to four-day-old mice (one or two litters) are inoculated intracranially with 0.02 ml of inoculum. Mice are held for seven days; dead mice are collected daily and frozen at -70°C. A second passage is made only if virus cannot be identified from mice that die following inoculation.

The chicken embryo is considered less sensitive than the newborn mouse when used for primary isolation of EEE and WEE viruses. Tissue suspensions can be inoculated by the yolk-sac route into 6- to 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 seven days, but deaths usually occur between two and four days post-inoculation. Usually only one passage is made, unless there are dead embryos from which virus cannot be isolated. Newly hatched chicks 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, which are generally considered to be less sensitive than are newborn mice. The most commonly used cell cultures are primary chicken or duck embryo fibroblasts, continuous cell lines of African green monkey kidney (VERO), or baby hamster kidney (BHK21). Isolation is usually attempted in 25-cm² cell culture flasks. Confluent cells are inoculated with 1 ml of tissue suspension.

Following a one-hour absorption period, cells are washed and maintenance medium is added. Cultures are incubated for seven days, and one blind passage is made. EEE and WEE viruses will produce a cytopathic effect (CPE) in cell culture. Cultures that appear to be infected are frozen. The fluid from the thawed cultures is used for virus identification.

When the 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 buffer; egg and cell culture fluids are used undiluted or diluted 1:10 in veronal buffer. The fluid or suspension is centrifuged at 9,000 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 (8). 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.

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
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to 14 days apart. Most horses infected with WEE virus have a high antibody titre when clinical disease is observed. Horses infected with EEE virus usually have antibody titres in the acute stage of the disease, but the titre is lower than with WEE virus. Consequently, a presumptive diagnosis can be made if an unvaccinated horse with appropriate clinical signs has antibody against only EEE or WEE virus (2). The detection of IgM antibody by the ELISA test can also provide a presumptive diagnosis of acute infection. The plaque reduction neutralisation (PRN) test or, preferably, a combination of PRN and HI tests are the procedures most commonly used for the detection of antibody against EEE and WEE viruses. CF antibody against both EEE and WEE virus appears later and does not persist; consequently, it is less useful for the serological diagnosis of disease.

a) Enzyme-linked immunosorbent assay

The ELISA is performed by coating flat-bottom plates with anti-equine IgM capture antibody and adding the following reagents sequentially to each plate: horse test serum, purified viral protein, EEE or WEE hyperimmune mouse ascitic fluid, goat anti-mouse IgG conjugated with horseradish peroxidase, and substrate (2). Reagents are diluted with phosphate buffered saline (PBS) with 10% fetal bovine serum and plates are incubated for 60 minutes after adding each reagent and then washed six times with PBS containing 0.05% Tween 20. An alternate procedure is to incubate overnight after anti-equine IgM capture antibody and the purified viral protein are added. Plates are read at an optical density of 409 nm.

b) Plaque reduction neutralisation

The PRN test is very specific and can be used to differentiate between WEE and EEE virus infections. There are cross-reactions between antibody against EEE and WEE virus on the CF and HI tests. The PRN test is performed in duck embryo fibroblast, VERO, or BHK21 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 onto confluent cell culture monolayers in 25-cm² flasks or dishes 6 cm in diameter. The inoculum is adsorbed for one hour, followed by the addition of 6 ml of overlay medium. The overlay medium consists of two solutions that are prepared separately. Solution 1 contains medium with antibiotics; solution 2 contains 2% Noble agar. Equal volumes of solutions 1 and 2 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, as compared with the virus control flasks, which should have 100 plaques.

c) Haemagglutination inhibition

For the HI test, kaolin-treated serum is diluted from 1:10 to 1:320. Four to eight haemagglutinating units of EEE and WEE antigen (see point d below) are added to the serum, and the plates are incubated at 4°C overnight. Washed goose erythrocytes, at the optimum pH for the antigen, are added and the plates are incubated at 37°C for 30 minutes.
d) Complement fixation

The EEE and WEE antigens for the CF and HI tests are produced from brains of infected mice by extraction with sucrose and acetone. The antigen is inactivated by treatment with 0.3% β-propiolactone. The CF test procedure is as outlined for virus identification except that the antigens are infected and normal mouse brain extracted with sucrose and acetone. The antibody titre is the reciprocal of the highest dilution that will produce 100% haemolysis.

3. Pathology

Gross lesions are rarely observed 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 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 (5, 10).

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. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Commercial inactivated vaccines against EEE and WEE viruses are available. Live attenuated EEE and WEE virus vaccines have not proven satisfactory. The vaccines licensed for use in the United States 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 influenza 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 (4).

1. Seed management

a) Characteristics

Reference strains of EEE and WEE viruses that were isolated over 20 years ago have been used for vaccine production and have proven to produce good immunity. Strains of EEE that differ antigenically and in molecular structure
have been identified from different geographic regions (6). Strains of WEE virus isolated from different countries have been found to be similar both by monoclonal antibody testing and RNA oligonucleotide fingerprinting analysis (7). 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) Culture

Primary chick embryo fibroblasts have been used for propagation of viruses used for vaccine production (4). 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 each subculture should be tested for extraneous agents including bacteria, fungi, mycoplasma, and viruses. The master seed viruses should also be tested before inactivation to ensure identity and freedom from bacteria, fungi, mycoplasma, and extraneous viruses. The residual free formalin content in the inactivated vaccine should not exceed 0.3% formaldehyde.

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

2. Manufacture

The details of the manufacture of currently available vaccines are not available. The virus and cell culture system should be selected so that a high virus titre, ≥ 10^6 median tissue culture infective doses (TCID$_{50}$) per ml, is obtained in less than 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 1:2,000 (0.05%) and holding at 37°C for 24 hours. Residual formalin is neutralised by sodium bisulfite (4).

3. In-process control

Cultures should be examined daily for virus-induced CPE. 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 of contamination of biological materials may be found in the chapter on General Information.

b) **Safety**

The inactivated vaccine is safety tested by inoculating at least ten 6- to 12-hour-old chickens by the subcutaneous route with 0.5 ml of the vaccine. The chickens are observed each day for 10 days for unfavorable reactions that are attributable to the vaccine (1). Safety testing can also be done by inoculating at least eight one- to four-day-old mice intracerebrally with 0.02 ml of the vaccine and observing for seven days.

c) **Potency**

Potency testing is performed by inoculating each of 10 guinea pigs with one half the horse dose on two occasions, 14-21 days apart, by the route recommended for the horse. Fourteen to 21 days following the second dose, serum is collected and tested using the PRN test. The EEE titres should be $\geq 1:4$, and the WEE titres should be $\geq 1:32$ (1).

**REFERENCES**


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 can remain viraemic carriers for life and will yield a positive serological result. Antibody response usually persists and antibody-positive animals are identified as virus carriers. They can potentially transmit the infection to other horses (6, 7).

Identification of the agent: Virus from a horse can be isolated by inoculating suspect blood into a susceptible horse or onto leucocyte 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 immunodiffusion tests. A successful virus isolation in horse leucocyte cultures is confirmed by the detection of specific EIA antigen in an immunodiffusion test, by immunofluorescence assay, or by the inoculation of culture fluids into susceptible horses. Virus isolation is rarely attempted because of the time and expense involved.

Serological tests: Agar gel immunodiffusion (AGID) tests are simple, accurate tests for the demonstration of EIA virus infection. Although other serological tests such as enzyme-linked immunofluorescent assay (ELISA) (20) or indirect fluorescent antibody tests (22) will detect antibodies somewhat earlier and at lower concentrations, these tests do not have a significant advantage over AGID. This is because precipitating antibody increases rapidly in infected animals. AGID also has the advantage of distinguishing between EIA and non-EIA antigen-antibody reactions by lines of identity. The precipitating antigen is prepared from infected tissue cultures (1, 6).

Requirements for biological products: There are no biological products available.

A. DIAGNOSTIC TECHNIQUES

Equine infectious anaemia (EIA) occurs worldwide. The disease is characterised by recurrent febrile episodes, 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. Once a horse is infected with the virus its blood is liable to remain 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 (3, 13).
Transmission occurs by transfer of blood cells 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 (11, 18).

Immunodiffusion provides an accurate and reliable test for the detection of EIA in the horse, except for animals in the early stages of infection and foals of infected dams (6, 8). 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 get high enough to be detectable (21).

The EIA virus is a lentivirus, a sub-family of the retroviruses which also includes maedi-visna virus, caprine arthritis-encephalitis virus and the human immunodeficiency viruses (2, 14). Nucleic acid sequence comparisons have demonstrated a marked relatedness (4, 19, 23).

1. Identification of the agent

Virus isolation is usually not necessary to make a diagnosis.

Isolation of virus from suspect horses may be made by inoculating their blood onto leucocyte cultures prepared from horses free of infection. Any virus produced in such cultures can be confirmed by the detection of specific EIA antigen, by ELISA (20), by immunofluorescence assay (22), or by sub-inoculation into susceptible horses.

When the state 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 and shown to be free of infection is given an immediate blood transfusion from the suspect horse and its antibody status and clinical condition 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 leucocytes from such a volume (5).

2. Serological tests

Precipitating antibody is rapidly produced as a result of EIA infection and can be detected by AGID. Specific reactions are indicated by reactions of identity between the positive reference serum and the test serum. Horses in the first 2-3 weeks after infection will give negative serological reactions.

a) Preparation of antigen

Specific EIA antigen may be prepared from the spleen of acutely infected horses (6), from infected equine tissue culture (12), or from a persistently
infected canine thymus cell line (1). Preparation from infected cultures gives a more uniform result and allows for a better standardisation of reagents.

To obtain a satisfactory antigen from spleen, a horse must be infected with a virulent strain of EIA virus. The resulting incubation period should be 5-7 days, and the spleen should be collected 9 days after infection, when the virus titre is at its peak and before any detectable amount of precipitating antibody is produced. Neat spleen pulp is used in the immunodiffusion test as antigen (7, 9).

Equine fetal kidney or dermal cells are infected with a strain of EIA virus adapted to grow in tissue culture (American Type Culture Collection VR-778). Virus is collected from cultures by precipitation with 8% polyethylene glycol or by pelleting using ultracentrifugation. The diagnostic antigen p26 is released from the virus by treatment with detergent or ether (12).

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 (10, 15).

b) Preparation of reference 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 identity with a known reference serum. It is essential to match 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.

c) Carrying out the AGID test (9, 16, 17)

Immunodiffusion reactions are carried out in a layer of agar either on slides or in petri dishes. Three millilitres of 1% Noble agar is used for slides. For petri dishes 100 mm in diameter, 15 ml of 1% (or 18 ml of 0.7%) Noble agar is used. Six wells are punched out of the agar surrounding a central well of the same diameter. The wells are all 2 mm apart on slides. In petri dishes the wells are 2.4 mm apart and 5.3 mm in diameter. The antigen is placed in the central well and the reference antiserum is placed in alternate exterior wells. Suspect serum samples are placed in the remaining 3 wells. (It is also possible to place the reference antiserum in two opposite exterior wells and the suspect serum samples in the remaining 4 wells.)

The tests are maintained at room temperature in a moist environment. After 24-48 hours the precipitation reactions are examined over a narrow beam of intense 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 reference reagents. A weakly positive reaction is indicated by a slight bending
of the reference 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 and to fade with time. These are not difficult to recognise because they will often dissolve the reference line about half-way across its normal position. Such reactions can be confirmed as specific for EIA by dilution prior to retesting, when a more distinct line of identity will be obtained. Sera devoid of EIA antibody will not form precipitation lines and will have no effect upon the reactions of the reference reagents.

d) Interpretation

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 in a week or two. 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 must be allowed for the maternal antibody to wane; the foal is then re-tested to determine whether any initial positive reaction was due to maternal antibody.

Reagents for EIA serological tests such as AGID or ELISA are available commercially in various countries. A reference antiserum for immunodiffusion which contains the minimum amount of antibody that should be detected by laboratories is available from the OIE Reference Laboratories: Dr J.R. Pearson, Veterinary Services Laboratories, USDA, P.O. Box 70, Ames, Iowa 50010, USA; Prof. B. Toma, Veterinary School of Alfort, 94701 Maisons-Alfort, France; or Dr. H. Sentsui, National Institute of Animal Health, Hokkaido Branch, 4 Hitsujigaoka, Toyohira-ku, Sapporo, Japan 004.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

No biological products are available.

REFERENCES


SUMMARY

Equine influenza is caused by two members of the genus Influenzavirus of the family Orthomyxoviridae. Diagnosis of influenzavirus infections of horses is based on virus isolation from horses with acute respiratory illness or on the demonstration of increased antibody titres of paired sera taken in the acute and convalescent stages of the disease. Ideally, both methods are used.

Identification of the agent: Embryonated chicken eggs or cell cultures can be used for virus isolation from nasal swabs or washings from guttural pouches. Subtype 2 strains grow equally well in both systems but subtype 1 strains are quite refractory to propagation in cell cultures.

Viral growth is monitored by haemagglutination (HA) or in cell cultures by haemadsorption (HAD) using chicken or guinea pig erythrocytes (RBC). If HA or HAD are positive, isolates can be typed by haemagglutination inhibition (HI) using strain-specific antisera. Isolates should always be sent to International Reference Laboratories (OIE or WHO) for typing and strain collection. Samples which yield negative results (i.e. showing no HA or HAD after one passage) should be repassaged.

Serological tests: Diagnosis of influenzavirus infections can usually only be done by tests on paired sera; the first sample should be taken as soon as possible after the onset of clinical signs and the second about two weeks later. Sera are heat-inactivated and pre-treated to reduce non-specific reactions. Antibody titres are determined by HI or single radial haemolysis (SRH). For SRH, serial dilution of sera is usually not required. HI and SRH give comparable results but SRH is superior if large numbers of sera are to be tested.

Requirements for biological products: Equine influenza virus vaccines contain inactivated strains of subtypes 1 and 2 and induce humoral antibody responses after vaccination.

A. DIAGNOSTIC TECHNIQUES

Equine influenza is caused by two members of the genus Influenzavirus of the family Orthomyxoviridae and consists of two subtypes, subtype 1 (H\textsubscript{7}N\textsubscript{7}) and subtype 2 (H\textsubscript{3}N\textsubscript{8}). These are not genuine pathogens for man, although horses can be infected with human influenza virus subtypes. These infections are unusual but can represent a potential biohazard to laboratory personnel.

All influenza viruses are highly contagious for susceptible hosts, including embryonated chicken eggs and cell cultures. Care must therefore be taken during
the handling of infected eggs or cultures (1). Reference strains should not be propagated in the diagnostic laboratory, at least never at the same time or in the same place as where diagnosis is done. 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 nasal swabs or washings, swabs taken at endoscopy or washings from the gullet pouches. Swabs consist of cotton wool on applicator sticks which should be transferred to a vial containing transport medium immediately after use. This medium consists of phosphate buffered saline (PBS) containing 40% glycerol. 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 -60°C or below. Samples should also be transported at these temperatures.

Only one sample is processed at a time. The liquid is expelled from the swab, which is suitably disposed of, and 0.1 ml of an antibiotic solution is added to 0.9 ml of the fluid. The remainder of the fluid is stored at -60°C. The antibiotic solution contains gentamycin (1 mg/ml), or penicillin (1000 units/ml) and streptomycin (500 µg/ml). Samples treated with antibiotics are centrifuged at 1,500 g for 15 minutes to remove bacteria and debris; the supernatant fluids are used for inoculation.

1. Identification of the agent

Embryonated chicken eggs

Fertile eggs are set in a humidified incubator at 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 drilled through the shell. Three eggs/sample are inoculated (0.1 ml) into the amniotic cavity. The syringe is withdrawn about 1 cm and a further 0.1 ml inoculated into the allantoic cavity. The hole is sealed with wax and the eggs incubated at 34-37°C for 2-3 days.

The eggs are then transferred to 4°C overnight to kill the embryos and to reduce bleeding at harvest. The shells are disinfected and the allantoic fluid is harvested by pipette, each harvest being kept separate. These are tested for HA activity using a 0.5% suspension of chicken erythrocytes (RBC) or 0.4% guinea pig RBC. If there is no HA activity, aliquots of each harvest are pooled and re-passaged into further eggs. All HA positive samples are divided into aliquots and stored at -70°C; one aliquot is titrated for HA immediately and if it has a titre of 1:16 or more, the isolate is typed. Non-agglutinated RBC form a "stream" when the tube or plate is tilted.

Cell cultures

Cultures of the Madin-Darby canine kidney cell line (MDCK, ATCC CCL34) may
be used to isolate equine influenza viruses. The cells are grown in tubes and infected in triplicate when grown to confluence with 0.25-0.5 ml of each sample, processed as described above. The cultures are maintained with medium containing 2 μg/ml trypsin, and examined daily for evidence of cytopathic effect (CPE). If positive, or after 7 days in any case, the supernatant fluids are tested for HA. Fluids with titres of 1:16 or more are typed immediately. Negative fluids are repassaged.

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 washed with PBS. One to two drops of a 50% suspension of chicken or guinea pig RBC are added, the tubes rotated carefully, and kept at room temperature for 30 minutes. Unbound RBC are washed off with PBS and the cultures examined microscopically for evidence of HAD.

Typing of new isolates of equine influenza viruses is best done by HI using specific antisera, prepared preferably in ferrets. Isolates may first be treated with Tween-ether, which destroys viral infectivity and reduces the risk of cross-contaminations.

For HI, both macro- and microtests may be used. The antigens are diluted to final HA titres of 1:4 or 1:8. Antisera, treated to reduce non-specific haemagglutinins, are serially diluted in PBS, antigen is added and the mixtures reacted for 30 minutes at room temperature. The RBC (0.5% chicken or 0.4% guinea pig) are added, gently mixed and allowed to stand at room temperature for 30 minutes. The volumes of the microtest are 0.025 ml for PBS, sera and antigen, respectively, followed by 0.05 ml for the RBC suspension. In the macrotest, 0.5 ml volumes are used throughout. The HI titres are read as the highest dilution of serum giving a complete inhibition of agglutination, i.e., the RBC form a "stream" when the plates are tilted. Incomplete inhibition is always scored as agglutination. All isolates should be sent to the International Reference Centres/Laboratories designated by OIE and WHO.

Standard antigens must be titrated in parallel with these tests and should include: subtype 1 (A/eq/Prague/56) and subtype 2 (A/eq/Miami/63; A/eq/Fontainebleau/79; A/eq/Kentuckay/81; or A/eq/Solvalla/79). Additionally, recent isolates from the same geographical area should be included if available. The standard antigens should be treated with Tween-ether to avoid cross-contamination. Test antigens and standard antigens are always back-titrated to confirm their antigen content.

Neuraminidase

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

2. Serological tests

Infections are detected by conducting 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 methods exist, each equally efficient and widely used. The complement fixation (CF) test can also be applied but the technique is not standardised.

a) Haemagglutination inhibition (HI) test

The standard antigens are described above. If available, isolates from recent cases should be included. The antigen is first treated with Tween-ether. Lyophilised treated antigens for equine influenza viruses are available from the OIE Reference Laboratories. The test is best done in microtitre plates using the appropriate dilution equipment.

Sera are pre-treated to remove non-specific haemagglutinins and are inactivated by heating at 56°C for 30 minutes. Pre-treatments include the use of one of the following: (i) Kaolin and RBC absorption, (ii) Potassium periodate, or (iii) V. cholerae receptor-destroying enzyme (RDE). All three procedures yield similar results. The treated sera are diluted in PBS, antigen with a titre of 1:4 or 1:8 is added, and these are kept at room temperature for 30 minutes after gentle mixing. RBC are added and the test is read 30 minutes later. The HI titres are the highest dilutions of serum that completely inhibit HA. Titre increases of 4-fold or more between paired sera indicate recent infections.

b) Single radial haemolysis (SRH)

In this test, viral antigens are coupled to fixed RBC 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 RBC, resulting in a clear, haemolytic zone around the well; the size of this zone can be measured.

Special immunodiffusion plates (Hyland; Miles Scientific) may be used for the assay, but simple petri dishes are also suitable. Sheep RBC are collected into Alsever's solution and washed 3 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 RBC by potassium periodate or, in the case of purified preparations, by chromic chloride. The coupled antigen-RBC preparations are mixed with C', together with a 1% solution of agarose (low melting grade) in PBS. Care must be taken that the temperature not be allowed to rise above 42°C at any time. The mixture is poured into plates and left overnight at 4°C. Wells of 2-3 mm in diameter and 12 mm apart are punched in the solidified agarose 6 mm from the edge of the plates. Such plates may be stored at 4°C for several weeks. Plates are prepared for each antigen and pre-tested with known positive and negative antisera.

For safety reasons, all sera are heat-inactivated (at 56°C for 30 minutes) but no further treatment is necessary. Paired sera should be assayed in duplicate on
the same plate; all sera are tested in a plate containing all components except virus. Sera that show haemolytic activity for sheep RBC must be pre-absorbed with sheep RBC. Zones of lysis should be clear, and not hazy or translucent. All clear zones should be measured and the area of haemolysis calculated. Differences in diameter between duplicate samples must be less than 1.5-fold. In paired sera, differences in diameters of 2-fold or more are considered significant for infection. Sera with differences between 1.5-fold and 2-fold should be retested.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

Equine influenza virus vaccines consist of inactivated virus subtypes 1 and 2, or their subunits, that induce sufficient humoral antibody responses in seronegative horses that are susceptible to these agents (2).

1. **Seed management**

   a) **Characteristics**

   The vaccine must contain the following influenza virus strains:

   **Subtype 1:** A subtype 1 strain, such as A/eq/Prague/1/56; but other subtype 1 strains may be used.

   **Subtype 2:** Two subtype 2 strains are available:

   i) One classical strain (i.e. isolated before 1979), such as A/eq/Miami/1/63; but other strains may be used if a very close antigenic relationship to the above strain has been demonstrated.

   ii) One strain isolated following the antigenic drift of 1979-81, such as A/eq/Fontainebleau/79, A/eq/Kentucky/81 or A/eq/Solvalla/79. Other strains may be used if they are shown to be either closely related to the three preceding strains or of local importance.

   The prevalence and antigenic variation of current isolates of equine influenza viruses must be monitored continuously, preferably by OIE or WHO Reference Laboratories. If the results of such studies indicate a significant antigenic drift, drifted strains should also be included.

   b) **Culture**

   Virus strains may be obtained from OIE or WHO Reference Laboratories or from the ATCC (American Type Culture Collection, Rockville, MD 26852, USA). The strains are propagated in the allantoic cavity of 10-day-old embryonated chicken eggs. All manipulations must be conducted separately for each strain. Viral growth is monitored by HA tests. Passaged virus is identified by serological tests, such as HI or SRH. Seed virus is divided into
aliquots and stored in freeze-dried form or at -70°C.

2. 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- to 11-day-old embryonated chicken 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.

3. In-process control

In-process controls include:

a) Identity of virus strains (tested by HI),

b) Sterility,

c) Virus titre (titration in embryonated eggs), and

d) Haemagglutination (tested by HA).

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

b) Safety

Using not less than two horses, each horse is inoculated intramuscularly at two different sites with the dose of vaccine specified by the manufacturer; these are repeated four weeks later. The animals are kept under observation for 10 days after the second injections. No abnormal local or systemic reactions should ensue.

To test for completeness of inactivation, inoculate 0.2 ml of the vaccine into the allantoic cavity of each of 10 embryonated chicken eggs, 9-11 days old. Incubate at a suitable temperature for 3 days. The death of any embryo within 24 hours of inoculation is considered to be non-specific and the embryo is discarded. The allantoic fluids are collected, pooled and passaged into further eggs in the same way. After 4 days’ incubation, the allantoic fluids of these eggs should possess no HA activity.

c) Potency

Horses

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 stated on the label as that between the first and second injection, inject a volume corresponding to the second dose of vaccine.

Collect two blood samples from each animal, the first one week after the first vaccination and the second two weeks after the second vaccination. Separate the serum from each sample. Inactivate each serum by heating at 56°C for 30 minutes. To one volume of each serum add 3 volumes of PBS pH 7.4 and 4 volumes of a 25% (w/v) suspension of kaolin R in the same buffer solution. Shake each mixture at room temperature for 10 minutes. Centrifuge, collect the supernatant fluid and mix it with a concentrated suspension of chicken RBC. Allow to stand at 37°C for one hour and centrifuge. The dilution of serum that results is now 1:8. Other treatments of sera may also be used (see section A2).

Perform tests on each serum using, respectively, the antigen or antigens prepared from the strain or strains used in the production of the vaccine. Using each diluted serum, prepare a series of 2-fold dilutions. To 0.025 ml of each of the latter, add 0.025 ml of a suspension of each antigen respectively treated with ether or Tween-ether and containing 4 haemagglutinating units (HAU). Allow the mixtures to stand at room temperature for 30 minutes and add 0.05 ml of a 0.5% suspension of chicken RBC. Allow to stand at room temperature for one hour and note the last dilution of serum that still completely inhibits HA.

The antibody titre of each serum taken after the second vaccination in each test calculated for the original serum is not less than 1:64, taking into account the pre-dilution of 1:8. If the titre found for any horse after the first vaccination indicates that there has been an anamnestic response, that 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 tests for potency in horses have been carried out with satisfactory results on a representative batch of vaccine, this test 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.

Guinea pigs

Into each of 10 guinea pigs free from specific antibodies, inject the dose stated on the label. Twenty-one days later, collect blood samples and separate the serum. Inactivate each serum by heating at 56°C for 30 minutes and treat the sera as described above for horse sera.

Perform the tests on each serum using, respectively, the antigen or antigens prepared from the strain or strains used in the production of the vaccine. Using each diluted serum, prepare a series of 2-fold dilutions. To 0.025 ml of
each dilution, add 0.025 ml of a suspension of each antigen respectively treated with ether or Tween-ether and containing 4 HAU. Allow the mixtures to stand at room temperature for 30 minutes and add 0.05 ml of a 0.5% suspension of chicken RBC. Allow to stand at room temperature for 1 hour and note the last serum dilution that still completely inhibits HA. The antibody titre of each serum in each test before mixture with the suspension of antigen and RBC should not be less than 1:16, taking into account pre-dilutions by treatment.

The European Pharmacopoeia may also be used as a reference for the control of equine influenza virus vaccines.

REFERENCES

Review articles


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

Parasites occur in the blood of infected animals. The introduction of carrier animals into areas where tick vectors are prevalent can lead to an epidemic 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 Babesia 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 B. equi are less than 2-3 μm long, and are round or ameboid. A characteristic of B. equi is the arrangement of 4 parasites in a tetrad or "Maltese cross".

When equivocal results are encountered in serological tests the subinoculation 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 cells examined for parasites. Alternatively, a specific tick vector is fed on a suspect animal and Babesia may then be identified either in the vector or through their 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 by the United States Department of Agriculture for the importation of horses. Other serological techniques are the indirect fluorescent antibody (IFA) tests and enzyme-linked immunosorbent assays (ELISA). The IFA test can be used to distinguish between B. equi and B. caballi infections. Differentiation between weak positive and negative reactions requires considerable experience. ELISA may be used to detect antibodies to both species in infected horses, although cross-reactions between B. equi and B. caballi occur, so that this cannot yet be recommended as a differential diagnostic test.
Requirements for biological products: There are no biological products available.

A. DIAGNOSTIC TECHNIQUES

Equine piroplasmosis or babesiosis is a tick-borne protozoal disease of horses, mules, donkeys and zebra. The aetiological agents of equine piroplasmosis are *Babesia 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 *B. equi*, while eight of these species were also able to transmit *B. caballi* infections trans-ovarially (2, 16, 21, 22). Infected animals may remain carriers of these blood parasites for long periods and will act as sources of infection for vector ticks.

The parasites occur in southern Europe, Asia, the USSR, Africa, Cuba, South and Central Americas and certain parts of the southern United States. *B. equi* has also been reported from Australia, and is now believed to have a wider general distribution than *B. caballi*.

1. Identification of the agent

Horses that are already infected may be identified by demonstrating the parasites in stained blood or organ smears. For this, Romanovsky-type staining methods such as the Giemsa method usually give the best results (20).

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 (12).

An accurate identification of the species of the parasite is sometimes desirable, as mixed infections of *B. equi* and *B. caballi* probably occur frequently.

a) Clinical signs

The clinical signs of equine piroplasmosis are often non-specific, 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 smaller and drier faecal balls 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 animal may show signs of mild colic. Mild oedematous swelling of the distal part of the limbs sometimes occurs.

Chronic cases usually present non-specific 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 (10).

b) The parasites

During the life cycle of Babesia, the merozoites invade erythrocytes where they transform into trophozoites (5, 18). 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 erythrocytes and the division process is then repeated.

Babesia caballi: the merozoites in the erythrocyte are pear-shaped, 2-5 µm long by 1.3-3.0 µm in diameter (9). The paired merozoites joined at their posterior ends are considered to be a diagnostic feature of B. caballi infection (13).

Babesia equi: the merozoites of this organism are relatively small, less than 2-3 µm long (9), and are round or ameboid. Often 4 parasites are to be found together in the form of a tetrad or so-called "Maltese cross" arrangement. This is a characteristic feature of B. equi (8).

It has been shown (19) that sporozoites inoculated into horses via a tick bite invade lymphocytes. The sporozoites undergo development in the cytoplasm of these lymphocytes and eventually form Theileria-like schizonts. Apparently, merozoites released from these schizonts enter other erythrocytes. The taxonomic position of B. equi appears to be uncertain at present, and further investigation into its life cycle is necessary.

2. Serological tests

It is extremely difficult to diagnose the organisms in carrier animals by means of the microscopical 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 specifications of that laboratory. Horses for export that have been subjected to serological tests and shown to be free of infection, should be kept free of ticks to prevent accidental infections.
A number of serological techniques have been used in the diagnosis of babesiosis, such as CF, IFA, and ELISA.

a) Complement fixation

The CF test is the primary test used by some countries to qualify horses for importation (23). A detailed description of antigen production and test protocol has been given by inter alia the United States Department of Agriculture (1, 4, 25). 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, the IFA test has been accepted by the USDA for use as a supplementary test.

Antigen production: Blood is collected, in an equal volume of Alsever's solution as an anticoagulant, from horses at the height of the reaction (3-7% parasitaemia for B. caballi and 60-85% for B. equi). The plasma-Alsever's supernatant and buffy coat is removed when the red blood cells have settled to the bottom of the flask. The red blood cells 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 (1-5% w/v) is added as stabiliser and the preparation is mixed on a magnetic stirrer for 30 minutes, strained through two thicknesses of sterile gauze, dispensed into 2 ml amounts and freeze-dried. The antigen can then be stored at below -50°C for several years.

Test procedure: The specificity and potency of each batch of antigen should be checked against reference antisera of known specificity and potency. Optimal antigen dilutions are also determined in a preliminary block titration.

Test sera are inactivated for 30 minutes at 60°C and tested in dilutions of 1:5 to 1:5,120. Veronal buffer is used for all dilutions. Complement is prepared and titrated spectrophotometrically to determine the 50% haemolytic dose (C'H50) (25). In the test 3.125 C'H50 are used. The haemolytic system consists of equal parts of a 3% sheep red blood suspension and veronal buffer with 5 minimum haemolytic doses (MHDs) of haemolysin (amboceptor) (25).

The test has been adapted to microtitration plates (7). The total volume of the test is 0.125 ml, made up of equal portions (0.025 ml) of antigen, complement (3.125 C'H50) and diluted serum. Incubation is performed for 30 minutes at 37°C. A double portion (0.05 ml) of the haemolytic system is added and the plates incubated for a further 30 minutes at 37°C on a shaker. The plates are centrifuged for one minute at 200 g before being read over a mirror.

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 red blood cells.

Anti-complementary samples are examined by the IFA test. Donkey sera are frequently anti-complementary.

b) Indirect fluorescent antibody test

The IFA test has been successfully applied (11) to the differential diagnosis of B. equi and B. caballi infections. 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 (11, 15).

Each sample of serum is tested against an antigen of B. caballi and of B. equi. Blood for antigen is obtained from horses with a rising parasitaemia, ideally 2-5%. The red cells are washed 3 times in phosphate buffered saline (PBS), pH 7.2. The supernatant fluid and the white cell layer are removed. After the last wash, the packed red cells are reconstituted to normal volume with 4% bovine serum albumin fraction V made up in PBS. That is, the original packed cell volume = 30%, so that a third consists of red cells. If the original red cell volume is 15 ml, then 5 ml of packed red cells plus 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 (15). These slides are allowed to dry, wrapped in soft paper and sealed in plastic bags, and stored at -20°C for up to one year.

Prior to use, the antigen smears are removed from storage at -20°C and incubated at 37°C for 10 minutes. The antigen slides are then removed from their protective covering and fixed in cold dry acetone (-20°C) for one minute. Test sera are applied at appropriate dilutions to the different wells on the antigen slide, incubated at 37°C for 30 minutes, and washed several times in PBS. An anti-horse immunoglobulin prepared in rabbits is conjugated with fluorescein isothiocyanate and applied to the slide, incubated and washed as before. After the final wash, 2 drops of a solution containing equal parts of glycerin and PBS are placed on each slide and mounted with a coverslip. The slides are then examined for the fluorescing parasites. Sera diluted 1:80 or more that show strong fluorescence are usually considered positive. Negative and positive control sera are included in each test; due consideration to the patterns of fluorescence in such controls is important.

c) ELISA

In recent years the ELISA has also been used to detect antibodies to both species of the parasite in experimentally infected horses (6, 14). It is apparent that cross-reactions between B. equi and B. caballi occur (6, 14, 26). More investigation therefore appears to be required before ELISA tests can be recommended as a diagnostic method to differentiate between species of Babesia.
d) DNA probes

Recently, sensitive and specific DNA probes have been isolated for both \textit{B. equi} and \textit{B. caballi} (26). In this test parasite DNA is extracted from a blood sample, spotted onto a nylon membrane and then examined with the relevant radiolabelled DNA probe. These probes have been shown to detect parasites in some carrier animals and could provide a possible solution for the problems experienced with serological tests, where horses are destined for the export market and need to be free of the parasites. However, these tests need further refinement and the sensitivity must be increased before they can be used to certify horses free of either \textit{B. equi} or \textit{B. caballi}.

3. Biological tests

\textbf{Subinoculation of blood:} False negative or false positive reactions may be encountered in the course of serological tests (3, 4, 24). 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 red cells.

In an additional technique, a specific vector tick 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 vector tick to another susceptible animal.

\section*{B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS}

No biological products are available.

\section*{REFERENCES}


SUMMARY

Infection of horses by equine herpesvirus 1 (EHV-1) is worldwide in distribution. The majority of horses possess detectable antibodies by the end of their first year of life. Isolation and identification of virus is required for the diagnosis of EHV-1 infection. Serological tests on individual horses may not provide a definitive diagnosis, except for foals suffering a primary respiratory disease, because clinical evidence of reinfection (abortion or neurological disease) can be observed after the maximum secondary antibody response.

Identification of the agent: Equine herpesvirus 1 exists as two antigenically, biochemically and biologically distinguishable subtypes, designated subtypes 1 and 2 respectively.*

Both subtypes exhibit characteristic tropisms for cells in culture. Subtype 1 infects a whole spectrum of cells whereas subtype 2 replicates preferentially in cells of equine origin. Certain isolates of subtype 2 virus may be adapted to replicate in subcultivable cell lines of porcine or African green monkey origin. Both subtypes produce a cell-associated viraemia and both will produce respiratory disease and abortion. Only subtype 1 has been isolated from cases of neurological disease. This subtype is the more common cause of infections which result in abortion. Subtype 2 is found more commonly in acute respiratory disease of young horses.

The subtypes may be distinguished by their cell preferences, by electropherograms developed from restriction endonuclease dissection of their DNA, or by the use of polyvalent or monoclonal antisera to either or both of the two type-specific envelope glycoproteins.

The virus can be isolated from the nasopharynx of infected horses, but most reliably from the lung, liver or thymus of aborted fetuses. It is not commonly recovered from tissues of the central nervous system, but in the case of horses with neurological disease it can more usually be isolated from the leucocyte fraction of the blood.

Viral antigen may be detected in frozen histological sections by immunofluorescence using a polyclonal antiserum. The specific subtypes can be distinguished by the use of conjugated monoclonal antibody specific for their particular glycoproteins. Infection of fetal tissues may be demonstrated by the

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* According to the International Committee for Viral Taxonomy, they have been designated since 1987 (adopted at the International Congress for Virology, Edmonton, August 1987) as EVH1 and EVH4 viruses, respectively. The former is the more common cause of abortion and the latter is found more commonly in acute respiratory disease of young horses.
occurrence of typical herpesviral intranuclear inclusions in epithelial cells.

Virus isolated in cell cultures is identified by neutralisation tests with known positive antisera or by use of conjugated monoclonal antibodies specific for unique epitopes of the subtypes.

Serological tests: Virus neutralisation, complement fixation and enzyme-linked immunosorbent assay (ELISA) can be used to detect specific antibodies; however, serological tests are of limited value in diagnosis unless there is a significant rise in titre within paired samples of serum. This is because most horses over one year of age will almost certainly have experienced previous infection, and most animals will therefore possess demonstrable antibodies. Complement-fixing antibody titres may persist for only relatively short periods of time (60 days).

Requirements for biological products: Inactivated virus vaccines are available for the control of equine herpesvirus 1 infections in horses.

A. DIAGNOSTIC TECHNIQUES

The isolation and identification of equine rhinopneumonitis virus is necessary for the diagnosis of most infections caused by equine herpesvirus 1 (EHV-1). Since clinically recognisable manifestations or reinfections (abortion or neurological syndromes) may occur subsequent to a maximal secondary antibody response, serological tests on individual horses may fail to provide a definitive diagnosis except in the case of immunologically negative foals suffering a primary respiratory infection.

Infections of horses with EHV-1 are worldwide in distribution. In most equine populations that have been investigated, specific antibodies are demonstrable in the majority of horses by the end of their first year of life.

1. Identification of the agent

Equine herpesvirus 1 exists as two antigenically, biochemically and biologically distinguishable subtypes, designated subtypes 1 and 2 respectively.* These subtypes exhibit characteristic tropisms for cells in culture. Whereas subtype 1 infects a whole spectrum of cell, subtype 2 replicates preferentially in cells of equine origin but may be adapted to replicate in subcultivable porcine or African green monkey cell lines.

The virus may be isolated from the nasopharynx of infected horses, and it can be recovered most reliably from the lung, liver or thymus of aborted fetuses. It is uncommon to recover it from the tissues of the central nervous system, but it may often be isolated from the leucocyte fraction of the blood of horses with herpesviral

* See the footnote on page 1 of this chapter.
neurological disease. Both subtypes produce a cell-associated viraemia and both can produce respiratory disease and abortion. Only subtype 1 has been isolated from neurological disease. Subtype 1 has also been found to be the more common cause of infections which result in abortion. Subtype 2 is more commonly associated with acute respiratory disease in young horses.

In the absence of virological facilities, the virus may be identified in situ in cryostat sections of tissues by immunofluorescence techniques using conjugated polyclonal antisera. Any infection of fetal tissues by EHV-1 may be confirmed by demonstrating typical herpesviral intranuclear inclusions in epithelial cells. Each subtype may be identified by the use of labelled monoclonal antibodies specific for the glycoproteins unique to each subtype. Any viruses isolated in cell cultures can be identified by serum neutralisation tests. The subtypes may be distinguished by their cell culture specificities, by electropherograms developed from restriction endonuclease dissection of their respective DNA, or by the use of either polyvalent or monoclonal antisera to either one or both the type-specific glycoproteins present in the virus envelope.

2. Serological tests

Virus neutralisation, complement fixation and ELISA techniques can be employed to demonstrate antibodies specific for EHV-1. It should be noted that neutralising antibody titres persist for a long time. In the interpretation of the results of serological tests, it must be recognised that most horses aged more than one year are likely to have experienced infection, and most will have demonstrable antibodies. Unless a significant rise in titre between paired samples of serum can be found, the results of serological tests must be regarded as being of little value in diagnosis.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Inactivated viral vaccines designed for the protection of horses against infection with EHV-1 are prepared by formalin inactivation of a virulent strain of subtype 1 that has been propagated in cell cultures prepared from the normal dermal and subcutaneous connective tissues of an equine fetus. An adjuvant can be added to this preparation.

1. Seed management

a) Characteristics

The seed for the vaccine is prepared from a cloned identified strain of EHV-1.

b) Validation as a vaccine

The seed virus and cell system must be shown to be free of equine mycoplasmas and viral contaminants by appropriate techniques. Special tests must be performed for equine viral arteritis, equine infectious anaemia, equine
cytomegaloviruses (EHV-2) and coital exanthema virus (EHV-3).

2. **Manufacture**

Roller bottle cultures are inoculated with sufficient seed virus to produce a one-step viral growth curve. After 6 hours of incubation the medium, which contains serum, is decanted from the cultures. These are then washed with phosphate buffered saline and the medium replaced with serum-free medium. Incubation is continued for 16 to 18 hours longer to allow for the maximum release of virus. Both cells and supernatant medium are harvested, pooled and frozen at -60°C. After thawing, both intact cells and cell debris are removed by low-speed centrifugation and the virus is inactivated by the addition of formalin to a final concentration of 0.2%. After 18 hours, any residual formalin is neutralised by the addition of sodium bisulfite and the pH adjusted to 7.0. It is concentrated 10-fold by vacuum ultrafiltration, after which thimerosal is added as a preservative, together with bovine serum albumin as a stabiliser.

The final product is prepared by the addition of an equal amount of an adjuvant.

3. **In-process control**

The inactivated virus pool is tested prior to concentration to determine its freedom from residual infectious virus.

4. **Batch control**

   a) **Sterility**

   Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

   b) **Safety**

   Safety tests should be performed on a sufficient number of horses. If the vaccine is to be used to prevent abortion in mares, it should be tested on pregnant mares in the second half of gestation.

   c) **Potency**

   Vaccine potency is tested preferentially on horses and, in the case of anti-abortion vaccine, on pregnant mares challenged intranasally with a virulent strain of virus. Potency tests in horses may be replaced, in the case of vaccines containing inactivated EHV-1 subtype 1 virus, by protection of hamsters (*Cricetus auratus*), in antigen extinction titrations, evaluating results by protection of vaccinated hamsters against challenge by a multiple hamster lethal dose of virus. Because the subtype 2 virus is apparently incapable of becoming adapted to hamsters, potency tests for vaccines containing subtype 2 virus may be conducted by demonstrating seroconversion (virus neutralising antibodies) in horses or laboratory animals.
d) Duration of immunity

The vaccine is recommended for the prevention of abortigenic EHV-1 infection of mares by the administration of 2 ml intramuscularly during the fifth, seventh and ninth month of pregnancy. It is recommended for use in non-pregnant horses for the prevention of respiratory disease. Satisfactory immunisation requires repeated vaccinations, commencing during the first year of life. Two injections given 6 to 8 weeks apart constitute the primary series. A third injection should be administered 6 months later, and further single injections given every 4 to 6 months throughout the period in which the horse is actively employed.

e) Stability

The vaccine will maintain its original potency after storage at 2-7°C for at least one year. At least three batches must be tested for shelf life before a conclusion on its stability can be reached.

REFERENCES

Review articles


Glanders is a contagious and fatal disease of horses, donkeys and mules and is caused by infection with the bacterium Pseudomonas mallei (previously classified generically as Pfeifferella, Loefflerella, Mallemomyces or Actinobacillus mallei). 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 easily transmitted to man, in whom 95% of cases are fatal. All infected or potentially infected material must be handled in the laboratory under conditions of biological containment.

Identification of the agent: Smears from fresh material may reveal Gram-negative non-sporulating, non-encapsulated rods. They grow aerobically and prefer media containing glycerol. It is the only Pseudomonas species which is non-motile. Guinea pigs are highly susceptible and males are used for testing of 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. 0.1 ml of 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 is the only serological test found to be of sufficient accuracy to aid diagnosis.

Requirements for biological products: There are no vaccines. Mallein PPD is currently available commercially from institutes in the Netherlands and Romania.

A. DIAGNOSTIC TECHNIQUES

Glanders is a bacterial disease of solipeds with zoonotic potential and has been known since ancient times. Carnivores may become infected by eating infected meat, but cattle, sheep and swine are resistant (8). 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 in 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 inoculation (e.g. via harness). The incubation period is from a few days to many months (9).

Glanders is transmissible to man by direct contact with sick animals or infected materials. In the untreated acute disease there is 95% mortality within three weeks. A chronic form with abscessation also occurs. When handling infected animals or fomites stringent precautions should be taken to prevent self-infection or transmission of the bacterium to other equids. In the laboratory all manipulations with potentially infected material must be performed in conditions of biohazard containment.

Glanders has been eradicated from many countries by statutory testing, elimination of infected animals, and import restrictions. It persists in some Near Eastern, Asian, and African countries.

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). Glanders should positively be excluded from suspected cases of epizootic lymphangitis (caused by *Histoplasma farcinosus*) with which it has many clinical similarities. In man in particular, glanders should be distinguished from melioidosis (*Pseudomonas pseudomallei* infection), which is caused by an organism with close similarities to *Pseudomonas mallei* (8).

a) **Morphology of Pseudomonas mallei**

In smears from fresh lesions the organisms are fairly numerous but in older lesions they are scanty (11). They should be stained by methylene blue or Gram stain. They are mainly extracellular, fairly straight Gram-negative rods with rounded ends, 2-5 μm x 0.5 μm, often stain irregularly and do not have capsules or form spores. *P. mallei* is the only organism in the *Pseudomonas* group without flagellae and is non-motile (5). In tissue sections the organisms may have a beaded appearance and are difficult to demonstrate (7). 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 (7).
The organism is aerobic and facultatively anacrobic, growing optimally at 37°C (6). It grows well but slowly on ordinary culture media; 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 on gelatin growth is poor (10).

Alterations in virulence may occur in vitro, so fresh isolates should be used for identification reactions. Litmus milk is slightly acidified and coagulation may occur after long incubation. Nitrates are reduced. Although some have claimed that glucose is the only carbohydrate which 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 *P. mallei* (3). Indole is not produced, horse blood is not haemolysed and no diffusible pigments are produced in cultures (5). An API test kit (made by Bio Merieux) can be used for easy confirmation that an organism belongs to the *Pseudomonas* group. Lack of motility is then of special relevance.

In contaminated samples supplementation of media with substances which inhibit the growth of Gram-positive organisms has proved helpful (e.g. crystal violet, proflavine), as has pretreatment with penicillin (1,000 units/ml for 3 hours at 37°C) (11). Xie et al. (12) have developed a selective medium comprised of 1,000 units polymyxin E, 250 units bacitracin and 0.25 mg actidione incorporated into 100 ml nutrient agar containing 4% glycerine, 10% donkey or horse serum, and 0.1% ovine haemoglobin or trypton agar.

Outside the body the organism has little resistance to drying, heat, light or chemicals, so that survival beyond two weeks is unlikely. Under favourable conditions, however, it can probably survive a few months. It can remain viable in tap water for at least a month (10). For disinfection, benzalkonium chloride or 'roccal' (1:2,000), sodium hypochlorite (500 ppm available chlorine), iodine, mercuric chloride in alcohol and potassium permanganate have been shown to be highly effective against *P. mallei* (6). Phenol is less effective and Lysol is ineffective.

c) Laboratory animal inoculation

Although guinea pigs, hamsters and cats have been used for diagnosis, it is the male guinea pig which is most commonly used. Suspected material is inoculated intraperitoneally and causes 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 (4). 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.
2. Mallein and serological tests

a) The mallein test

The mallein purified protein derivative (PPD) which is commercially available is a solution of water soluble protein fractions of heat-treated *P. 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).

i) The intradermo-palpebral test

This is the most sensitive, reliable and specific test for detecting infected solipeds and has largely displaced the ophthalmic and subcutaneous tests (2). 0.1 ml of concentrated mallein PPD is injected intradermally into the lower eyelid and the test read at 24 and 48 h. 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.

ii) The ophthalmic test

This is less reliable than the above. 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.

iii) The subcutaneous test

This test interferes with subsequent serological diagnosis and so the other two mallein tests are preferred. The test may also not be recognised as acceptable by some countries. The horse's temperature has to be less than 102°F (38.8°C) on the day before the test, at the time of the injection and at 9, 12 and 15 h afterwards. A 10 cm square skin patch in the middle of the neck is clipped and disinfected. 2.5 ml of dilute mallein is 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 h and a firm painful swelling with raised edges develops within 24 h at the injection site. In non-glandered horses there is no, or minimal, transient local swelling. Doubtful reactors may be retested after 14 days using a double dose of mallein.
b) Serological tests

i) Complement fixation test (CFT)

Although not as sensitive as the mallein test, the CFT is the most accurate of the serological tests which have been used for glanders diagnosis (2). It is reported to be 90-95% accurate, serum being positive within one week of infection and remaining positive for a long time in chronic cases (10). The antigen for the CFT has been prepared from young cultures by growing the organism on glycerol-agar slopes for 12 hours and washing off with normal saline. The suspension is then heated for 1 h at 65°C.

Serum is diluted 1:5 in veronal buffered saline containing 0.1% gelatin (VBSG) and inactivated for 35 minutes at 58°C. Serum of equidae other than horses should be inactivated at 63°C for 35 minutes. Two-fold dilutions of the sera are prepared in 96-well round-bottom microtitre plates. Guinea pig complement is diluted in VBSG and 5.5 complement haemolytic units-50% (CH50) are used. Sera, complement and antigen are reacted in the plates and incubated for one hour at 37°C. A 2% suspension of sensitised washed sheep red blood cells is added and the plates are incubated for 45 minutes at 37°C, 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 some strains cross react with *P. pseudomallei* (2).

ii) Agglutination and precipitin tests

The accuracy of these tests is unsatisfactory for use in control programmes. Horses with chronic glanders and those in a debilitated condition give negative or inconclusive results.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

No vaccines are available.

Mallein PPD for use in performing the intradermo-palpebral and ophthalmic tests is produced commercially by two institutes:

1) Centraal Diergeneeskundig Instituut (CDI), Edelhertweg 15, 8219 PH, Lelystad, Netherlands.

2) Min Agr Si Industr. Institut Die Cercetari Si Biopreparate 'Pasteur', R-7000 - Bucuresti, 77.826 SOS Giulesti Nr 333, Romania.

The CDI has provided the following information on requirements for mallein PPD.
1. **Seed management**

Three strains of *Pseudomonas (Actinobacillus) mallei* are employed for production of mallein PPD, namely strain Bogor (originating from Indonesia), strain Mukteswar (India) and strain Zagreb (Yugoslavia). The seed material is kept as a stock of freeze-dried cultures. The strains are subcultured on glycerol-agar at 37°C for one to two days. For maintaining their virulence and antigenicity, they may be passaged through guinea pigs.

2. **Manufacture**

For production of mallein PPD Dorset-Henley medium, enriched by the addition of trace elements, is used. The liquid medium is inoculated with a thick saline suspension of *P. 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 three 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-greyish precipitate settles.

The supernatant fluid is pipetted off and discarded. The precipitate is centrifuged for 15 minutes at 3,000 r.p.m. 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 under 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 division into ampoules.

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 *P. 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.

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.

The examination for safety is conducted on five to ten normal healthy horses by carrying out the intradermo-palpebral test. The resulting swelling should be at the most hardly detectable and transient, without any signs of conjunctival discharge.
Preparations containing phenol as a preservative should not contain more than 0.5% w/v. The protein content should be not less than 0.95 mg per ml and not more than 1.05 mg per ml.

Potency testing is performed in guinea pigs and horses. The animals are sensitised by subcutaneous inoculation with a concentrated suspension of heat-killed *P. mallei* in paraffin oil or incomplete Freund's adjuvants. Cattle can also be used instead of horses. The production batch is bioassayed against a reference 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 of skin thickness is measured by calipers. The results are statistically evaluated, using standard statistical methods for parallel-line assays.

**REFERENCES**


SUMMARY

Equine viral arteritis is caused by a virus belonging to the family Togaviridae, genus Arterivirus, whose natural host range is restricted to equids.

Identification of the agent: Virus isolation from suspected cases should be attempted from nasopharyngeal and conjunctival swabs, citrated blood samples, and semen in the case of stallions considered putative carriers of the virus. Where only necropsy material is available, isolation of equine arteritis virus can be attempted from a wide range of tissues, or placental and fetal fluids from virus-related cases of abortion. Although not always successful in natural cases of equine arteritis virus infection, virus isolation should be attempted from clinical specimens or necropsied tissues in equine, rabbit or monkey kidney cell culture. The identity of isolates of equine arteritis virus should be confirmed by direct immunofluorescence or in a neutralisation test.

Where mortality is associated with a suspect outbreak of equine viral arteritis, a wide range of tissues should be examined for histological evidence of a panvasculitis that is especially pronounced in the small arteries throughout the body. The characteristic vascular lesions present in the mature animal are not a feature of abortions related to equine arteritis virus.

Serological tests: A variety of serological tests, including neutralisation, complement fixation, indirect fluorescent antibody, agar gel immunodiffusion and enzyme linked immunoabsorbent assay (ELISA), have been used for the detection of antibody to equine arteritis virus. The test currently in widest use is a microneutralisation test in the presence of complement. It is a sensitive assay used to diagnose acute infections and is especially valuable in seroprevalence studies. The complement fixation test, although less sensitive, has also been used for diagnosing recent infections.

Requirements for biological products: Tissue culture equine arteritis virus vaccine is a live vaccine prepared from virus that has been attenuated for horses by multiple serial transfers in horse and rabbit cell cultures. Vaccination of foals under six weeks of age and of pregnant mares in the final two months of gestation is contraindicated.

The vaccine should be tested for safety in at least two horses seronegative for neutralising antibodies to equine arteritis virus inoculated by the intramuscular route, neither of which should exhibit any clinical sign of disease other than a mild pyrexia.

Potency of vaccinal virus in the final containers is determined by a plaque infectivity assay in cell culture and by a vaccination-challenge test in horses.
Lyophilised 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.

A. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Equine viral arteritis is caused by a virus belonging to the family Togaviridae, genus Arterivirus (25), whose natural host range is restricted to equids (12).

In the presence of characteristic clinical signs of the disease or where subclinical infection is suspected, virus isolation should be attempted from nasopharyngeal and conjunctival swabs, citrated blood samples, and semen in the case of stallions considered putative carriers of the virus (21). To optimise the chances of isolation of virus, the relevant specimens should be obtained as soon as possible after the onset of clinical illness. Short- and long-term carrier stallions, however, appear to shed equine arteritis virus constantly in the semen, but not in the respiratory secretions or urine (22). It has also not been demonstrated in the buffy coat of the blood. Virus is associated with the sperm-rich but not the pre-sperm fraction of semen.

Where only necropsy material is available, isolation of equine arteritis virus can be attempted from a variety of tissues, especially the lymphatic glands associated with the alimentary tract and related organs (15). In suspect cases of equine arteritis virus-related abortion, placental and fetal fluids and a wide range of placental, lymphoreticular and other tissues can be productive sources of virus (21).

Although not always successful in natural cases of equine arteritis virus infection (17, 24), virus isolation should be attempted from clinical specimens or necropsied tissues in equine, rabbit or monkey kidney cell culture (15). Either primary cell culture or selected cell lines, e.g. RK-13, VERO and LLC-MK_2, can be used. Inoculated cultures are examined daily for the appearance of cytopathic effect which is usually evident within six days. The development of non-viral cytotoxicity has not constituted a major problem in cultures inoculated with decimal dilutions of seminal plasma from putative carrier stallions. In the absence of visible cytopathic effect, culture supernatants should be subinoculated into fresh cell monolayers. The identity of isolates of equine arteritis virus should be confirmed by direct immunofluorescence (8) or in a neutralisation test (22).

Viral antigen can be detected in the tissues of infected horses by immunofluorescence using conjugated equine antiserum to equine arteritis virus (3). A polyclonal rabbit antiserum to the virus has also been used in an indirect fluorescent antibody assay to confirm infection in inoculated cell cultures (8). Mouse monoclonal antibody directed at the 14K nucleocapsid protein of equine arteritis virus has been developed and this allows immunofluorescent detection of virus in RK-13 cells as early as 12 hours after infection (2).
Where mortality is associated with a suspect outbreak of equine viral arteritis, a wide range of tissues should be examined for histological evidence of a panvasculitis that is especially pronounced in the small arteries throughout the body, particularly in the caecum, colon, spleen, associated lymphatic glands and adrenal cortex (3, 9, 5). The presence of a disseminated necrotising arteritis involving endothelial and medial cells of affected vessels is considered pathognomonic of equine viral arteritis. The characteristic vascular lesions present in the mature animal are not, however, a feature of abortions related to equine arteritis virus.

2. **Serological tests**

A variety of serological tests - including neutralisation (microneutralisation (20) and plaque reduction (12)), complement fixation (6), indirect fluorescent antibody test (3), agar gel immunodiffusion (3) and ELISA (10) - have been used for the detection of antibody to equine arteritis virus.

The test currently in widest use is a microneutralisation test in the presence of complement. Besides the neutralisation assay, the complement fixation test, although less sensitive, has also been used for diagnosing recent infections, since complement fixing antibody titres are relatively short-lived (6). Although the ELISA has been used for serosurveys (10), the specificity of this test is unacceptable in horses previously immunised with tissue culture vaccines.

**Virus neutralisation**

The microneutralisation test in the presence of complement (20) is a sensitive assay used to diagnose acute infections and is also valuable in seroprevalence studies, since detectable neutralising antibody titres to equine arteritis virus can persist for several years after initial infection. A standardised virus neutralisation test procedure has been developed by the National Veterinary Services Laboratories of the United States Department of Agriculture in an attempt to ensure uniformity of results in those laboratories in the United States engaged in the serological testing of horses for export (20).

The test is carried out in RK-13 cells using a low passage level of the Bucyrus strain of equine arteritis virus and results are read after 48-72 hours of incubation. A serum dilution is considered positive if there is an estimated 75% or greater reduction in the amount of viral cytopathic effect in the serum test wells compared to that present in the wells of the lowest virus control dilution. A titre of 1:4 or greater is considered positive. In view of the subjective nature of endpoint determinations, serum titres may, on occasion, be difficult to reproduce since partial neutralisation may be observed over a range of several dilutions. There is evidence that the sensitivity of the microneutralisation test can, in certain instances, be increased by using the attenuated Bucyrus strain of equine arteritis virus (i.e. vaccine strain) of high passage history in cell culture as test virus.
B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

The product for which the following details are provided is a live tissue culture equine arteritis virus vaccine prepared from a virus that has been attenuated for horses by multiple serial transfers in horse and rabbit cell culture.

1. Seed management

a) Characteristics

The most widely used vaccine virus is a derivative of the prototype Bucyrus strain of equine arteritis virus that has been attenuated by serial passage in primary cultures of horse kidney (HK-131), rabbit kidney (RK-111) and a diploid equine dermal cell line, ATCC CCL 57 (ECID-24) (4, 7, 11). Available evidence points to the existence of only one serotype of the virus, and strain variation is not considered of significance in relation to vaccine efficacy (11).

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 and induces a very good protective immunity (4, 7, 11, 13, 14, 16, 18, 23). In spite of the vaccine’s high degree of safety, vaccination of foals under six weeks of age is not recommended except when the latter run a high risk of natural exposure. In addition, pregnant mares should not be vaccinated during the final two months of gestation because of the slight possibility of fetal invasion by vaccinal virus during this period (4).

Suitable lots of master seed virus should be maintained in liquid nitrogen or the equivalent.

b) Culture

The vaccine virus should be grown in appropriate cell cultures, such as equine dermal cells, using the most suitable medium supplemented with sterile fetal bovine serum or bovine serum albumin as a replacement for bovine serum in the growth medium. Cell monolayers should be washed prior to virus inoculation to remove traces of bovine serum. Good virus growth should be obtained in two to three days.

c) Control methods

Vaccine virus should be grown in appropriate cells that have been officially approved for vaccine production and confirmed free from extraneous bacterial, fungal, mycoplasmal and viral agents (19). The identity of the vaccine virus in the master seed should be confirmed by neutralisation. Incomplete neutralisation of equine arteritis virus by homologous horse or rabbit antisera has been scientifically documented (20, 1) and is a problem when screening master seed virus for extraneous viruses and 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 passages in susceptible cell lines. No evidence of cytopathic viruses, haemadsorbing virus, or non-cytopathic strains of bovine virus diarrhoea virus by immunofluorescence, should be found. When cells of equine origin are used, the absence of infectious anemia virus should be demonstrated.

2. **Manufacture**

Cell monolayers should be washed prior to inoculation with seed virus to remove traces of bovine serum in the growth medium. Inoculated cultures are maintained on an appropriate maintenance medium. Harvesting of infected cultures should take place when almost the entire cell sheet shows characteristic cytopathic effects. Supernatant fluid and cells are harvested and treated to remove cell debris and unwanted material through appropriate purification processes.

3. **In-process control**

In addition to the pre-production testing of the master seed virus and the cell cultures for adventitious contaminants, the cells infected with the vaccine virus should be examined macroscopically for evidence of microbial growth during the incubation period. If growth in a culture vessel cannot be reliably determined by visual examination, judgment should be confirmed by subcultures, microscopic examination, or both.

4. **Batch control**

   a) **Sterility**

   Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

   b) **Safety**

   Besides the testing of a representative sample of each production lot of vaccine 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 equine arteritis virus with one vaccine dose of lyophilised virus each (19). None of the inoculated horses should exhibit any clinical sign of disease other than a mild pyrexia during the ensuing two-week observation period. In addition, nasopharyngeal swabs should be collected daily from each horse for virus isolation studies, white blood cell counts should be made daily, and diurnal temperatures recorded.

   c) **Potency**

   Potency of vaccine virus 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 (19). Each vaccine lot must be tested in triplicate in cell culture, 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 equine arteritis virus. A one-stage vaccination-challenge test using 20 vaccinated and 5 control horses or a two-stage test using 10 vaccinates and 5 controls for each stage constitutes the animal potency test.

d) Duration of immunity

Detectable neutralising antibody titres to equine arteritis virus should develop in the majority of horses within three weeks of vaccination (14, 16, 18, 23). Responses to primary vaccination are characterised by a rapid fall in antibody titres with a significant number of animals reverting to seronegativity one to three months after vaccination (23). Revaccination with the attenuated equine arteritis virus vaccine results in an excellent anamnestic response, however, with the development of high antibody titres that remain relatively undiminished for at least 9 to 12 months (23).

e) Stability

Lyophilised vaccine can be stored for at least three to four years at 2 to 7°C without loss in infectivity, provided it is kept in the dark (7). Infectivity should be preserved for much longer periods if vaccinal virus is frozen at -20°C or below. Once rehydrated, however, the vaccine should be used within one hour or else destroyed.

REFERENCES


Equine viral arteritis (B44)


JAPANESE ENCEPHALITIS
(B45)

SUMMARY

Japanese encephalitis virus is a mosquito-borne Flavivirus that causes encephalitis principally in horses. It also infects man, 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. Since 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 which showed the clinical signs of encephalitis. Isolation procedures include the inoculation of suckling 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 suckling mice 2-4 days of age. A positive isolation is where the mice show neurological signs followed by death within 14 days. The virus is also isolated in cell cultures made from chick embryos, in porcine or hamster kidney cells, VERO cells, the MD-BK cell line, and mosquitoes.

A cytopathic effect (CPE) appears in some cell cultures, but it is usually unclear. Identification of the virus in suckling mice or in tissue cultures is confirmed by serological methods.

Serological tests: Antibody assay is a useful technique to determine the prevalence of infection in a horse population, and also to diagnose Japanese encephalitis in diseased individuals. The assay methods include haemagglutination inhibition (HI), indirect fluorescent antibody, complement fixation and virus neutralisation (VN) tests.

The HI test is used most extensively, and antigen for this test is prepared by sucrose-acetone extraction of the brains of suckling mice inoculated with the virus. The antigen agglutinates the red cells of geese and day-old chicks within a pH range of 6.6-7.0. The VN test is the most specific because of its ability to differentiate Japanese encephalitis virus infection from other Flavivirus infections.

Requirements for biological products: There is an inactivated vaccine prepared by the inactivation of a virus suspension derived from infected mouse brains or infected cell cultures.
A. DIAGNOSTIC TECHNIQUES

Japanese encephalitis is a disease of horses caused by a mosquito-borne Flavivirus that elicits clinical signs of encephalitis in infected animals and can lead in turn to their death. It also infects man, and causes abortions in pigs. Pigs act as amplifiers of the virus, and birds can also be involved in its spread.

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).

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.

1. Identification of the agent

Samples of brain and spinal cord are homogenised into a 10% suspension in buffered saline, pH 7.4, containing 2% calf serum or 0.75% bovine serum albumin, streptomycin 100 μg/ml and penicillin 100 units/ml. The calf serum should be free from Japanese encephalitis antibodies. The suspension is centrifuged at 1,500 g for 15 minutes and the supernatant fluid removed for inoculation. Suckling mice aged 2-4 days are inoculated intracerebrally with 0.02 ml. The inoculated mice are kept under clinical observation for 14 days afterwards. 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 from pinkish colour to dark red, and convulsions develop immediately before they 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. This antigen is checked for its ability to agglutinate the red cells of day-old chicks or of geese at different pH levels between pH 6.0-7.0 at intervals of pH 0.2, according to the method described (2). If the antigen is able to haemagglutinate, this is used in an HI test using a Japanese encephalitis antiseraum. If the antibody titres of the serum are not significantly different between the newly prepared and a standard antigen, the isolated virus is considered to be that of Japanese encephalitis.

Primary cultures of chick 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 pathological materials and the brain suspension of the isolated virus are inoculated into the cultures. In this case, monoclonal antibodies specific to Flavivirus and Japanese encephalitis virus may be used to identify the virus by the indirect fluorescent antibody test.
2. Serological tests

These 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 borne in mind that horses in an endemic area may have been inapparently infected with the virus for some time and 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.

In some regions of the world, there is a need to perform additional tests before an unequivocal diagnosis of Japanese encephalitis can be made. For example, in Australia and Papua New Guinea the antigenically closely-related virus of Murray Valley encephalitis occurs.

The HI test is the most widely used test 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 erythrocytes to remove any non-specific haemagglutinins. The red cells of geese or of day-old chicks are used at the optimum pH. The test should be conducted with the treated sera and 8 units of standard antigen; this is commercially available.

Complement fixation (CF) is also used for serological diagnosis, but less often. The antigen for this test is extracted with acetone-ether from the brain of inoculated mice.

There are several methods of performing VN tests. Neutralisation tests in mice are both time consuming and laborious, and VN tests in tubes using hamster kidney cells lacks sensitivity. However, a plaque reduction test using chick embryo primary cultures, VERO cells or BHK cells is considerably more sensitive and reliable. In the latter, a cell monolayer is grown in petri dishes. A virus dose of 100 plaque-forming units is mixed with an equal volume of serially diluted serum, and the mixtures incubated at 37°C for 90 minutes on the monolayer. The monolayer is then overlaid with medium consisting of maintenance medium with agar or carboxymethylcellulose. After 4 days incubation at 37°C in a CO₂ incubator, any plaques that form can be visualised either by staining with a second agar overlay containing neutral red, or by fixation with formalin followed by staining with crystal violet. The antibody titres are expressed as the maximum dilution of the serum that reduces more than 50% of the plaque number of the virus dose in serum-free control plates.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

The vaccine for Japanese encephalitis in horses is prepared by the inactivation of a virus suspension derived from infected mouse brains or cell cultures.
1. **Seed management**

a) **Characteristics**

The Nakayama strain of Japanese encephalitis virus is used for vaccine production in Japan. The strain must be lethal for mice when inoculated intracerebrally and be able to grow in a primary culture of porcine kidney. This strain has the capacity to haemagglutinate the red cells of geese, day-old chicks or pigeons. The virus must be able to be neutralised by a standard antiserum to Japanese encephalitis virus.

b) **Culture**

The original and seed viruses should be grown in mice brains or cell cultures. The passage levels should not exceed 3 times that of the original virus and 2 times 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. The product should retain its potency for at least 12 months when kept at 4°C.

2. **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, from which the brains of those showing severe clinical signs of encephalitis are collected. These are homogenised in phosphate buffered saline, centrifuged at 1,500 g for 30 minutes, and the supernatant fluids processed as the virus suspension.

Seed virus is inoculated onto cell cultures and the fluids 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 processed as the virus suspension.

Formalin is added to the suspension to inactivate any live virus; this is considered as the undiluted virus suspension. Adjuvant may be added to this 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 undiluted virus suspension should be
re-examined for contamination by culture and by microscopy after staining, and 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 the Chapter on General Information.

b) **Safety**

The essential safety test is mouse inoculation to ensure complete inactivation of live virus.

c) **Potency**

The final product must be checked for immunogenicity by mouse protection tests. The product is diluted in phosphate buffered saline to 1:10. Thirty mice aged 3-4 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. Eight days following the first inoculation, all mice are challenged with graded doses of live virus, 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%.

***REFERENCES***


Mites causing mange in domestic animals belong to the burrowing (Sarcoptidae), non-burrowing (Psoroptidae), and hair follicle mites (Demodicidae). The species in Sarcoptidae include Sarcoptes scabiei, of which there are several physiological varieties which are specific for their host. The mite occurs in pigs, cattle, sheep, goats, and horses. Species of the genus Cnemidocoptes occur on poultry and include Cnemidocoptes mutans and C. gallinace. The family Psoroptidae includes two genera. The genus Psoroptes has two species – P. ovis, causing body mange particularly in sheep and cattle, and P. cuniculi, causing ear mange particularly in goats. The genus Chorioptes has one species, C. bovis, the foot mange mite, which is mildly pathogenic in sheep, goats, cattle and horses. The family Demodectidae is represented by the genus Demodex, with several species according to the host. Demodex occurs in all domestic mammals but demodectic mange is mainly restricted to goats and cattle.

The mites are obligate parasites and the life cycle occurs exclusively on the host, being completed in 2-3 weeks. Adult mites produce eggs which hatch to larvae, followed by nymphal stages, and finally adults. Transmission is usually by direct contact, though contaminated grooming tools, housing or fencing may remain infectious for a limited period.

Mange mites, in particular Sarcoptes and Psoroptes, cause production losses resulting in economic damage. In domestic mammals mange mites, in particular Sarcoptes, Psoroptes and Demodex, downgrade hides and skins resulting in leather damage. Sarcoptes scabiei in the pig may generalise to a severe infection. Psoroptes ovis is the cause of sheep scab, which may generalise with fatal effect.

Identification of the agent: Except for the worm-like Demodex, mange mites are globose or oval in outline. The adults have four pairs of legs and anteriorly-placed mouth parts, consisting of chelicerae and pedipalps used for feeding. The surface is striated in a fingerprint-like pattern and bears a variety of spines or setae. The legs are supported by distinct chitinous rods (epimeres or apodemes) and end terminally in bristles or bell-shaped suckers (caruncles). The distribution of bristles and suckers on the various legs is characteristic for the sex and species of mite. For exact species identification a text on parasitology should be consulted.

The parasitological diagnosis is made by microscopical examination of deep skin scrapings and identification of the parasite. In demodectic mange the contents of the typical skin lesions are examined. Crusts and skin scrapings are macerated and cleared in hot 10% sodium or potassium hydroxide or in
hot chlorallactophenol. Sedimentation or flotation techniques enhance the chance of finding the parasites when they are scarce. For large scale surveys a vacuum cleaner sampling method can be used.

**Serological tests:** There are no serological tests applicable to the diagnosis of mange.

**Requirements for biological products:** There are no commercial vaccines available to protect domestic animals against mange mites.

## A. 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 (3, 5) or in samples collected by a vacuum cleaner (1, 2).

1. **Identification of the agent**

Four genera are of importance in domestic mammals, namely, *Sarcoptes*, *Psoroptes*, *Chorioptes* and *Demodex*, and one in poultry, namely *Cnemidocoptes* (*Knemidokoptes*).

**Sarcoptes scabiei:** Varieties of this species occur in different host species – e.g. pigs, cattle, goats, sheep, and horses – but morphologically they are similar. The mites are globose; the females are 350-600 μm, and the males are slightly smaller. The body surface is striated in a fingerprint pattern, and spines and setae are present. There are four pairs of short, stumpy legs and a capitulum bearing the mouth parts; larval stages have three pairs of legs. Distinct chitinous rods (epimeres), which are extensions of the coxae of the legs, have a characteristic distribution. The legs terminate in either bell-shaped tarsal suckers on stalks (pedicels) or bristles. The adult has characteristically long, unsegmented pedicels, and the anus is located at the posterior end of the body.

**Psoroptes ovis and P. cuniculi:** These non-burrowing mites are oval in outline. There are no spines or setae on the dorsal surface. The four pairs of legs are longer than in the genus *Sarcoptes* and project well beyond the margins of the body. Similarly, the mouth parts are elongated. A characteristic diagnostic feature is the long three-jointed stalk or pedicel of the tarsal suckers. The posterior margin of the abdomen of the male bears two triangular lobes on which are borne copulatory suckers. *P. ovis* occurs on the body and *P. cuniculi* in the outer ear canal.

**Chorioptes bovis:** This resembles *P. ovis* but the tarsal suckers have short unjointed pedicels on the first, second and fourth pairs of legs of the female and on all legs of the male. The male has marked rectangular abdominal lobes bearing hairs which are spatulate at the base. Several varieties occur in horses, goats and sheep, but it is considered that all are of the same species (6).
**Demodex spp.:** The hair follicle mite is worm-like in outline and therefore easily distinguishable from other genera. The four short pairs of legs are situated directly behind the gnathosoma. In cleared preparations even several mites can be overlooked because they lack contrast.

**Cnemidocoptes:**

*C. mutans (Knemidokoptes mutans):* A diagnostic feature is that this species causes marked keratinisation of the legs ('scaly leg') of poultry. The mites are globose in outline (375-475 μm), with the male being much smaller than the female. Adults have four pairs of short legs which have suckers. The legs of the female mite are very short, whereas those of the male are much longer. On the dorsal surface just behind the gnathosoma two parallel sclerotic rod-like structures are present. A less sclerotic transversal area between the caudal ends of these rods results in a U-shaped sclerosis. The dorsal surface striations in *C. mutans* are interrupted in the mid-region to form rounded scales.

*C. gallinae (Knemidokoptes laevis):* A diagnostic feature is that this species causes the loss of feathers ('depluming itch') in poultry. The mites resemble *C. mutans*, but differ from the latter in having a non-interrupted dorsal striation. In the mid-region the dorsal striation shows indentations.

The clinical manifestations of mange include pruritis, which is often intense, loss of hair or feathers, thickening of the skin and scab formation according to the species of mite and the animal infected. Pruritis is mild or absent in demodectic mange, which is characterised by typical skin nodules up to several mm in diameter.

Mites are demonstrated in skin scrapings taken from the periphery of the lesions. The lesion is scraped with a scalpel until it oozes blood. Scrapings should be collected in a water-based medium, such as glycerol, in order to facilitate any subsequent clearing or digestion of debris. Crusts and skin scrapings are macerated and cleared by boiling for a few minutes in 10% sodium or potassium hydroxide or in chlorallactophenol. Instead of boiling, scrapings may be left overnight in these solutions. The cleared material is microscopically (x 100) examined directly or the sediment is examined after centrifugation. Flotation of the cleared material or sediment in saturated salt or sugar enhances the chance of demonstrating the parasites when they are scarce. In sarcoptic mange in pigs it is often only possible to demonstrate the parasite in ear scrapings, despite the presence of lesions elsewhere on the body. In demodectic mange the skin nodules are squeezed and their contents are carefully examined. In the case of depluming itch in poultry, *C. gallinæ* may be demonstrated by pulling out feathers at the periphery of the lesion and examining the shaft for mites.

Samples from domestic mammals can be collected by a vacuum cleaner with a milk filter or tissue filter insert (1, 2). This is an easy and reliable method for large scale surveys. The material collected on the filter is examined as described for skin scrapings.
2. **Serological tests**

A few serological tests for mange infections have been developed for experimental work (4). There are, however, no serological tests available for the routine diagnosis of mange infections.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

There are no commercial vaccines available to protect domestic animals against mange mites.

**REFERENCES**


SUMMARY

Trypanosomiasis*, when caused by Trypanosoma evansi, is known as surra and principally affects camels and horses, although other animals are susceptible. It is an arthropod-borne disease.

The diagnosis of surra is based on the demonstration of the parasites in the blood, supplemented by haematological, biochemical and serological tests.

Identification of the agent: Since the general clinical signs of the various forms of T. evansi infection are not pathognomonic, laboratory diagnosis is required. When there is a heavy 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 concentration and the inoculation of laboratory animals, are recommended.

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 estimates of the packed blood cell volume and immunoglobulin levels.

Serological tests: Infection gives rise to specific immune responses, and several serological tests are of use. Under field conditions the indirect fluorescent antibody test or enzyme-linked immunosorbent assay can be employed. It may become possible to carry out card agglutination tests, such as the Testryp (R) CATT for Gambian sleeping sickness adapted for the diagnosis of T. evansi infection or the card agglutination test for veterinary use based on an early variant antigen type RoTat 1.2. T. evansi; this is specific, sensitive, and easy to perform for mass screening.

Requirements for biological products: No vaccines are available for the disease.

* Nomenclature of parasitic diseases: see the note in Chapter 36 (Trypanosomiasis – B22).
A. DIAGNOSTIC TECHNIQUES

The diagnosis of *Trypanosoma evansi* infection, which is an arthropod-borne disease, is based on clinical signs and on the demonstration of the presence of the parasites by direct or indirect methods.

1. **Clinical diagnosis**

The clinical signs of surra, the disease caused by *T. evansi*, are familiar but are not sufficiently pathognomonic to enable a definite diagnosis to be made. Diagnosis is confirmed by laboratory methods. 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 (9). There are indications that the disease causes immunodeficiencies.

The duration and course of the disease may continue for weeks and vary according to the host species. There is also individual variation in response. The disease is often rapidly fatal in camels, buffaloes, horses, llamas, and dogs, but it can be mild and subclinical in cattle, donkeys, goats, sheep, pigs, and capybara. Generally draught animals are more sensitive.

2. **Identification of the agent**

The classical direct method for the diagnosis of trypanosomiasis 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 areas of *T. evansi* distribution, where the animals can also be infected with *T. brucei* or *T. equiperdum*, specific identification can be carried out on blood smears using a non-radioactive kDNA, if these strains possess a kinetoplast (14).

**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.

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 cleanse instruments with alcohol between individual animals so that infection cannot be transmitted in residual blood.

ii) **Wet blood films**

A small drop of blood is placed onto a clean glass slide and covered with a coverslip to spread the blood as a monolayer of cells. This is examined by light microscopy (x 200) to observe any motile trypanosomes.

iii) **Thick stained smears**

A large drop of blood is placed on the centre of a microscope slide and spread with another slide so that an area of about 1.0-1.25 cm in diameter is covered. This is air-dried for an hour or longer, and at the same time it is protected from insects. The unfixed smear is stained with Giemsa (1 drop of Giemsa + 1 ml phosphate buffered saline (PBS) pH 7.2) for 25 minutes. After washing, the slides are examined under high magnification of the light microscope (x 500-1,000). The advantage of the thick smear technique is that it concentrates the drop of blood into a smaller 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 therefore loses its value for species identification in cases of mixed infections.

iv) **Thin stained smears**

A drop of blood is placed 20 mm from one end of a clean microscope slide and a thin film drawn out in the usual way. The film is air-dried briefly, fixed in methyl alcohol for two minutes and allowed to dry. The smears are then stained by Giemsa (1 drop Giemsa + 1 ml PBS pH 7.2) for 25 minutes. This is poured off, the slide washed in tap-water and dried. Unfixed smears can be stained by May-Grunwald Giemsa by covering them with May-Grunwald stain for two minutes, then adding an equal volume of PBS pH 7.2; this is left for a further three minutes. This is poured off and diluted Giemsa added for 25 minutes. This is again poured off, the slides washed with tap-water, and dried. All slides are examined by high magnification using a light microscope (x 500-1,000). This technique permits detailed morphological studies and identification of the trypanosome species.

v) **Lymph node biopsies**

Samples are usually obtained from the prescapular or precrural lymph nodes. A suitable node is selected by palpation and the site cleansed with alcohol. The node is punctured with a suitable gauge needle and lymph node material aspirated into the syringe. This material is then expelled onto a slide, covered with a coverslip and examined as for the fresh blood preparations.
b) Concentration methods

In most hosts *T. evansi* induces 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) from a vein into heparinised capillary tubes (75 x 1.5 mm), which are then sealed at one end. These are centrifuged, sealed end down, at 3,000 g for 5 minutes. Two pieces of glass (25 x 10 x 1.2 mm) are glued to a slide and the capillary tube is placed between them. A coverslip is 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 by high magnification light microscopy (x 100-200); if water is used, the examination is carried out with a dry objective (x 60).

ii) Dark-ground or phase contrast

Blood is collected into heparinised capillary tubes and centrifuged as before. The tube is broken 1 mm below the buffy coat layer, thus including the top layer of red cells, and 1-2 mm above the buffy coat layer, to include plasma. The contents of this piece of tube are expelled onto a slide, covered with a coverslip and examined under dark ground or phase contrast illumination.

As an alternative to the haematocrit centrifuge requiring electrical supply as a power source, a simple, hand-powered micro-centrifuge has been developed for field use. This latter gave results comparable with the microhaematocrit centrifuge for detection of trypanosomiasis in cattle and camels (6, 7).

iii) Haemolysis techniques

Sodium dodecyl sulfate (SDS) is used as reagent to haemolyse red blood cells and to facilitate detection of motile trypanosomes in parasitised blood samples. As SDS is toxic, contact with skin, inhalation and ingestion should be avoided (it must not be pipetted out by mouth). SDS solution can be stored for several months at ambient temperature and for a longer time at 4°C. 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.

This method might be applicable to a wide range of mammalian host species infected with either salivarian or stercorarian trypanosomes. The haemolysis techniques are under evaluation with promising results for the diagnosis of human and animal trypanosomiases in field conditions (Van Meirvenne N., personal communication).
Two general procedures, namely wet blood film clarification and haemolysis centrifugation, are presented.

Wet blood film clarification method

This method uses the partial lysis of red blood cells to facilitate detection of motile trypanosomes. The method requires SDS solution (1% SDS dissolved in Tris-glucose-saline, pH 7.5), inoculating loops (10 μl), slides and coverslips (24 x 24 mm), and a drop of fresh neat or heparinised blood.

Put 10 μl of blood on a slide. Add 10 μl of SDS solution using a dip inoculation loop and mix gently. Apply a coverslip. Read the entire preparation without delay at low magnification (≤100 x).

Haemolysis centrifugation technique

Nearly complete lysis of red blood cells 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, graduated tubes and fine tapering tubes with attached rubber teats to draw out the sediment, pipettes or syringes, slides, coverslips (24 x 24mm or 24 x 32 mm), heparinised blood.

Using a pipette or syringe, transfer 9 volumes (max. 6.3 ml) of SDS solution into an ordinary test tube. Aspirate one volume (max. 0.7 ml) of heparinised blood and expel it by compressing the attached rubber teat completely just above of the surface of the SDS solution and mix quickly and thoroughly. Avoid foam formation which may result in destruction of the trypanosomes. Wait for 10-20 minutes.

Pour the lysed blood suspension into a conical centrifuge tube and spin at 2,000 rpm for 10 minutes. With a clean pipette remove as much supernatant as possible without disturbing the sediment. Again with a fine tapering tube draw out more supernatant, leaving 10-20 μl of undisturbed sediment at the bottom. Then very carefully collect the entire sediment and put it onto a microscope slide. Apply a coverslip and read the entire preparation without delay at low magnification (≤100 x).

iv) Mini-anion-exchange centrifugation technique

When the blood of humans and animals infected with salivarian trypanosomes, mixed with buffer of appropriate ionic strength and pH, is passed through an anion-exchange column, the host blood cells, being more negatively charged than the trypansomes, are adsorbed onto the anion-exchanger cellulose, while the trypanosomes are eluted from the column, retaining viability and infectivity. A simplified method for detection of low parasitaemia and for field use has been developed (8, 11, 17).
Preparation of phosphate buffered saline glucose (PSG) pH 8.0:

<table>
<thead>
<tr>
<th></th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄</td>
<td>13.48 g</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>0.78 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>4.25 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 litre</td>
</tr>
</tbody>
</table>

Solutions of different ionic strength are made by diluting the stock PBS (pH 8.0) and adding glucose to maintain a suitable concentration. For infections of mice, domestic and wild ruminants, and dogs, add 4 parts of PBS to 6 parts of distilled water and adjust the final glucose concentration to 1%. For infections of pigs and rabbits add 3 parts of PBS to 7 parts distilled water and adjust the final glucose concentration to 1.5%. The phosphate buffer-saline-glucose solution stored at room temperature must be sterile.

Equilibration of DEAE-cellulose: Suspend 500 g of DEAE-cellulose in two 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 DEAE-cellulose 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 (20G x 1.5 inch). A disc of no. 41 filter paper is placed at the bottom and moistened by adding a few drops of PSG. Pour 2 to 2.5 ml of the slurry of equilibrated cellulose into the syringe and allow to pack. Again wash twice with PSG.

Adsorption and elution: Gently add 100 to 300 μl of heparinised blood above the surface of the cellulose column and an equal amount of PSG. Discard the first drops and place the needle into a tapered pasteur pipette. Centrifuge at 2,000 rpm for 15 minutes. Examine the bottom of the pasteur pipette (x 200) or with objective 40/0.85 Oil D = 1.5.

c) Animal inoculation

Laboratory animals may be employed to reveal subclinical parasitaemias in domesticated animals. While they may be more susceptible than domesticated or wild animals, the susceptibility of laboratory animals depends on the species selected. Both T. evansi and T. brucei have 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 respectively 15.2% and 17% more positive results than were obtained by thick smears alone (5, 16).

Blood treated with an anti-coagulant is inoculated intraperitoneally into rats (1-2 ml) or mice (0.25-0.5 ml). Inoculation of a minimum of two animals is
recommended; these are bled from the tail three times each week to detect evidence of parasitaemia. The incubation period before appearance of the parasites and their virulence depend on the strain of trypanosome, their concentration in the original inoculum, and the strain of laboratory animal used. Subclinical infections in the field may perhaps be detected more easily by the prior use of immunosuppressive techniques on the laboratory animals. Drugs such as cyclophosphamide or hydrocortisone acetate are used, or X-irradiation or splenectomy performed for this purpose.

d) Recombinant DNA probes

Specific DNA probes to detect parasite DNA in infected blood or tissue, are being evaluated (19).

Indirect methods

These methods involve haematological, biochemical and serological (see below) 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 expressed as a percentage of packed red cells to total blood volume.

b) Biochemical tests

Biochemical tests include flocculation, formol-gel, mercuric chloride precipitation and thymol turbidity tests. Several older methods considered outdated still have some use in the field because of the simplicity with which they can be performed. The tests all depend on increases in serum globulins as the result of infection, but these increases are not specific for *T. evansi* infection. The formol-gel and mercuric chloride tests are the best.

For the formol-gel test, about 3-5 ml of blood are collected into a dry tube and allowed to clot. About 1 ml of cell-free serum is transferred to a smaller tube and two drops of concentrated formalin solution (40% w/v) 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, about 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 dilution of mercuric chloride that will not precipitate normal serum. The strongest recommended solution of mercuric chloride is between 1:20,000 and 1:30,000. A solution of mercuric chloride (1 ml) in distilled water is transferred to a small tube, and one drop of serum, free from red cells, is added with gentle shaking. 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 (4).

3. Serological tests

Methods to detect specific humoral antibodies to trypanosomes include complement fixation, indirect haemagglutination and precipitin tests. They have not been applied in large-scale surveys. More recently, indirect fluorescent antibody tests (10, 13, 18), ELISAs (3, 10, 21), and card agglutination tests (1, 2, 20) have been employed. These are more useful for general surveys. Currently, the indirect fluorescent antibody test is the most widely used.

It may be added that the ELISA method for the detection of circulating antigens in animals infected experimentally and naturally with *T. evansi*, using *T. brucei* group-specific monoclonal antibody, is under evaluation with promising results (15).

a) Indirect fluorescent antibody test

The antigen consists of dried blood smears containing 5-10 *T. evansi* trypanosomes per field at x 500 magnification, collected from a heavily parasitaemic rat. Smears are dried at room temperature for one hour and fixed in acetone for 15 minutes. The fixed smears may be stored at -20°C for several weeks.

For staining, the slides are first washed in PBS pH 7.2 at room temperature for 15 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; distilled water to one litre. This is stored at 4°C and replenished each month. It is diluted 5-fold in distilled water before use.

After washing, specific antiserum (control serum) or field sera to be tested, diluted 1:20 to 1:80 or more, are added and allowed to react for 30 minutes in a moist chamber. The slides are rewashed three successive times in PBS for 10 minutes each time. A fluorescein-conjugated antiserum specific to the animal species tested is then added at a suitable dilution (from 1:30 to 1:100) and left for 30 minutes. The slides are rewashed in PBS for 10 minutes, mounted with 50% buffered glycerol pH 8.0 and examined by fluorescence microscopy. The glycerol solution should be stored at 4°C and renewed every two weeks.

The fluorescein conjugate should be stored at -20°C in small aliquots to avoid repeated freezing and thawing. The tube should be wrapped in aluminium foil or shielded from light in some way. The conjugate is diluted in PBS pH 7.2 for the test. A 1:1,000 (w/v) solution of Evans blue may be used as a counterstain to discriminate between positive (green) and negative (red) fluorescence.
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 conjugate binds to the antigen-antibody complex and then reacts with a substrate specific to the enzyme to yield a characteristic colour change.

The antigen for coating the plates is derived from the blood of a heavily parasitaemic rat: the parasites are separated on a DEAE-cellulose column and washed three times in PBS pH 8.0. They are then ultrasonicated for 30 seconds. This preparation is diluted in 0.05 M carbonate-bicarbonate buffer pH 9.6. The buffer consists of 25 ml of solution A (anhydrous Na$_2$CO$_3$ 0.4 M, 21.2 g in 500 ml of water), 100 ml of solution B (NaHCO$_3$ 0.4 M, 16.802 g in 500 ml water), 0.1 g NaN$_3$ and distilled water to 475 ml, adjusted to pH 9.6, filtered and stored at 4°C.

The antigen is added (200 µl) to each well of a microtitre plate and incubated overnight at 4°C or for one hour at 37°C. Excess antigen is removed, the plates washed with 0.01 M PBS containing 0.05% Tween 20 (PBS-Tween), and serum samples added (200 µl) in two-fold dilutions in PBS-Tween. The plates are re-incubated at 37°C for 30 minutes and washed three times with PBS-Tween.

The specific conjugated antiglobulin (200 µl) with appropriate dilution (1:30-1:100) is added. The plates are re-incubated at 37°C for 30 minutes and washed three times with PBS-Tween.

The substrate is ortho-diphenylenediamine (OPD) in citrate buffer (35 mg OPD; 100 ml citrate buffer pH 6.0; 0.167 ml 30% hydrogen peroxide). The citrate buffer consists of 36.85 ml of solution A (citric acid 0.1 M, 21.01 g/l), 65.15 ml of solution B (Na$_2$HPO$_4$ 0.2 M, 35.59 g/l) and 100 ml of distilled water.

The substrate is added (200 µl) and the plates incubated at room temperature for 20 minutes. The reaction is stopped by the addition of 7 M sulphuric acid (50 µl). The absorption of each well mixture is read on a spectrophotometer at 492 nm. All tests should include a known positive and negative control serum as well as a buffer control.

Other enzyme labelled systems can also be used (phosphate, biotin/avidin) as long as they are specific for the immunoglobulin being assayed. For closely related species, cross-reacting agents may often be used (e.g. antibovine immunoglobulin for buffaloes).

c) **Card agglutination tests (CATT)**

It is well known that certain variable antigen types (VATs) are expressed in common in different strains of salivarian trypanosomes from different areas. A field test for the diagnosis of Gambian sleeping sickness, the card agglutination test for trypanosomiasis (CATT), was developed at the Laboratory of Serology,
Institute of Tropical Medicine, Antwerp (ITMAS) (12). This test is based on a well defined selected VAT of *T. b. gambiense*.

For the diagnosis of *T. evansi* infections a slightly modified CATT has been used and a similar test system for veterinary use based on a well defined VAT of *T. evansi* has been developed (1, 2).

**Modified CATT**: The test is based on the use of a selected serotype, LiTat 1/3 of *T. gambiense* antigen. The modification consists of the addition of a specific anti-immunoglobulin (Coombs reagent) to amplify the reaction chain. The addition of Coombs reagent induces a prozone phenomenon and for this reason high dilution of the serum is recommended.

**Card agglutination test for veterinary use**: The test is performed as above (CATT) with or without Coombs reagent. It is based on the use of an early variant antigen type of *T. evansi*, RoTat 1/2, which is found to be ubiquitous. This test seems to be more sensitive than the Testryp (R) CATT for *T. evansi* infection.

The test requires lyophilised stained antigens, PBS (pH 7.4), plastic-coated cards, heparinised capillary tubes, and a rotator. The lyophilised antigen may be stored at 2-8°C for up to one year. Reconstituted antigen stored at 2-8°C should be used within two weeks. At room temperature in tropical conditions, the lyophilised and reconstituted antigens should be used within two weeks and eight hours respectively.

Put the diluted serum (1:2-1:4) and antigen suspension (45 µl) in buffer solution containing 10% of specific antiserum on one of the circles of the test card. Spread the mixture. After mixing the test card is rotated for five minutes. A positive reaction is revealed by blue granular deposits visible within five minutes to the naked eye.

This test system has been developed at the Institute of Molecular Biology, Free University of Brussels, and a CATT/*T. evansi* kit produced in collaboration with the Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium is available.

**REFERENCES**


**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

No vaccines are available for this disease.


VENEZUELAN EQUINE ENCEPHALOMYELITIS
(B49)

SUMMARY

Venezuelan equine encephalomyelitis (VEE) viruses cause reactions ranging from mild febrile ones to fatal encephalitic zoonoses in equine animals and humans (3, 4, 6, 8). 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. Neither infective VEE viruses nor their antigens must ever 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 with facilities for moderate biocontainment and an adequately filtered air exhaust system.

VEE viruses are divided into 6 antigenic subtypes (I-VI). Within subtype I there are 5 antigenic variants (variants AB-F). Antigenic variants I-AB and I-C are associated with epizootic activity in equine animals and humans. The other 3 variants of subtype I (I-D, I-E, I-F) and the other 5 subtypes of VEE have been associated with natural enzootic cycles. These variants and subtypes are non-pathogenic for equidae and do not produce clinical disease, although they can cause clinical disease in humans. These are known as sylvatic VEE viruses; they cycle between rodents, mosquitoes and birds.

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 display clinical signs of encephalomyelitis in tropical or subtropical areas when 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. It is recovered less frequently from the blood or brains of encephalitic animals.

VEE virus can be identified by complement fixation (CF), haemagglutination inhibition (HI), plaque neutralisation (PN), immunofluorescence (IF), or radioimmunoassay (RIA) tests. Specific identification of epizootic VEE variants can be made by short-incubation HI tests, a differential PN test using subtype or variant specific antisera, or by molecular biology.

Serological tests: Specific antibodies may be demonstrated by plaque neutralisation tests against epizootic VEE virus variants, or by IgM capture enzyme-linked immunosorbent assay (MAC-ELISA).
Any diagnosis of VEE in an individual that is based on seroconversion in the absence of an epizootic should be made with care. Although sylvatic subtypes and variants are non-pathogenic for equine animals, infection will stimulate antibody production to epizootic VEE virus variants.

Infection of equine animals with sylvatic VEE viruses will produce a low level viraemia accompanied by antibody development, but without clinical disease.

Requirements for biological products: The only acceptable vaccines against Venezuelan equine encephalomyelitis are a live attenuated virus vaccine, strain TC-83, or inactivated virus preparations made from this strain. It is safe and immunogenic at the established passage levels and induces a long-lasting immunity in vaccinated animals.

Formalin-inactivated virulent VEE virus preparations should never be used in equine animals 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. DIAGNOSTIC TECHNIQUES

The Venezuelan equine encephalomyelitis (VEE) complex of viruses are mosquito-transmitted, zoonotic pathogens that produce from mild to severe febrile, occasionally fatal, encephalitic diseases in equine species 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 should be vaccinated (1, 4, 5). Successful immunisation results in the production of VEE virus-specific antibodies. All procedures producing aerosols from VEE virus materials should be conducted in biological safety cabinets with biocontainment and efficient filtration of the exhaust air from the laboratory (5).

The VEE virus complex is comprised of 6 subtypes (I-VI). Within subtype I, there are 5 antigenic variants (AB-F) (2, 3, 4, 6, 8). These were originally considered distinct variants, but subtypes I-A and I-B are now considered identical (I-AB). Within subtype III, there are 3 antigenic variants (A-C). Antigenic variants I-AB and I-C have been associated with epizootic VEE in equidae and concurrent epidemics in humans (3, 4, 6, 7, 8). These equine pathogens are known as epizootic variants; they have been isolated from equine animals, humans and haematophagous insects (primarily mosquitoes) only during equine epizootics. Epizootic VEE virus variants have not been isolated since 1973 and may no longer exist in naturally occurring cycles (8).

The variants of subtype I (I-D, I-E, and I-F) and the other 5 subtypes (II-VI) have been associated with naturally occurring cycles and do not produce clinical encephalomyelitis in the equine species (7). These sylvatic variants and subtypes can however produce clinical disease in humans (3, 4, 6, 8). Infections with both epizootic and sylvatic variants and subtypes have been acquired by laboratory workers.
Historically, epizootic VEE was limited to northern and western South America (Venezuela, Colombia, Ecuador, Peru and Trinidad) (4). From 1969-1972, however, epizootic activity occurred in Guatemala, El Salvador, Nicaragua, Honduras, Costa Rica, Belize, Mexico and the US (Texas). Epizootics of VEE have not occurred in North America, Mexico and South America since 1972, nor in Venezuela since 1973. There have been no isolates of epizootic VEE virus variants since 1973. In contrast, sylvatic VEE variants and subtypes exist continuously as enzootic foci in 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 Guyana (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 US (Colorado and South Dakota) in an unusual bird-swallow bug cycle (3, 4, 6, 8).

The enzootic foci of sylvatic 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. Sylvatic viruses cycle among rodents and perhaps birds by the feeding of mosquitoes (3, 4, 6, 8).

A tentative diagnosis of viral encephalomyelitis in equine animals can be based on the occurrence of acute neurologic disease during the summer in temperate climates or in the wet season in tropical or subtropical climates. These are the seasons of haematophagous fly activity. Virus infection will result in clinical disease in many equine animals concurrently rather than isolated cases. Epizootic activity can move vast distances through susceptible populations in a short time (3, 4, 6, 8).

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 appearance of clinical neurological signs. Frequently, VEE viruses cannot be isolated from the brains of infected equidae. Blood samples for virus isolation should be collected from febrile animals that are closely associated with clinical encephalitic cases (3, 4, 6, 7, 8).

Virus may be isolated from the blood or sera of infected animals by inoculating suckling 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), baby hamster kidney (BHK-21), or duck or chick embryo fibroblasts; or by inoculation of embryonated hens' eggs (3, 4, 6, 8).
Isolates can be identified as VEE virus by CF, HI or PN tests; immunofluorescence; or solid phase radioimmunoassay. Antigenic characterisation of VEE virus isolates may be done by a number of methods: (a) a short incubation HI test using antisera produced in spiny rats, with convalescent sera from other species, or with antisera produced to the E2 glycoproteins; (b) hydroxyapatite chromatography; (c) fingerprinting of ribonuclease T1 resistant oligonucleotide; or (d) differential viral PN tests in equine animals or mice (3, 4, 6, 8).

2. **Serological tests**

Diagnosis of VEE virus infection in equine animals requires the demonstration of specific antibodies in paired serum samples collected in the acute and convalescent phases. After infection, PN 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 (3, 4, 6, 7, 8). The serological procedures are described in detail in the chapter on Eastern and Western equine encephalomyelitis (B37).

Any diagnosis of VEE in an individual that is based on seroconversion in the absence of an epizootic should be made with care. Although sylvatic subtypes and variants are non-pathogenic for equine animals, infection will stimulate antibody production to epizootic VEE virus variants.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

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, 6, 8).

Formalin-treated preparations of epizootic VEE virus should never be used in equine animals. Residual virulent virus can remain after formalin treatment and result in severe illness. Epizootics of VEE have occurred in Latin America from the use of such preparations (6, 8).

1. **Seed management**

   a) **Characteristics**

   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 a long-lasting immunity in vaccinated equine animals. 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.
b) **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 plaque neutralisation tests against hyperimmune serum. For inactivated vaccines of cell culture origin, strain TC-83 virus is treated with formaldehyde.

Attenuated vaccine should be reconstituted with physiological saline and used immediately. Multiple dose vials are kept on ice while vaccine is being used. Any vaccine not used within 4 hours of reconstitution should be discarded. Foals less than 2 weeks old and pregnant mares should not be vaccinated. Animals are vaccinated subcutaneously in the cervical region with a single dose. Annual revaccination is recommended.

Inactivated vaccine should be administered in 2 doses with an interval of 2-4 weeks between doses. An annual revaccination is recommended.

The vaccine should be protected at all times against improper storage or handling, and kept under refrigeration at 2-7°C until required.

2. **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 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.

* Note: Sections on Seed management, Manufacture, In-process control, and Batch control are from the Veterinary Biologies Division of the USDA Animal and Plant Health Inspection Service (APHIS).
4. **Batch control**

a) **Sterility**

Tests for sterility and freedom from contamination of biological materials may be found in the Chapter on General Information.

b) **Safety**

Safety tests are conducted in mice. A 0.5 ml dose is injected intraperitoneally or subcutaneously into each of 8 mice and the animals kept under observation for 7 days. If unfavourable reactions attributable to the product occur during this period, the product is considered unsatisfactory.

c) **Potency**

Potency is 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 $2.5 \log_{10} \text{TCID}_{50}/ml$. 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 not less than $2.5 \log_{10} \text{TCID}_{50}$/dose.

The final product must not contain bacterial, fungal, mycoplasmal or extraneous viral contaminants.

**REFERENCES**


ATROPHIC RHINITIS OF PIGS

(B50)

SUMMARY

Atrophic rhinitis is an infectious disease of pigs characterised by shortening or twisting of the snout, atrophy of the turbinate bones and reduced productivity.

A severe progressive form of the disease is caused by infection by toxigenic strains of Pasteurella multocida, and is usually associated with reduced productivity. A less severe form with a mild or moderate turbinate bone atrophy, generally without significant snout changes, is associated with combined infections with Bordetella bronchiseptica, and perhaps other components of the nasal flora. The severity of the disease can be enhanced by the effects of intensive production, poor management and environmental conditions.

Virtually all apparently normal pig herds are infected with B. bronchiseptica and non-toxigenic P. multocida, often with evidence of some degree of turbinate atrophy. Infection with toxigenic P. multocida often results in clinical evidence of severe atrophic rhinitis. This may occur enzootically, or eventually more sporadically, depending on the herd immunity.

Turbinate bone atrophy may only become obvious at slaughter, or may be detected in the live animal by radiography.

Identification of the agent: The diagnosis of atrophic rhinitis in pigs depends on clinical and post-mortem observations assisted by the recovery and characterisation of P. multocida and B. bronchiseptica in affected pigs. The isolation of P. multocida is often complicated by the more profuse growth of other organisms. This may be obviated by passaging the material in mice or by using a selective culture medium, usually blood agar containing antibiotics. P. multocida can be identified by biochemical tests; any ability for toxin production can be demonstrated by testing for dermonecrotic activity in guinea pigs, for lethality in mice, or for cytotoxicity in cultured cells.

Isolates can be characterised by their capsular and somatic antigens. Capsular types A and D are usually isolated from pigs; toxigenic strains are often type D, but toxigenic type A strains also occur. Capsular antigens may be distinguished serologically by indirect haemagglutination or immunofluorescence tests, chemically by flocculation in acriflavine, or by susceptibility to hyaluronidase. Somatic antigens are detected by precipitation or agglutination tests.

B. bronchiseptica is usually isolated by the use of selective culture media. It forms characteristic colonies and can be identified by slide agglutination tests.

Serological tests: Since non-toxigenic strains of P. multocida share cross-reactive antigens with toxigenic strains, and since B. bronchiseptica can
be isolated with ease from pigs of most herds, the use of serology for identifying diseased or carrier animals is of no value with the tests available. Also, some infected pigs do not develop antibodies to P. multocida toxins. However, a serological test based on detection of antibodies to the toxin has been described, which may prove to be useful on a herd basis.

Requirements for biological products: Several vaccines are available commercially which contain bacterins of B. bronchiseptica and a mixture of toxigenic and non-toxigenic strains of P. multocida or a P. multocida toxoid.

A. DIAGNOSTIC TECHNIQUES

Atrophic rhinitis is an infectious disease of pigs characterised by shortening or twisting of the snout, turbinate atrophy and reduced productivity. Two forms of the disease have been recognised:

a) a severe progressive form caused by toxigenic isolates of Pasteurella multocida, and

b) a less severe form with mild to moderate turbinate atrophy, generally without significant snout changes, and associated with combined infections with Bordetella bronchiseptica and perhaps other components of the nasal flora (8, 9).

Increased severity of these infections is associated with intensive production, overstocking, and poor management, housing and environment. Reduced productivity has generally been associated with the severe progressive form of atrophic rhinitis, and therefore by implication with toxigenic P. multocida. However, the precise relationship between infection with these bacteria and reduced weight gains has not been adequately elucidated. While this view is currently widely held, others hold that the major factors influencing the severity of the disease in a herd are managemental and environmental rather than microbiological.

Since virtually all pig herds are infected with B. bronchiseptica and non-toxigenic P. multocida, the presence of some degree of turbinate atrophy can be expected in most apparently normal herds. However, infection with toxigenic P. multocida appears to occur in herds with clinical evidence or a history of the more severe form of atrophic rhinitis. This may occur either as a herd problem or sporadically in a few slaughtered pigs; such herds are important because of the risk of transferring infection to other herds previously free of toxigenic P. multocida and the risk of their progressing to the severe form of the disease.

The diagnosis of atrophic rhinitis of pigs, therefore, depends on clinical, pathological and microbiological investigations. The latter are particularly important for herds capable of spreading the disease because they are subclinically infected with toxigenic P. multocida.
Clinical signs include sneezing, nasal discharge, shortening or twisting or the snout, accompanied by atrophy of the nasal turbinate bones, a reduced growth rate and, in severe cases, additional difficulty in eating. Turbinate atrophy may only become obvious at slaughter when snout sections at the level of the second premolar tooth are examined, but radiography as well as endoscopy have also been used to detect this change in live animals (3, 13). Several scoring systems for these gross changes are in use and are particularly useful for monitoring disease.

Diagnosis is assisted by histopathological examination of any turbinate atrophy, and the isolation and characterisation of *P. multocida* and *B. bronchiseptica*. The histopathological changes include replacement of the bony plates of the vertical conchae with varying degrees of inflammatory and reparative changes (4). Demonstration of toxigenic *P. multocida* either by culture or by detection of its toxin can confirm the diagnosis of progressive atrophic rhinitis (1, 6) or may diagnose subclinically infected pigs.

1. **Identification of the agent**

Nasal swab samples are collected from pigs aged 8-10 weeks with clinical signs of atrophic rhinitis or from pigs whose carrier status is being investigated. These should be placed in charcoal transport medium if there is to be any delay.

Cultures are made on selective medium or the material is injected into mice. The cultures should produce individual colonies of *B. bronchiseptica* on Bordet-Gengou agar that contains furantadone, penicillin, and streptomycin (12). MacConkey agar with added glucose (5) is also used for the isolation of *B. bronchiseptica*, or 5% horse blood agar with added neomycin, bacitracin and actidione for *P. multocida* (12). *P. multocida* may also be selectively cultured in mice by the intraperitoneal inoculation of 0.2 ml of the transport medium in which the swab has previously been dissolved, with occasional mixing, at 37°C for up to 4 hours. The livers of any mice which die, or are killed after 3 days, should be cultured on horse blood agar or on selective medium.

Isolates of *P. multocida* are identifiable by their colony morphology. Gram staining reveals Gram-negative bipolar pleomorphic rods; these fail to grow on MacConkey agar, but yield a positive oxidase reaction and produce indole. Isolates producing the type D capsule form a heavy flocculate in 1/1,000 aqueous acriflavine, and the growth of *P. multocida* with the type A capsule appears to be inhibited in the presence of hyaluronidase which digests the capsule (see 10). *B. bronchiseptica* is identified by its characteristic colony morphology on Bordet-Gengou medium and by a slide agglutination test using hyperimmune rabbit serum (9).

Toxigenic isolates of *P. multocida* can be differentiated from non-toxigenic strains by the toxicity of the supernatant fractions of broth cultures for embryonic bovine lung (EBL) cells (11). The bacteria are grown in brain heart infusion broth incubated at 37°C for 24 hours. These are centrifuged and the supernatant fractions are sterilised by filtration and titrated in EBL monolayer cultures in microtitre trays. These are incubated for 2-3 days, after which the cells are stained with crystal violet and examined microscopically to detect cytopathic effects.
In a rapid test, colonies resembling *P. multocida* can be selected from the medium used for primary isolation and subcultured on an agar overlay of EBL cells. After 18 hours’ incubation, toxigenic isolates produce sufficient toxin to diffuse through the agar and cause the cells to separate from each other (1). This method has the advantage that isolates do not need to be identified unless they are toxigenic, and that many isolates per sample with a suitable colony morphology may be tested. A guinea pig skin test may be used to demonstrate the dermonecrotic toxin. There is also an enzyme-linked immunosorbent assay (ELISA) based on monoclonal antibodies which can detect toxin in cultures of toxigenic *P. multocida* (6).

2. **Serological tests**

Many animals infected with toxigenic *P. multocida* fail to produce antibodies to the toxin. Consequently, there are at present no satisfactory serological tests that can be relied on to detect animals infected with toxigenic *P. multocida* that could be capable of developing or spreading the disease. Non-toxigenic strains of *P. multocida*, which share many cross-reacting antigens with toxigenic strains, and *B. bronchiseptica* can be isolated from most pig herds. Thus, serological tests for the differentiation of diseased or carrier animals are of limited value. However, a serological test based on detection of antibodies to the *P. multocida* toxin has been described (6).

Infection with *B. bronchiseptica* can be detected serologically by agglutination tests (9), but most pig herds are infected and have antibodies to this organism.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

There are several commercially available vaccines which contain bacterins of *B. bronchiseptica* and a mixture of toxigenic and non-toxigenic strains of *P. multocida*, or a *P. multocida* toxoid. *B. bronchiseptica* used for vaccine production should be phase I virulent cultures, and *P. multocida* isolates should be toxigenic. *P. multocida* toxin is produced extracellularly after 16 hours’ incubation in fluid culture, but no production criteria have been established to provide a vaccine known to give complete protection against *B. bronchiseptica* and toxigenic *P. multocida*. However, some available vaccines assist in establishing a protective herd immunity when combined with improved managerial measures. Vaccines based on a *P. multocida* toxoid have become available and seem likely to offer specific protection against the action of the toxin (2, 7).

**REFERENCES**


TRANSMISSIBLE GASTROENTERITIS
(B53)

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 pathogen but it has greatly complicated the diagnosis of TGE, particularly by means of serology.

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. An alternative diagnostic method is the oral dosing of susceptible piglets with suspect intestinal contents. This is a very sensitive method that may be appropriate for laboratories that lack facilities for other specified tests. However, if experimental disease is induced in this way, some form of laboratory testing is still necessary to confirm TGE.

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 (ELISA) on faeces and fluorescent antibody tests (FAT) on cryostat sections of intestine. Detection of virus by reversed passive haemagglutination has also been described. Another enteric disease, porcine epidemic diarrhoea, is caused by a serologically distinct coronavirus, which nevertheless has an identical electron microscopic appearance. Diagnostically, immune electron microscopy circumvents this problem.

Serological tests: The most widely used methods are virus neutralisation (VN) tests and ELISAs. Only in the latter case is differentiation from PRCV possible, since TGEV and PRCV antibodies show complete cross-neutralisation. Passive haemagglutination and haemagglutination inhibition tests have also been developed for TGE serology.

Requirements for biological products: There are no commercial biological products available internationally. However, in the United States, licences have been issued authorising the production and distribution of monovalent and combined vaccines.
A. DIAGNOSTIC TECHNIQUES

Transmissible gastroenteritis virus (TGEV) multiplies in and damages the epithelium of the small intestine, producing villous atrophy and enteritis. Extra-intestinal sites of virus multiplication include the respiratory tract and mammary tissues (16), but the virus is most readily isolated from the intestinal tract. By contrast, porcine respiratory coronavirus (PRCV) is most readily isolated from the upper respiratory tract, the tonsils or the lungs, and little if any enteric multiplication of virus occurs (24, 21, 9). PRCV is probably a deletion mutant of TGEV (25).

Since TGE is a contagious disease that can occur as explosive epizootics, rapid diagnostic methods for its confirmation are particularly important.

1. Identification of the agent

Virus may be identified by virus isolation in tissue culture (33), electron microscopy, various immunodiagnostic assays, and more recently by specific detection of viral RNA (29, 3). The most commonly employed rapid assays are probably the immunodiagnostic ones, particularly enzyme-linked immunosorbent assays (ELISA) on faeces (4, 30) and fluorescent antibody tests (FAT) on cryostat sections of intestine (23). Detection of virus by reversed passive haemagglutination has also been described (1). Another enteric disease, porcine epidemic diarrhoea, is caused by a serologically distinct coronavirus, which nevertheless has an identical electron microscopic appearance. Diagnostically, immune electron microscopy circumvents this problem (27, 31).

a) Virus isolation in tissue culture

Apart from the inoculation of live piglets (10), this is the most definitive method of diagnosis. However, for routine use it is slow and laborious.

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 phosphate buffered saline (PBS), pH 7.2, with added antibiotics 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 effects of the material and it is then used to inoculate susceptible tissue cultures, such as 3- to 4-day-old primary or secondary pig kidney monolayers. Other low passage porcine cultures (such as thyroid or testis) and some cell lines (18, 13) may also be used for primary virus isolation. After incubation at 37°C for one hour the cell sheets are overlaid with a medium such as Earle's yeastolate lactalbumin (EYL) balanced salt solution, containing sodium bicarbonate, antibiotics and 1% fetal calf serum.
Incorporation of trypsin into the culture medium may enhance primary viral recovery (5, 13). Uninoculated control cultures are established concurrently and all cultures are incubated at 37°C.

Viral cytopathic effects (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. Wild-type TGEV does not grow readily in tissue culture so several sub-passages 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 (6). If suitable monoclonal antibodies are available they can be used to distinguish between TGEV and PRCV by immunostaining methods (11). Differentiation of TGEV from PRCV can also be accomplished by TGEV-specific cDNA probes (2).

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 less than 4 weeks old and just starting to show clinical signs of the disease (that is, within 24–28 hours of infection). Two centimetre lengths from 4 different regions of the posterior part of the small intestine should be removed within 30 minutes of death. 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 coverslips, 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, the sections are stained with a diluted solution of FITC-conjugated anti-TGEV antibody, and placed in a moist incubator at 37°C for 30 minutes. Any unbound stain is removed by washing in Tris buffer. The sections are counterstained with a 10⁻⁵ 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 in both frozen and paraffin-embedded tissues has also been described (15).

c) ELISA detection of faecal virus antigens

A double antibody sandwich system is used, for instance with a monoclonal capture antibody and a polyclonal enzyme-linked detector antibody. The anti-TGEV monoclonal antibody is directed towards a conserved epitope on the viral nucleoprotein and is in the form of clarified ascitic fluid. It is applied to 96-well microplates in a bicarbonate buffer, pH 9.6, and incubated overnight at 4°C. Faecal samples are prepared in McIlvaine’s buffer, pH 6.0, and then
diluted in PBS, pH 7.2, with 0.05% Tween 20, 1% gelatin, 1% Nonidet and a proprietary dried milk product. Plates are washed twice with washing buffer (PBS plus 0.05% Tween 20) before adding the prepared faecal samples. The plates are incubated at 37°C for 2 hours. After a further wash, a peroxidase-linked, porcine, anti-TGEV antiserum is added in a buffer of PBS plus 0.05% Tween 20, 1% gelatin and 5% normal pig serum; this mixture is incubated at 37°C for 2 hours. The plates are washed 6 times before adding the enzyme substrate which is 0.1 mg/ml tetramethyl benzidine in 0.1 M acetate buffer, pH 6.0, with 0.00225% hydrogen peroxide. After five minutes the reaction is stopped by the addition of 2.0 M sulphuric acid, and the optical densities of the well contents are assessed. A control negative and a control weak positive antigen well are included on each plate.

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. Since 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, antiviral antibodies can be detected in serum from 6 or 7 days post-infection and such antibodies persist at least for many months. Although anti-PRCV and anti-TGEV antibodies show complete neutralisation of either virus, there are differences in the specificities of some of the non-neutralising antibodies (7, 11), since PRCV lacks certain epitopes present on TGEV. Monoclonal antibodies to such regions can be incorporated into competition ELISAs to detect serum antibody that is entirely TGEV specific. Whilst such tests are reliable in respect of not producing false positive results with PRCV antisera, false negatives may occur due to i) a reduced sensitivity compared to neutralisation tests, and ii) because of strain variation amongst TGE viruses, such that no single TGEV-specific monoclonal antibody will recognise all strains (32, 6). The problem of insensitivity can be reduced by using the test on a group or herd basis.

a) TGEV/PRCV tests

These tests detect antibody to both TGEV and PRCV, and include VN tests, indirect ELISAs (19, 12, 26, 14) and competitive ELISAs based on TGEV/PRCV group-specific monoclonal antibodies (22). Haemagglutination-based tests so far described (17, 28, 20) would probably also fall into this category.

VN tests can be performed with a variety of cell types and viral strains. 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 (33) 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 is incubated with test sera and
neutralisation is indicated by absence of CPE after further incubation with 2 x 10^5 cells/ml in Leibovitz 15 medium with added antibiotics, 10% fetal calf serum and 1% L-glutamine. The sera should be unhaemolysed, sterile and inactivated by heating at 56°C for 30 minutes.

25 μl volumes of virus and serum are added to the test wells. Test sera are screened in duplicate at doubling dilutions, beginning with undiluted serum (this gives a final dilution of 1:2 when mixed with an equal volume of virus). Controls must include a back titration of the virus without serum, a known negative serum, a titration of a known positive antiserum, and serum control wells without virus for each test serum dilution. Where virus or serum is omitted, a 25 μl volume of tissue culture medium without fetal calf serum is substituted.

After the addition of the sera and virus, the plates are agitated briefly on a shaker and incubated at 37°C for one hour. Finally, 100 μl of the suspension of A72 cells is added to each well and the plates are sealed and again incubated at 37°C. After 3-7 days the plates are examined for CPE. The virus control back titration is calculated and should be within the range of 50-200 TCID_{50}/unit volume. Inhibition of viral CPE by the test sera is assessed after checking that the serum controls are free of evidence of cytotoxicity or contamination. The serum dilution that gives 50% protection against CPE in paired wells represents the neutralisation titre.

b) TGEV-specific tests

These are ELISAs employing a monoclonal antibody that recognises TGEV but not PRCV (8, 32, 6). Test sera from pigs previously infected with a strain of TGEV recognised by the monoclonal antibody 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 mock infected. 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 monoclonal antibody to all wells. Bound monoclonal antibody is detected by a peroxidase-conjugated anti-mouse antibody which induces a colour reaction in the presence of an appropriate substrate. The colour changes are measured by 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 cutoff value for the test must be determined by previous testing of known negative and positive populations.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

There are no commercial biological products available, although monovalent and combined vaccines have now been licensed for production in the United States.
REFERENCES


SUMMARY

Diagnostic tests for swine trichinellosis fall into two categories: 1) direct detection of first-stage larvae encysted in striated muscle tissue, and 2) indirect detection of parasitism by tests for specific antibodies.

For ante-mortem tests, serodiagnostic methods are preferable to biopsy. The sensitivity of serological methods is equal to or better than direct methods. However, in light or moderate infections in pigs, a serological response is often not detected for up to 3 weeks after muscle larvae become infective. In such cases, a false negative serological result is obtained. A low rate of false positive results has also been reported for serological tests.

Identification of the agent: Two general methods, compression or digestion of muscle tissue, are used for the direct detection of Trichinella spiralis infestation in pigs. Both methods test for the presence of parasites in tissues where infestation is heaviest, these being in descending order of incidence, the diaphragm (crus), tongue, masseter and abdominal muscles; although this partially depends on the degree of infestation. The selection of an appropriate method for the direct inspection of pork for trichinellosis depends on the facilities available and the number of samples to be tested.

The first method is by visual inspection of compressed pieces of muscle tissue for the presence of larvae in situ. This requires a specialised microscope, the trichinoscope, which has an estimated efficiency of detecting as few as 3 larvae per gram of tissue. It has the disadvantage of requiring considerable time for the inspection of multiple samples from each carcass.

The second method involves the digestion of individual or pooled muscle tissue samples, followed by selective screening filtration, or sedimentation procedures. Finally these samples are inspected microscopically for the presence of larvae. Digestion methods may be mechanically assisted by stirring or homogenising the digest mixture. Digestion methods have an efficiency of approximately one larva per gram of tissue examined when tissue sample size is one gram.

Serological tests: The enzyme-linked immunosorbent assay (ELISA) is the best method for the ante-mortem diagnosis of trichinellosis. It is comparable in sensitivity to the best direct methods. Infection levels as low as one larva per 100 grams of tissue have been detected. The specificity of ELISA for trichinellosis is directly linked to the type and quality of the antigen employed in the test. Secretory antigens collected by short-term maintenance of T. spiralis muscle larvae in vitro currently provide the most specific and economical source. There are no known cross-reactions using these antigens, although a low rate of false positive results have been obtained.
The use of secretory antigens is recommended for herd surveillance programmes by ELISA. As a confirmatory test the digestion of up to 100 grams of tissue will ensure an accurate diagnosis. A low rate of false negative ELISA results may be obtained with recently infested animals that have low grade infestation.

Requirements for biological products: No biological products are available.

A. DIAGNOSTIC TECHNIQUES

A number of diagnostic tests are available for the detection of *Trichinella spiralis* infection (trichinellosis) in swine. These are either:

1. the direct demonstration of the parasite in tissue samples or digests, or
2. indirect demonstration of the parasite by the detection of specific antibodies by serological methods.

1. **Identification of the agent**

The direct demonstration of the parasites is generally limited to inspection of carcasses post-mortem although methods of ante-mortem biopsy have been reported (6). The sensitivity of direct testing relates to the amount of tissue examined. Commonly employed procedures estimate the sensitivity of direct methods at 3 larvae/gram (lpg) of tissue for the trichinoscope technique (8), and approximately one lpg of tissue for the pooled sample digestion method. Direct methods will identify infected pigs as early as 17 days after exposure, coincident with the time that muscle larvae become infective for a new host. Direct methods remain effective as long as muscle larvae remain viable. Where large quantities of tissue (up to 100 grams) are available for digestion, the sensitivity of this test is greatly increased. The disadvantages of direct inspection methods, particularly by trichinoscopy, are the time, labour and expense required.

Two methods are generally employed for the direct diagnosis of trichinellosis:

a) **The trichinoscope or compression method**

Trichinoscopy for the inspection of pork has been described elsewhere (1, 2).

Samples are taken for examination from the pillars of the diaphragm (crus) and cut into 14 pieces each about 2 x 10 mm in size. Alternative sites for tissue collection include the tongue, masseter and abdominal muscles, but larger samples are required for comparable sensitivity. The tissues are compressed between glass plates until they become translucent. They are then examined for larvae through a specially constructed projection microscope, the trichinoscope, or a conventional microscope at 15-40 x magnification. In case of doubt, further tissues should be examined.
b) The digestion method

Muscle tissue can be digested with digestive fluid. This releases live trichinae from the muscle cysts. Four digestion procedures are recommended within the European Economic Community (EEC) (1, 2), and EEC Directives should be consulted for more complete details. The procedures include:

i) the artificial digestion method for individual or pooled samples,

ii) the mechanically assisted pooled sample digestion sedimentation technique,

iii) the mechanically assisted pooled sample digestion method on filter isolation technique, and

iv) the magnetic stirrer method for pooled samples.

The magnetic stirrer method for pooled samples can be employed in a variety of circumstances with a minimum of equipment. An example of this method, as used in the USA, is as follows:

Sampling: Muscle samples are taken from the diaphragm pillars or tongue; other sites generally have lower numbers of larvae. Sample sizes can vary, but individual samples of 100 grams may be taken from one pig, or multiple samples from a number of animals are collected to make a 100-gram pool. The size of the samples which make up the latter will determine the sensitivity of the method. A minimum sample size of 5 grams, a requirement in the United States, is recommended, with 20 animals involved in each 100-gram pool. The samples are ground or diced to facilitate digestion.

Digestion and recovery: Each 100 grams of tissue is digested in one litre of artificial gastric fluid containing 1% w/v pepsin (1:10,000 National Formulary) and 1% v/v hydrochloric acid (0.12 N final). The ground or diced sample is added to the digestion fluid, pre-warmed to 37°C, and the mixture stirred on a magnetic stirrer for 3-4 hours at 37°C. The digest is allowed to settle for 15-20 minutes and the upper two-thirds of the fluid decanted. The remaining fluid and deposit are allowed to settle for a further 15-20 minutes. The maximum possible supernatant fluid is aspirated without disturbing the sediment: the latter is washed with warm (37°C) tap water and allowed to settle once more for 15-20 minutes. The washing step is repeated until the supernatant fluid is clear. The washed sediment is transferred to a 50 ml tube, allowed to settle, and aspirated down to a final volume of 10 ml. For enumeration purposes, all 10 ml is poured into a gridded petri dish and examined for T. spiralis larvae with a dissecting microscope. If the counts are high, appropriate dilutions must first be made. When larvae are detected in pooled sample digests, the entire procedure must be repeated on the individual samples comprising the pool.

Use of a thermostatically controlled tissue blender shortens the time required for digestion to about 12-15 minutes. Details of other methods using a
Stomacher Lab Blender 3500 have been described (2) and an alternative protocol for the magnetic stirrer method for pooled samples, approved by the EEC (84/319/EEC), can also be recommended (2).

2. Serological tests

The use of ELISA to detect the presence of parasite-specific antibodies provides a rapid method which can be performed on blood or serum collected before or after slaughter. Infestations as low as one larva/100 grams of tissue can be detected by ELISA. Several antigen preparations have been developed that provide a high degree of specificity for swine trichinellosis (3, 4, 9). In abattoir testing, the ELISA yielded less than 0.3% false positives and was nearly 100% sensitive in detecting infected hogs with >1 larvae per gram of tissue (7).

A disadvantage of serology in trichinellosis is the occurrence of a low rate of false negative results in the case of infested pigs. Such results are due to a lag in the kinetics of antibody responses in lightly or moderately infested animals (3). This slow rate of antibody production means that infested pigs cannot be diagnosed for several weeks.

Diagnosis of trichinellosis by ELISA can be accomplished by using secreted stichosome antigens collected from T. spiralis larvae. The antigens recognised in worm secretions consist of a group of structurally related glycoproteins with molecular weights of 45-55 kilodaltons (4). To prepare the antigen (5) T. spiralis muscle-stage larvae are recovered from skinned, eviscerated, ground rat carcasses by digestion in 1% pepsin with 1% HCl for 3 hours at 37°C. These larvae are washed in Dulbecco's Modified Eagle's Medium (DMEM) with penicillin (500 units/ml) and streptomycin (500 µg/ml) and placed in DMEM supplemented with HEPES (10mM), glutamine (2mM), pyruvate (1mM), and penicillin (50 units/ml)-streptomycin (50 µg/ml) (complete DMEM) for 18-20 hours at 37°C in 10% carbon dioxide in air. Culture medium is recovered, worms removed by filtration and fluid concentrated under pressure with a 5,000 dalton molecular weight retention. Secretory antigens thus recovered may be stored frozen for short periods at -20°C or for longer at -70°C; they comprise approximately 25 protein components as determined by SDS-polyacrylamide gel electrophoresis.

An example of an ELISA for trichinellosis is given below. It is essential that all reagents used in the assay be standardised for optimal concentration to obtain reliable results. Typical values are indicated in the example.

In the indirect ELISA for trichinellosis (3), antigen is diluted to 5 µg/ml in 0.1 M carbonate buffer (pH 9.6) and used to coat microtitre plates (100 µl/well) at 4°C overnight or at 37°C for one hour. The wells are washed 3 times with phosphate buffered saline containing 0.5% Tween 20 (PBS-Tween) at this stage and between all subsequent steps. Each of the following reagents is added (100 µg/ml) sequentially and incubated for 30 minutes at room temperature between each step: swine serum (diluted 1:10 or 1:100 in PBS-Tween); rabbit anti-swine IgG heavy-chain (diluted 1:1,000 in PBS-Tween); goat anti-rabbit IgG conjugated to horseradish peroxidase (diluted 1:1,000 in PBS-Tween). Bound enzyme is visualised.
by the addition of 5'-aminosalicylic acid (0.8 mg/ml) with 0.005% hydrogen peroxide (pH 5.6-6.0) or other suitable peroxidase substrate.

The plates are read for colour density. Values 4 times that of normal serum pool controls are considered positive. Values 3 times higher than normal are classified as suspect. Commercial adaptations of ELISA make use of a double antibody format with a peroxidase-labelled anti-swine serum.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

No biological products are available.

REFERENCES


SUMMARY

The clinical diagnosis of avian infectious bronchitis (IB) is complicated by the variety of its clinical forms (respiratory disease, nephritis, or loss of egg production). The use of live virus vaccines may lead to their reisolation in the course of attempts at virus isolation. Antibodies arising from large scale vaccination may complicate the interpretation of serological reactions. The natural occurrence of antigenic variant strains may break any immunity induced by conventional vaccines, although their importance is still controversial.

Laboratory diagnosis is made by virus isolation in chick embryos or in tracheal organ cultures. This can be supplemented by immunofluorescence, electron microscopy, haemagglutination inhibition tests (HI) or enzyme-linked immunosorbent assays (ELISA).

Identification of the agent: IB virus may be isolated from tracheal mucosa during the acute phase of the respiratory form of the disease. Faeces or caecal tonsillar tissue are better sources of virus at other times.

Chick embryos originating from specific pathogen free flocks are used. The inoculation of the allantoic cavity of chick embryos of 9 to 11 days' incubation with IB virus results in embryo stunting or death within about 3 serial passages. 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.

Tracheal organ cultures have the advantage that IB virus produces stasis of the tracheal cilia on initial inoculation. No blind passages are required as in embryos. Antigenic typing of IB virus 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. Contradictions may arise when these tests are repeated in other laboratories which may stem from the presence of antigens common between strains. The use of monoclonal antisera may prove to be a useful method of distinguishing vaccine strains from field strains.

Serological tests: Regular monitoring of sera from flocks for IB antibody levels assists the diagnosis of suspected disease outbreaks. The HI test is rapid; also, commercially produced kits are available for ELISA assays which are very sensitive but appear to lack type specificity.
A positive diagnosis of IB is made by virus isolation together with tests to demonstrate significant rises in specific antibody. Following preparation of antiserum to a virus isolate, a serological comparison can be made with existing strains. One-way serological tests on field sera are unreliable indicators of infection with particular variants, because they may be confused by the presence of group-reactive antibody.

Requirements for biological products: Both live attenuated and oil emulsion inactivated vaccines are available. Live vaccines have been attenuated by serial passage in chick embryos and confer a better local immunity of the respiratory tract. The use of some live vaccines carries the risk of residual pathogenicity, and this consideration may be avoided by using inactivated vaccines instead.

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; inactivated vaccines are available with Newcastle disease, infectious bursal disease and EDS76 viruses.

A. DIAGNOSTIC TECHNIQUES

Avian infectious bronchitis (IB) was first described in the United States in the 1930s as an acute respiratory disease of mainly young chicks. The aetiology was established as avian infectious bronchitis virus (IBV). It affects chickens of all ages which are the only species naturally affected, with the possible exception of pheasants. It occurs world-wide and assumes a variety of clinical forms, the principal one being a classical respiratory syndrome. Infection of the oviduct can lead to cessation of egg-laying, or production of thin-walled and misshapen shells with loss of shell colour. It can be nephrotropic causing acute nephritis, urolithiasis and mortality (1). After apparent recovery, persistent nephritis can produce sudden death, especially in brown birds. The virus persists in the intestinal tract and is excreted in the faeces for long periods. This occurs with vaccine strains as well as natural field strains (3).

Extensive use is made of vaccines, but this does not completely prevent IB infections. It complicates diagnosis by serological methods since vaccinal immunity and field infections cannot be distinguished. Persistence of live vaccine virus also confuses attempts at recovering the causative agent (21).

1. Identification of the agent

a) Sampling

In acute respiratory disease, swabs made from the trachea, or tracheal and lung tissues, from a recently killed bird should be despatched on ice in a transport medium containing penicillin (10,000 IU/ml) and streptomycin (10 mg/ml).
Samples may also be selected from the kidneys or oviduct, but the highest success rate of virus recovery is from samples of large intestine, particularly the caecal tonsillar tissue or faeces (3).

Suspensions of tissues (20% w/v) are prepared in sterile broth with antibiotics for egg inoculation, or in tissue culture medium for organ culture inoculation (15). The suspensions are first clarified by low-speed centrifugation and filtered through membranes of 0.3-0.65 μm average pore diameter.

b) Culture

Embryonated hens' eggs are capable of generating high yields of IBV. They are widely used to titrate the virus or to make primary isolations of virus. This is nevertheless a slow means of isolating IBV and other ways are now gaining acceptance. Cell cultures do not show cytopathic effects of virus infection until and unless IBV has first been adapted to growth in chick embryos.

Eggs used in all culture work with IBV must originate from birds that have neither been infected nor vaccinated. Such eggs should preferably be from specific pathogen free (SPF) hens; white eggs are best for candling purposes. The allantoic sac of embryos aged 9-11 days is the best route for inoculation, using 0.05-0.5 ml of inoculum, 0.1 ml being the most common. Eggs should be examined daily thereafter. Any deaths that occur within 24 hours are assumed to be non-specific and the eggs are discarded. True effects take up to 1 week to be apparent. The initial inoculum usually has no effect on the embryo unless the strain is already egg-adapted. Normally the allantoic fluids of all eggs are pooled after harvesting; this pool is diluted 1:5 or 1:10 in antibiotic broth and passaged into further eggs. This is repeated as desired. Typically, a field strain will induce teratological changes in the embryo at the second or third passage with some mortality in later passages. A serum neutralisation or electron microscopy test will identify the agent as IBV. Infective allantoic fluids are stored in the long term at -60°C or below, or at 4°C after lyophilisation.

Tracheal organ cultures prepared from 20-day old embryos can be used to isolate IBV directly from field material without any prerequisite egg-adaptation (10, 13). An automatic tissue-chopper is essential for the large-scale production of suitable transverse sections or rings of the trachea for this technique (15). 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) on roller drums (15 rev/h) at 37°C. Infection of tracheal organ cultures produces a ciliostasis within 24-48 hours. A neutralisation test will confirm the identity of IBV. Electron microscopy of an ultracentrifuged deposit of the medium may also confirm it.

Antigenic variation amongst IBV strains is well reported (12, 17, 20, 21, 24, 25). However, there is still no agreed definitive classification system. The use of a closely type-specific method can be misleading, since the serum used will not react unless near the antigenic type of the new isolate. For this reason,
the less specific serum neutralisation test in embryonated eggs and the immunodiffusion technique are useful for identification purposes. Antisera prepared by multiple inoculations possess higher titres of group-reactive antibody.

2. Serological tests

A number of tests have been described (21). Those considered here include virus neutralisation (VN), agar gel immunodiffusion (AGID) (35, 36), HI (2), immunofluorescence (IF) (12) and ELISA (9, 29, 30, 31).

a) Virus neutralisation

In VN tests, all sera should first be heated at 56°C for 30 minutes (5). Virus is mixed with serum for up to 30 minutes at room temperature (17, 21, 32). Chick embryos are often employed especially when they have been used to isolate virus. In the so-called alpha method, logarithmic dilutions of the egg-adapted virus are reacted with a fixed dilution (usually 1:5) of known antiserum and the mixtures inoculated into groups of 5-10 eggs. The virus alone is titrated in parallel. End-points are calculated by Karber. The results are expressed as a neutralisation index (NI) which represents the $\log_{10}$ difference in the titres of the virus alone and that with the antiserum. 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 results of down to 1.5. This method is still often used for virus identification.

The test more widely adopted for antibody assay with chick embryos is the beta method. Two- or four-fold dilutions of antiserum are reacted with a dilution of virus often fixed at 100 median egg-infective doses (100 EID$_{50}$) per 0.05 ml and each mixture inoculated (0.1 ml) into a group of 5-10 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 $10^{1.5}-10^{2.5}$ EID$_{50}$. End-points of the serum titres are determined by Karber or Reed and Muench 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 5 tubes per serum dilution as is conventional with other viruses (16). 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 wide-spread adoption.

b) Agar gel immunodiffusion

Immunodiffusion can be used in diagnosis (18, 28, 35, 36). The antigen is prepared from a heavy homogenate of the chorio-allantoic membranes of infected chick embryos. The Beaudette embryo-lethal strain is often employed. The test is liable to yield inconsistent results.
c) **Haemagglutination inhibition**

A standard protocol for an HI test for IBV has been described (2, 4). The haemagglutinin antigen is preferably prepared from the Massachusetts M41 strain. Infective allantoic fluid is centrifuged at 30,000 g for 3 hours, the pellet resuspended at 100-fold concentration in 0.01 M Tris-HCl buffer (pH 6.5), and then mixed with an equal volume of phospholipase C type 1 enzyme (final concentration one unit per ml) in the same buffer. This mixture is incubated at 37°C for 2 hours. Based on a preliminary titration, this antigen is used in the test at 4 haemagglutinating units (HAU) per unit volume, mixed with doubling dilutions of test serum. End-points of antigen titrations are read at 100% haemagglutination, equal to 1 HAU, and at 100% inhibition in HI tests. Two positive control sera, of low and high titre respectively, must be included in each test, as well as known negative serum. Serum titres are expressed in log₂ reciprocals.

The HI test is widely used in diagnosis and routine flock monitoring of vaccine responses. Care must nevertheless be exercised in the interpretation of the results. There is good correlation between HI antibody titres in laying birds and protection against loss of egg production following infection (7, 19), but there is little or no correlation in birds in general between HI titres and protection against respiratory disease (34, 37). In assessing vaccinal responses, tests using a vaccine strain as HA antigen result in higher titres than those using the more standard M41 strain antigen (22). Strain typing of IBV may be done by HI tests (8, 26, 27) but it is requisite to perform chess-board titrations between viruses, as their own antigens, and their homologous antisera to obtain reliable results.

d) **Immunofluorescence**

The IF test is less used, but may identify newly isolated virus during blind passaging in eggs (11). It is preferable to prepare specific antisera against the isolate for conjugation with fluorescent dyes for this purpose. Suspect allantoic fluid is centrifuged at 1,000 g for 10 minutes and the pellet resuspended in a few drops of a buffer, air-dried and fixed with acetone at -20°C for 30 minutes. The smears are stained overnight at 4°C, washed for up to one hour in buffer and mounted in buffered glycerol saline for examination by dark field fluorescence microscopy.

e) **ELISA**

The ELISA technique is the most sensitive serological method and gives earlier reactions and higher antibody titres (30). It lacks type or strain specificity but is suitable for monitoring vaccination responses under field conditions (31). Commercial kits for ELISA are available. The use of monoclonal antibodies with ELISA may provide an improved typing system for IBV (23, 25).
B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

All viruses in live virus vaccines are first attenuated by serial passage in chick embryos. At present many countries only permit live vaccines based on the Massachusetts type. A single inoculation of an inactivated IB vaccine will not confer protection unless preceded by a primary response usually to a live one. Inactivated vaccines have to be administered to birds individually, whereas live vaccines can be given as aerosols or in the drinking water, or by eye drop. Live vaccines confer a better local immunity on the respiratory tract and may protect against a wider antigenic spectrum of field strains (14, 33).

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 (6).

1. Seed management

a) Characteristics

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 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 seed of live virus vaccines should be shown by histology not to damage the reproductive system of chicks when administered at one week old. The antigenic type incorporated in live vaccines requires justification if there is doubt as to its existence in a country.

b) Culture

All seed viruses are grown in the allantoic sac of developing chick embryos. The eggs should be from an SPF flock.

c) Validation as a vaccine

Every seed lot must be free from bacterial, mycotic, mycoplasmal and viral contamination.

For the detection of extraneous viruses, the seed is first treated with a high-titred 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 on past experience to be potential contaminants. The serum must not contain antibodies to adenovirus, avian encephalomyelitis, chicken anaemia agent, fowl pox, infectious laryngo-
tracheitis, influenza A, Newcastle disease, infectious bursal disease, leucosis, reovirus, Marek's disease, turkey herpesvirus, adeno-associated virus, egg-drop syndrome 76 (EDS76) virus or reticuloendotheliosis virus. The residual infectivity of the IBV component under test should be at least 10-fold the minimum field dose in each dose given to each unit of culture system used. These systems include:

1. SPF chick embryos, 9-11 days incubation, inoculated via both allantoic sac and chorio-allantoic membrane;

2. Chick embryo fibroblast cultures, for leucosis virus sub-groups A and B. The COFAL test or double antibody sandwich ELISA for group-specific leucosis antigen is performed on cell extracts harvested at 14 days. An IF test for reticuloendotheliosis virus is done on coverslip cultures after 2 passages.

3. SPF chick kidney cultures which are examined for cytopathic effects, cell inclusions and haemabsorbing agents passaged at intervals of not less than 5 days up to 20 days total incubation.

4. SPF chicks of minimum vaccination age inoculated with 100 field doses intramuscularly, and 10 field doses into the conjunctiva; repeated in 3 weeks together with 10 field doses into the foot pad and 10 intranasally. Observations are made for 6 weeks overall, and serum collected for tests for avian encephalomyelitis, infectious bursal disease, Marek's disease, Newcastle disease and *Salmonella pullorum* infection.

Safety tests should be done on seed viruses intended for live vaccines.

Tests on seed virus should include histology for detecting damage to the reproductive tracts of chicks as well as a test for any potential ability to revert to virulence.

Vaccines intended to protect against loss of egg production should be tested for duration of antibody responses up to at least 60 weeks of age. Mean HI titres should be greater than 6 log₂. 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 rearers up to the age when a booster vaccination would be administered (often at 16-18 weeks of age).

At least 3 batches should be tested for shelf life before stability is confirmed.

The stability of a live vaccine must be measured by maintenance of an adequate infectivity titre.
The stability of an inactivated vaccine is measured by batch potency tests at intervals. 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.

2. **Manufacture**

Requirements that generally apply to all vaccines will not be reiterated here. All virus strains destined for vaccines are cultured in the allantoic sac of SPF chick embryos. The pooled fluid is clarified and then titrated for infectivity. For live vaccines this is lyophilised in phials, whereas 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 chick embryos.

Live vaccine should contain not less than $10^{3.5} \text{ EID}_{50}$ per dose per bird until the expiry date indicated, and not less than $10^{2.5} \text{ EID}_{50}$ per dose per bird after incubation at $37^\circ\text{C}$ 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 β-propiolactone or formalin, any live leucosis 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 2 passages in cell cultures, embryos or chicks, using inoculations of 0.2 ml and 10 replicates per passage.

4. **Batch control**

Every batch of live vaccine should be tested for the absence of extraneous agents as for the seed virus. Live vaccines must be tested for safety, and for potency by titration of infectivity; inactivated vaccines are tested for safety, and for potency by measuring antibody production. The potency test for a batch of inactivated vaccine consists of vaccinating 20 SPF chicks, four weeks of age, and showing that their mean HI titre four weeks later is not less than $6 \log_2$.

a) **Sterility**

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.
b) Safety

There are no definitive schedules for determining reversion to virulence. A possible test would be to inoculate SPF chicks aged 3-4 weeks with 100 times the vaccine dose intratracheally, and to attempt to passage any virus recovered after 3 days from trachea or faeces in further chicks. This could be repeated up to 10 times.

c) Efficacy

To demonstrate protection following vaccination, a typical batch from each seed lot is subjected to challenge tests designed for the vaccine.

For live vaccines a minimum of 10 birds aged 3-4 weeks are vaccinated intranasally or intraocularly with the recommended dose. Ten non-vaccinated control birds from the same age and source are retained separately. Three or four weeks later, all birds of both groups are challenge inoculated intranasally or intraocularly each with $10^{3.0-3.5}$ EID$_{50}$ of the virulent Massachusetts M41 strain. A swab of the trachea is taken from each bird 4-7 days after challenge and placed in 3 ml antibiotic broth. Each fluid is tested for IBV by inoculation (0.2 ml) of 5 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 microscopically examine tracheal rings for ciliary activity (14). Failure to resist challenge is indicated by an extensive loss of ciliary motility. The live vaccine is suitable for use if at least 80% of the challenge vaccinated birds show no evidence of IBV in their trachea, whereas 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 birds 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 not later than 3 weeks of age. A further group of 30 controlled 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 chicks aged 4 weeks are vaccinated as recommended. An additional 20 control birds of the same age and origin are housed with them. Antibody responses are determined 4 weeks later; there should be no response
in the control birds. All birds are then challenged with $10^3$ median chick-infective doses (CID$_{50}$) of virulent virus and killed 4-7 days after for the examination of tracheal sections 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 are available in combination with Newcastle disease virus; inactivated vaccines containing Newcastle disease, infectious bursal disease and EDS76 viruses are also available. The efficacy of the different components of these vaccines must each be established independently.

REFERENCES


Avian infectious laryngotracheitis (ILT) is a respiratory disease caused by an alphaherpesvirus. It affects mainly chickens, although it can also affect pheasants and partridges. The clinical signs and pathological reactions may vary from an extremely severe to a very mild disease. The latter may be indistinguishable from other mild respiratory infections of chickens. The principal lesion is a tracheitis.

Laboratory diagnosis depends on the demonstration of the presence of the virus, viral antigen, or specific humoral antibodies.

Identification of the agent: The methods used include direct electron microscopy, immunofluorescence of tracheal exudate, agar gel immunodiffusion for the detection of viral antigen in tracheal samples or infected egg material, and the use of enzyme-linked immunosorbent assay (ELISA) to demonstrate viral antigen in mucosal scrapings. The methods of virus isolation by the inoculation of suspected material onto the dropped chorioallantoic membrane of embryonated eggs, or into avian embryonic cell cultures, are time-consuming. They may however be required if other, more rapid techniques give inconclusive results.

Serological tests: Antibodies to ILT virus can be detected by virus neutralisation tests conducted in eggs or in cell cultures, or by agar gel immunodiffusion reactions, indirect fluorescent antibody tests or ELISA.

Requirements for biological products: Vaccines against ILT are usually prepared from attenuated live virus. Those available at present afford some degree of protection but are not completely satisfactory.
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 tracheal obstructions. 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 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 10-30%. 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 may themselves be entirely mild. Incidence within a flock may be only 1-2%, but most affected birds will die of suffocation. An outbreak of mild ILT may extend over months with deaths occurring at irregular intervals. Signs include spasms of coughing and gasping with nasal and oral discharge and reduced production. Birds may appear unthrifty. On post-mortem examination, diphtheritic and caseous necrotic plaques and plugs are found in the trachea, larynx and mouth.

1. **Identification of the agent**

The virus may be demonstrated directly in tracheal exudate by electron microscopy (11). It can be isolated in chick embryo liver (8), chick embryo kidney (4) or in chick kidney (9) cell cultures. Chick embryo liver cells have been found to be the most sensitive (5). It can also be grown on the dropped chorioallantoic membrane (CAM) of 10- to 12-day old embryonated chicken eggs (6).

Viral antigen may be detected by immunofluorescence (3, 12), agar gel immunodiffusion (7), or ELISA, using mucosal scrapings (13). Histopathological examination of the trachea for typical herpesvirus intranuclear inclusions may also be helpful (10).

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. 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, or wrapped in moist tissue for electron microscopy. Any storage of infected tissues should be at -60°C or below to minimise loss in virus titre. Successive freezing and thawing must be avoided as this reduces virus infectivity.

Tracheal exudate and epithelial cells are 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 the supernatant fluid inoculated (0.1 ml) onto the dropped CAM of at least three embryonated chicken eggs of 10-12 days incubation. The eggs are sealed with paraffin wax and incubated for up to seven days. They are candled daily and the CAMs of dead embryos or of those surviving for seven days are examined for typical pocks. Alternatively, at least two confluent chick embryo kidney or chick embryo liver cell monolayer cultures are inoculated and allowed to adsorb for 1-2 hours. The cultures are then overlaid with fresh medium, incubated for up to seven days and examined daily for any evidence of a typical cytopathic effect (CPE) with syncytial cell formation.

In every instance, up to three passages of material may be necessary before a specimen is considered negative. The identity of a virus isolate may be confirmed as ILT by a neutralisation test in eggs or cell cultures using anti-ILT virus serum. The virus may be identified rapidly by electron microscopy.

b) Electron microscopy

To demonstrate the presence of virus by electron microscopy, tracheal exudate or epithelial scrapings are smeared on a microscope slide and mixed with a few drops of distilled water. One drop of suspension is placed on a grid and left for two minutes, after which excess moisture is removed with filter paper. One drop of 4% phosphotungstic acid (pH 6.4) is added and the excess removed after a further three minutes. The grid is allowed to dry thoroughly and examined under the electron microscope for typical herpesvirus particles.

c) Immunofluorescence

In immunofluorescence tests for viral antigen, epithelial cell scrapings from the trachea are smeared onto a glass slide. Alternatively, snap-frozen cryostat sections of the trachea 5 µm thick 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). Otherwise, they can be stained indirectly by first rinsing with 0.01 M phosphate buffered saline (PBS), pH 7.2, followed by the addition of an appropriate dilution of chicken anti-ILT antiserum for one hour. After a further rinse, an FITC-labelled anti-chicken immunoglobulin is applied for 30 minutes. The preparations are examined for specific intranuclear fluorescence in the epithelial cells using epifluorescent ultraviolet-blue illumination. Suitable controls should be included.

d) Agar gel immunodiffusion (AGID)

ILT viral antigen may be demonstrated by AGID tests on tracheal exudate, infected CAMs or infected cell culture material using hyperimmune ILT antiserum. Noble agar (1.5%) containing sodium chloride (8%) and sodium azide (1%) in distilled water are autoclaved at 15 lb/sq.in. for 15 minutes. Five ml of the molten agar is poured into a 5 cm petri dish. A pattern of wells is
punched in the agar, consisting of a central well into which is pipetted the hyperimmune serum, surrounded by six other wells which are filled with suspected virus samples under test. Dishes are incubated in a moist 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.

e) ELISA

When the monoclonal antibody ELISA is used for detecting viral antigen (13), tracheal exudate is mixed with an equal volume of a detergent such as Nonidet P 40 (BDH Chemicals Ltd., Poole, UK) then centrifuged at 12,000 rpm for one minute. The supernatant fluid is dropped in 50 μl volumes in wells of microtitre plates previously coated with rabbit IgG against virus diluted 1:200 in 0.05 M carbonate-bicarbonate buffer, pH 9.0, and incubated for one hour. Next, 50 μl monoclonal antibody against major glycoproteins of ILT virus diluted 1:50 in PBS is added to each well followed by 50 μl of a 1:1,000 dilution of affinity-purified goat anti-mouse IgG conjugated to horseradish peroxidase. The substrate, recrystallised 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 OD reading for each well is corrected by subtracting the reading obtained for wells containing diluting buffer instead of tracheal exudate.

f) Histopathology

Tracheas for histopathological examination should be placed in formol saline immediately after removal from the birds. Intranuclear inclusions may be seen in the epithelial cells in longitudinal sections stained by haematoxylin and eosin. These inclusions are the classical Cowdry type A inclusions of herpesviruses, but may only persist for about 3-5 days after infection.

2. Serological tests

Antibodies to ILT virus in chicken serum can be detected by virus neutralisation (VN), AGID, indirect fluorescent antibody (IFA), or ELISA tests (1).

a) Virus neutralisation

VN tests are conducted on the dropped CAM of chicken eggs of 9-11 days incubation, where antibody specifically neutralises pock formation due to ILT virus. Alternatively, the tests can be performed in cell cultures, where antibody neutralises the viral CPE. Doubling dilutions of serum are added to equal volumes of a constant concentration of virus. This concentration may either be 100 median egg infectious doses (EID₅₀) for egg inoculations, or 100 median tissue culture infectious doses (TCID₅₀) for the inoculation of cultures. The mixtures are incubated at 37°C for one hour to allow any neutralisation to take place.
When VN tests are performed in eggs, the virus-serum mixtures are inoculated onto the dropped CAMs. 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 performed in cell cultures, serum dilutions are prepared in 96-well flat-bottomed culture plates and virus is then added. After a period for neutralisation, freshly trypsinised chick embryo liver or chick embryo kidney cells are added to the mixture. The plates are sealed, incubated at 37°C and examined daily. Fifty per cent end points are read after about four days when the virus control titration indicates that 30-300 TCID$_{50}$ of virus has been used in the test.

b) **Agar gel immunodiffusion**

When immunodiffusion tests are used to detect ILT antibody, 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 10-day old fertile specific pathogen free (SPF) chicken eggs. The CAMs are harvested after four 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 chick embryo liver or kidney, or chick 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. For the test, the agar is prepared as described previously, but this time the CAM or cell culture antigen is placed in the central well with test sera in surrounding wells. Known positive and negative antisera are usually incorporated in the test, which is read after 24-48 hours incubation at room temperature or at 37°C. Immunodiffusion tests are simple, cheap to perform, and useful for flock screening, although they are less sensitive than the other methods.

c) **Indirect fluorescent antibody test**

For IFA tests, the antigen consists of chick embryo liver cell monolayers grown on teflon multi-spot microscope slides and infected with ILT virus. The cells are first fixed in acetone. Dilutions of test sera prepared in PBS are applied to each spot culture and the slides incubated at 37°C for one hour, washed in several changes of PBS, drained and treated with an appropriate dilution of a commercial FITC-labelled rabbit anti-chicken immunoglobulin. After incubation at 37°C for one hour, the slides are rewashed and coverslips are applied over a mountant of buffered glycerol. They are examined by epifluorescence with ultraviolet-blue illumination; end-point titres are read as the highest serum dilutions giving specific staining. This test is more sensitive than VN, but the interpretation of results may be subjective.

d) **ELISA**

The antigen for ELISA is obtained by the ultrasonication of ILT-infected cell cultures at the time of maximum CPE. The antigen is absorbed onto 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 two hours, the plates are washed and a 1:4,000 dilution of a rabbit anti-chicken IgG conjugated with peroxidase is added. This is followed by rewashing and a further incubation at 37°C for one hour. Finally a substrate consisting of 5-amino-salicylic acid is added to each well, and the absorbance of the fluid in each well read at 450 nm on a spectrophotometer. Results are expressed as the difference between the mean absorbance produced by the serum with the positive and negative antigens. The test is very sensitive and possibly the best available for survey purposes.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

ILT vaccines usually consist of live virus. They are most often prepared from eggs, or perhaps cell cultures, that have been infected with a suitably attenuated or naturally avirulent strain of ILT virus. Such vaccines are reconstituted from the lyophilised state immediately before use with a suitable liquid. Inactivated vaccines are occasionally used.

1. **Seed management**

   a) **Characteristics**

   Passage of a selected strain through embryonated chicken eggs establishes the master seed "X". The vaccine virus proper ranges between passages "X + 1" to "X + 5". In chickens, live virus vaccines should not cause mortality or severe respiratory reaction, although slight erythema of the conjunctiva and lacrimation may occur. However, severe reactions may occur in certain species of pheasant following routine ocular instillation of commercial live virus vaccines.

   b) **Culture**

   In large-scale vaccine preparation, the production seed virus will range between "X" and "X + 4"; that is, up to the fourth embryo passage after "X".

   c) **Validation as vaccine**

   For control tests on the seed virus, virus concentration will be equivalent to that specified for the final product.

   Twenty SPF chickens aged 1-4 weeks should be vaccinated with one field dose supraconjunctivally; 10-14 days later these birds, together with 10 controls of the same age and source, are challenged by administration of approximately $10^3$ EID$_{50}$ of a virulent strain of ILT virus.

   The vaccine passes the test if none of the vaccinated birds dies or shows severe signs of ILT, and not more than four birds show signs of clinical disease,
whereas at least 80% of the inoculated control birds die or display severe clinical signs of ILT.

The vaccine must be shown to be free of *Salmonella* spp., other bacteria, fungi and mycoplasmas by cultivation on suitable artificial media. Freedom from extraneous viruses must be demonstrated by inoculation of samples of the vaccine into 9- to 11-day-old embryonated chicken eggs via the allantoic cavity, CAM or yolk sac, and examination for any abnormalities.

Vaccination should confer life-long immunity. When stored under the prescribed conditions, the lyophilised vaccine can be expected to retain its potency for not less than one year; some products specify 1.5 years.

2. **Manufacture**

The vaccine is produced by the inoculation of the production seed virus in 9- to 11-day-old embryonated chicken eggs obtained from SPF flocks. Such flocks should therefore be free of evidence of infection with the following agents: avian adenoviruses, ILT virus, influenza virus type A, leukosis, Newcastle disease, infectious bursal disease and Marek's disease viruses, reoviruses, turkey herpesvirus, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, and *Salmonella* spp.

Eggs are inoculated with production seed virus through a hole in the shell onto the droppedCAM. They are then sealed and incubated at 37°C (± 2°C) for 4-6 days. All eggs are candled prior to harvest, and only those with viable embryos are used. To harvest the virus, the shells are disinfected and the eggs opened aseptically. The CAMs and fluids are pooled in sterile refrigerated containers. The membranes should show the thick greyish plaques typical of ILT virus growth. They are then homogenised in a sterile blender. The resulting homogenate is pooled, tested for purity, potency, safety and virus content, mixed with a stabiliser (usually beef peptone and sucrose), lyophilised and stored at 4°C.

3. **Batch control**

a) **Sterility tests**

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

b) **Safety**

Samples are inoculated intratracheally into SPF chicks, following which they are observed for 14 days for any signs of adverse reaction and their sera are tested for antibodies to extraneous avian pathogens.

c) **Potency**

The vaccine shall be titrated in SPF embryonated eggs and must contain not less than the amount of virus specified on the label.
REFERENCES


SUMMARY

Diagnosis of tuberculosis in birds depends on the demonstration of Mycobacterium avium in the dead bird, or the detection of an immune response, cellular or humoral, in the live bird.

It must be borne in mind that in man 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.

Identification of the agent: Where clinical signs of tuberculosis are seen in the flock, or where typical lesions of tuberculosis are present in birds at autopsy, 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 demonstrated, but typical signs 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, serological or chromatographic (thin layer chromatography of lipids) criteria. If possible, 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 autopsy 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 in a few minutes, while the bird is still being held.

Requirements for biological products: 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 (PPD) is the standard preparation for use in the tuberculin test of domestic poultry.
A. DIAGNOSTIC TECHNIQUES

Tuberculosis in birds is caused by *Mycobacterium avium*. 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.

It is essential to bear in mind that *M. avium* is capable of giving rise to a progressive disease in man that is refractory to treatment. All manipulations involving the handling of open live cultures or of material from infected birds must be performed with adequate biohazard containment.

1. Identification of the agent

Where there is a characteristic history of tuberculosis in the flock and typical lesions are found in birds post-mortem, the detection of acid-fast bacilli in smears or sections from affected organs, stained by Ziehl-Neelsen method, is normally sufficient to establish the diagnosis. Occasionally a case will be encountered, 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 retractile 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.

If there is a characteristic flock history, and suggestive lesions are found at autopsy, but no acid-fast bacilli are seen in smears or sections, an attempt must be made to isolate *M. avium* from the autopsy material. Liver or spleen are usually the best organs to use, but if the carcass is decomposed bone marrow may prove more satisfactory since it could be less contaminated. *M. avium* grows best on media such as Lowenstein-Jensen or Middlebrook and Cohn's 7H11, with 1% sodium pyruvate added. It may occasionally be necessary to incorporate mycobactin, as used for the isolation of *M. paratuberculosis*. Growth may be confined to the edge of the water of condensation. Typically *M. avium* produces "smooth" colonies, but rough variants do occur.

Typing of mycobacteria requires a specialist laboratory. Tuberculosis in birds is usually caused by *M. avium* types 1, 2 or 3, or by *M. intracellulare* serotype 6 strains with the characteristic lipid patterns of one of those three types. 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 *M. tuberculosis*. Hence, if rough colonies of mycobacteria are isolated from such birds, they should be tested for growth at 42°C. If the isolate will not grow at 42°C *M. tuberculosis* should be suspected.
If specialist typing facilities are not available, the likelihood that the organism isolated is the cause of the disease may be established by pathogenicity tests. For preference these should 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, sterilising and then weighing it. A loopful of culture is then placed on the foil and the whole 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 have extensive lesions filled with acid-fast bacilli by that time.

2. **Tuberculin and serological tests**

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 of tuberculin, using a needle of about 10 mm x 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 about 5 mm in diameter to gross oedema extending into the other wattle and down the neck. It will be found that 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 of waterfowl using the foot web has also been described; the test is not very sensitive and is often complicated by infections of the inoculation site.

b) **Stained antigen test**

The stained antigen whole blood plate agglutination test of Rozanska 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 venupuncture, 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. This 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 been claimed that in domestic fowl it is also more reliable than the tuberculin test.
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.

### B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

**Avian tuberculin** is a preparation made from the heat-treated products of growth and lysis of *M. avium*. It is used by intradermal injection to reveal delayed hypersensitivity as a means of identifying animals infected with or sensitised to the same species of tubercle bacillus.

An antigen stained with 1% malachite green is used for the rapid whole blood plate agglutination test.

1. **Seed management**
   
a) **Characteristics**

   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 that conforms to these norms.

   The strain used for preparation of the stained antigen must be smooth and not auto-agglutinate in saline suspension. It must conform to the characteristics of the species *M. avium*. Although at first sight it might appear that it would be best to use a strain of the serotype that is most likely to be encountered (in Europe serotype 2 for domestic fowl, serotype 1 for waterfowl), in practice it is often better to use a strain which will detect infection with any serotype. For special purposes, however, it may be preferable to use a strain which 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. On the whole, serotype 1 strains seem more often to detect a wide spectrum of infection (a serotype 1 strain is used at the Central Veterinary Laboratory, Weybridge, UK), and will often also detect infections with mycobactin-dependent or non-cultivable mycobacteria (e.g. the so-called "woodpigeon bacillus"). There is no reason why a culture containing more than one strain of *M. avium* should not be used, 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.

b) **Culture**

For **tuberculin**, the culture should be shown to be capable of producing a product that conforms to these norms. It must be free from ingredients known to cause toxic or allergic reactions.
For stained antigen, the organism should be grown in a suitable liquid medium, such as Middlebrook and Cohn's 7H11 medium; better growth will be obtained if the medium contains 1% sodium pyruvate. Good growth should be obtained in about 7 days.

c) Validation

Cultures for tuberculin or stained antigen should be checked by Gram staining for the presence of organisms other than mycobacteria.

Tuberculin: Liquid avian tuberculin should be protected from the light and held at 5°C (± 3°C). Lyophilised preparations may be stored at higher temperatures (but not exceeding 25°C) protected from the light. Periods of exposure to higher temperatures or to direct sunlight should be kept to a minimum. Liquid tuberculin should retain its potency for at least 2 years, and lyophilised tuberculin for at least 8 years.

Antigen: One or more batches for agglutinating antigen must be tested for efficacy in naturally or artificially infected tuberculous birds by comparison with a reference preparation of known potency. The potency relative to that of the reference 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 which give satisfactory agglutination reactions with the antisera can now be pooled and the antigen stained. This is done by the addition of 3 ml of 1% malachite green solution per 100 ml of suspension. If possible, the stained antigen should now be checked using whole blood just as the unstained antigen was tested with serum. The agglutinating antigen should keep for at least 6 months in the refrigerator at 4°C, and much longer if frozen at -20°C or below. If a batch has not been used for a long time it should be rechecked, especially for autoagglutination.

2. Manufacture

Tuberculin: Avian tuberculin may be made by the following three methods:

i) Old tuberculin (OT): The organism is cultivated in glycerol broth medium, killed by heating in flowing steam and filtered. The filtrate is concentrated by heat and sterilised by filtration.

ii) Heat-concentrated synthetic-medium tuberculin (HCSM): As for (i) but the glycerol broth medium is replaced by a synthetic medium.

iii) Purified protein derivative (PPD): As (ii) but, instead of being concentrated by heat, the protein in the filtrate is precipitated chemically, 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 5% w/v), may be added. Mercurial derivatives have been found to cause such reactions. Glycerol (not more than 10% w/v) may be added as a stabiliser. The product is dispensed aseptically into sterile, neutral glass containers which are sealed so as to preclude contamination. The product may be freeze-dried.

**Antigen:** Antigen for agglutination tests is best grown on solid medium, such as Lowenstein-Jensen or 7H11, in Roux flasks or large bottles; again the incorporation of 1% sodium pyruvate (rather than glycerol in Lowenstein-Jensen medium) is beneficial. The use of solid medium maximises the chances 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, the washing collected into a sterile bottle and re-incubated 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 which 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 10 times that which matches tube 4 on McFarland's scale.

3. **In-process control**

**Tuberculin:** One or more batches of tuberculin must be tested for specificity along with a reference preparation of bovine tuberculin by comparing the reactions produced in guinea pigs sensitised with *M. bovis* using a procedure similar to that described below under tests for potency. In guinea pigs sensitised with *M. avium* the potency of the reference preparation of bovine tuberculin must be shown to be not more than 10% of the potency of the reference preparation of avian tuberculin used in the test of potency. The pH should be not less than 6.5 and not more than 7.5.

**Antigen:** 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, likely contaminants being yeasts, and re-checked by culture to ensure that the formalin has killed the mycobacteria.
4. **Batch control**

a) **Sterility**

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

b) **Safety**

Two guinea pigs, each weighing not less than 250 g and which have not previously been treated with any material which will interfere with the test, are injected subcutaneously with 0.5 ml of the tuberculin under test. No abnormal effects should occur within seven days.

Tests on tuberculin for living mycobacteria may be performed either on the tuberculin immediately before it is dispensed into final containers or on samples taken from the final containers themselves. A sample of at least 10 ml must be taken and this must be injected intraperitoneally or subcutaneously into at least two guinea pigs, dividing the volume to be tested equally between the guinea pigs. It is desirable to take a larger sample, say 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea pigs are observed for at least 42 days and examined macroscopically post-mortem. Any lesions found are examined microscopically and culturally.

Each filled container must be inspected before it is labelled and any showing abnormalities must be discarded.

For antigen, the only safety test needed is the culture test of the unwashed antigen after seven days incubation, to ensure that all the bacilli are dead.

c) **Potency**

The potency of avian tuberculin is determined in guinea pigs sensitised with *M. avium*, by comparison with a reference preparation calibrated in International Units.

Use not fewer than 9 albino guinea pigs, each weighing 400-600 g. Sensitise them by administering to each, by deep intramuscular injection, a suitable dose of inactivated or live *M. avium*. Between 4-6 weeks later, the test is performed as follows. Shave the guinea pigs' flanks so as to provide space for 3-4 injections on each side. Prepare at least 3 dilutions of the tuberculin under test and at least 3 dilutions of the reference 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, the volume injected being 0.1-0.2 ml. After 24-28 hours, the diameters of the reactions are
measured and the results calculated using standard statistical methods taking the diameters to be directly proportional to the logarithms of the concentrations of the tuberculins. The estimated potency is 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 20,000 International Units/ml, giving a dose for practical use of 2,000 units/0.1 ml.

REFERENCES


DUCK VIRUS HEPATITIS

(B58)

SUMMARY

Hepatitis in ducks can be caused by at least three different viruses. The most common and internationally widespread is duck hepatitis virus (DHV) type I, an enterovirus, which causes a highly lethal, acute, contagious infection in ducklings less than six weeks of age and, frequently, less than three weeks old. It does not occur in older birds. This infection is often referred to simply as duck virus hepatitis (DVH).

DHV type II has been reported only in the United Kingdom. It occurs in ducklings from ten days to six weeks of age, and causes 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. 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 upon a) the characteristic disease pattern in the flock, b) gross pathological changes, c) the recovery of virus from dead ducklings, and d) the reproduction of the disease in susceptible ducklings.

Identification of the agent: It is not possible to distinguish between 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 I. These tests have been used for virus identification, assay of immune responses to vaccination, and epidemiological surveys.

Requirements for biological products: DHV type I infections can be controlled by the use of attenuated live virus vaccines. They are administered to breeder ducks to confer passive immunity to ducklings, or to susceptible day-old ducklings to actively immunise.

DHV type III infections can be controlled by the use of an attenuated live virus vaccine given to breeder ducks to confer passive immunity to ducklings.
A. DIAGNOSTIC TECHNIQUES

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 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.

1. Identification of the agent

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 less than six 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.

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 with 5% chloroform (v/v) for 10-15 minutes at ambient temperature.

DHV type I virus is resistant to this treatment.

The presence a DHV type I virus is usually confirmed through one or more of the following procedures:

a) subcutaneous (s/c) or intramuscular (i/m) 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 less than 24 hours. The ducklings should show the gross pathology attributable to DHV type I. Virus should be reisolated from the livers.
b) 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 24-72 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 which take longer to die the greenish colour of the allantois is more pronounced, and both the liver lesions and stunting become more evident.

c) inoculation of primary cultures of duck embryo liver (DEL) cells which are particularly sensitive (11). 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, 6, 11) include:

a) passive immunisation s/c of 1- to 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 i/m or s/c 24 hours later with at least $10^3$ LD$_{50}$ 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.

b) 1- to 7-day-old DHV type I-susceptible and DHV type I-maternally immune ducklings are challenged i/m or s/c with at least $10^3$ 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.

c) serial tenfold dilutions of the virus isolate are mixed with equal volumes of DHV type I specific hyperimmune serum diluted 1:5 to 1:10. The mixtures are allowed to react at room temperature for one hour and then inoculated (0.2 ml) s/c into susceptible ducklings, via the allantoic cavity (0.2 ml) of embryonated duck eggs, and onto 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 isolated in the United States only partially reacts with the classical type I virus in cross-VN tests (4). Other variants have been reported from India and Egypt, but nothing further is known about them.
Duck virus hepatitis (B58)

DHV type II

DHV type II infection of ducks has been reported only from the United Kingdom (1, 5). It is an acute fatal infection of ducklings, producing clinical and pathological signs similar to DHV type I. Affected birds may show signs of polydipsia, 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.

The virus can be recovered in 20% (w/v) homogenised liver suspensions in buffered saline. This can be used to inoculate:

a) 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 (5). This contrasts with the findings with DHV type I infection, which is more virulent and rapid in its effect.

b) embryonated chicken or duck eggs, via either the amniotic cavity or yolk sac. These may respond, erratically, after 4 passages, but no deaths may be seen during earlier passages. Embryos take 6-10 days to show evidence of infection with stunting, and green necrotic livers.

Immunological techniques have not been employed routinely, since the serological response to infection of both ducklings and duck embryos is poor. However, a neutralisation assay has been applied (5) 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- to 4-day-old ducklings (5): these are inoculated with antisera to types I and II, then challenged 3 days later with the virus isolate. Electron microscopy of liver and faecal preparations have revealed viral particles 28-30 nm in diameter with a morphology resembling that of astroviruses (5). This technique could distinguish DHV type II from types I and III.

DHV type III

This has been reported only in the United States. Losses of up to 20% occur in ducklings immune to DHV type I (7, 9). 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:
a) inoculating the isolate into susceptible ducklings i/m. The mortality rate may reach 20% with 60% morbidity. No deaths occur in the first 24 hours and all losses ensue between the second and fourth days after inoculation. Intravenous inoculation is more effective; type III infection is less virulent than type I.

b) inoculating the isolate onto the chorio-allantoic membrane of 10-day 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 detect the virus by inoculation of embryonated chicken eggs have not been successful.

Attempts to grow 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 (7).

2. **Serological tests**

These are not applicable for diagnosis as the clinical disease is too acute. VN tests in eggs have, however, been applied for all three DVH types. In the case of DHV type I, *in vitro* tests have been developed, and used to identify virus isolates, assay immune responses to vaccination, and for epidemiological surveys.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

DHV type I can be controlled by the use of an attenuated live virus vaccine. This is given to breeder ducks so that immunity is transferred via the yolk to newly-hatched birds. Vaccine can also be administered to susceptible newly-hatched ducklings. 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 under experimental conditions (5).

DHV type III infections have been controlled by the use of attenuated live virus vaccines given to breeder ducks, so that immunity is transferred via the yolk sac to the hatching ducklings.

1. **Seed management**

   a) **Characteristics**

   The type I virus vaccine seed used most commonly in Europe is derived from an isolate passaged in embryonated chicken eggs 53-55 times; that in the United States has been passaged 84-89 times.
The type II virus vaccine seed originated from an isolate attenuated by 25 serial passages in embryonated chicken eggs (1), and has been employed experimentally under field conditions (Gough, personal communication).

The type III virus vaccine seed has been attenuated by 30 serial passages in embryonated duck eggs inoculated via the chorio-allantoic membrane (CAM).

b) Culture

The seed viruses of types I and II are handled similarly. They should be prepared in 8- to 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 onto 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 of extraneous viruses that are pathogenic for ducks, chickens or turkeys. The seeds should also be free of all microbiological and fungal contamination.

In the case of newly-hatched ducklings, attenuated live virus replicates rapidly and results in an immunity within 48-72 hours of vaccination. This immunity persists throughout the susceptible period of life. 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 s/c or orally at about 10 days of age (8, 10). Alternatively, 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 virus.

Once frozen at -70°C or below, and subsequently kept at -20°C or below, the vaccine stores well with little loss in titre for at least 2-3 years in the case of types I and II viruses, and for at least one year in the case of type III virus. Once the vaccine has been issued, it should not be refrozen, and it is best stored at 4°C. Under these conditions it should be used within 7 days of issue.

2. Manufacture

Types I and II viruses are treated similarly. The vaccine is produced in 8- to 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.

The type III vaccine is prepared in 10-day-old SPF duck eggs inoculated on 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 chickens for egg yolk antibody production. Eggs are collected 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 not more than 0.20% formalin as a preservative. The dispensed product is stored at 4°C and has a shelf life of one year. Tests are carried out in the usual way for the absence of contaminants.

3. **In-process control**

Eggs in which embryos die within 24 hours of inoculation should be discarded as non-specific 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, in duck eggs. The antiserum should reduce the titre of the respective virus by at least $10^2 \text{ELD}_{50}$ (mean embryo lethal dose).

4. **Batch control**

a) **Sterility**

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

b) **Safety**

A group of 1- to 3-day-old ducklings, susceptible to the type of virus concerned, should be inoculated s/c or i/m (in the case of types I and II), or s/c (in the case of type III), with the vaccine at 10 times the recommended dose, and kept under observation for 10-21 days for any adverse reactions.
Safety tests on yolk antibody are done by inoculating 1 ml s/c into each of a group of ducklings, which are then kept under observation of 3 days for signs of adverse effects.

c) Potency

For types I and II viruses, the virus titre of the vaccine should be determined in 8- to 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 can be assessed by inoculating s/c a minimum of \(10^3.3\) ELD\(_{50}\) per duckling of the vaccine virus and challenging s/c with \(10^3\) LD\(_{50}\) per duckling of virulent DHV virus type I or II 72 hours later (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.

For type III virus, the titre of the vaccine should be determined in 10-day-old embryonated duck eggs inoculated onto 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 \(10^3\) is considered 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.

REFERENCES


**DUCK VIRUS ENTERITIS**  
*(B59)*

**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 the isolation and identification of the virus.

**Identification of the agent:** The virus may be isolated from the liver and spleen 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 (CAM); 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 immunofluorescence tests on infected cell cultures.

**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 two weeks of age. Ducks are vaccinated subcutaneously or intramuscularly for active immunity. Vaccine virus is not thought to spread from vaccinated to unvaccinated stock.

**A. DIAGNOSTIC TECHNIQUES**

Duck virus enteritis (DVE) is an acute, sometimes chronic, contagious virus infection which occurs naturally only in ducks, geese and swans, all members of the family Anatidae of the order Anseriformes. The aetiological agent is a herpesvirus. The infection has not been reported in other avian species, mammals or man.

In domestic ducks and ducklings, DVE has been reported in birds ranging from seven 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.

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 symptoms includes photophobia, polydipsia, 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 bloodstained vents.

At necropsy, there is little evidence of emaciation in adult ducks which have died. In mature males prolapse of the penis may occur. 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 (8).

1. **Identification of the agent**

Primary isolation of the virus is best achieved from samples of liver and spleen tissue which have been homogenised in buffered saline and clarified by low speed centrifugation. Isolation may be attempted by inoculating such homogenates into cell cultures, ducklings or duck embryos.

a) **Cell cultures**

Cell culture is the method of choice for isolation of DVE virus. Isolations may be made in primary duck embryo fibroblasts (DEF) (9) or preferably primary Muscovy duck embryo fibroblasts (MEF) (5, 7). The cytopathic effect is characterised by the appearance of rounded clumped cells which enlarge and become necrotic 2-4 days later. Cultures should be stained with fluorescent antibody conjugate to identify the virus. Cells can 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 MEF cells is favoured by incubation at temperatures between 39.5-41.5°C. However, high temperature is not always necessary for isolation.

b) **Ducklings**

When inoculated intramuscularly, 1-day-old susceptible ducklings die within 3-12 days. Muscovy ducklings (*Cairina moschata*) are more susceptible than White Pekin ducklings. Both macroscopical and microscopical lesions typical of DVE should be seen on post-mortem examination. Uninoculated ducklings should be maintained as controls at the same time. 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 (6).
c) Duck embryos

Primary virus isolations can be made by inoculating 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.

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 (2). Provided a hyperimmune antiserum of sufficiently high titre is used, a fluorescent antibody for DVE in DEF cells is the next most sensitive assay after isolation in 1- to 9-day old ducklings (4). A reverse passive haemagglutination test for DVE has been described (3) but it is reported to be less sensitive than immunofluorescence and plaque assays. The identity of the virus may also be confirmed by electron microscopy.

d) Strain variation

Although strains of DVE differ considerably in virulence there is little evidence of serological variation to date.

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 exposure to DVE in wildfowl.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

A live attenuated virus vaccine can be used to control DVE in birds over 2 weeks of age. Fattening or breeding ducks may be vaccinated subcutaneously or intramuscularly to produce an active immunity. The vaccine virus is thought not to spread by contact from vaccinated to unvaccinated ducks, since the unvaccinated birds remain susceptible to infection.

1. Seed management

a) Characteristics

DVE vaccine can be prepared from a strain of the virus which 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) Culture

The seed virus should be prepared in 8- to 11-day-old specific pathogen free (SPF) embryonated chicken eggs by inoculating 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 of extraneous viruses pathogenic to ducks, chickens and turkeys. It should also be free from bacterial, fungal and mycoplasmal contaminants.

The identity of the virus should be confirmed by a virus neutralisation test conducted with specific antiserum using the constant-serum varying virus method. This test should be performed in embryonated chicken eggs. The antiserum should reduce the virus titre by at least $10^{1.75}$ ELD$_{50}$.

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 subsequent batches the titre of the virus should be a sufficient indication of its potency.

Once frozen at -70°C or lower the vaccine stores well for at least one year with little loss in titre. Once issued the vaccine should not be refrozen, and it should be kept at 4°C and used as soon as possible.

2. Manufacture

The vaccine is produced in 8- to 11-day-old SPF embryonated chicken eggs inoculated onto the CAM and incubated at 37°C. Most embryo deaths occur between 48-96 hours after inoculation. The embryos, their CAMs and chorioallantoic fluids are harvested, pooled and homogenised in buffered saline and clarified by low-speed centrifugation. 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 which have died from non-specific causes.
4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

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 of 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- to 11-day-old embryonated chicken eggs inoculated onto the CAM and incubated at 37°C. It should contain a minimum of $10^3$ ELD$_{50}$ per dose at time of use.

REFERENCES


SUMMARY

Fowl cholera is a commonly occurring avian disease which can affect all types of birds and is distributed worldwide. It usually occurs as an acute septicaemia and is often fatal. Diagnosis depends upon identification of the causative bacterium, Pasteurella multocida, following isolation from birds with signs and lesions consistent with this disease. Presumptive diagnosis may be based on the occurrence of typical signs and lesions and/or on the microscopic demonstration of bipolarly stained bacteria in tissue smears such as blood, liver, or spleen.

Identification of the agent: Pasteurella multocida is a facultative anaerobic bacterium which grows best at 35-37°C. Primary isolation is usually accomplished using such media 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 to 24 hours incubation. They usually appear discrete, circular, convex, and butyraceous. The cells are coccobacillary or short rod shaped, usually 0.2-0.4 x 0.6-2.5 μm in size, stain Gram-negative, and generally occur singly or in pairs.

Isolation of the organism from visceral organs such as liver, spleen or heart blood of birds which 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.

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. 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 biological products: Inactivated vaccines are available commercially in some countries.
A. DIAGNOSTIC TECHNIQUES

Fowl cholera is a commonly occurring avian disease which can affect all types of birds and is often fatal (1, 3). In the peracute form, fowl cholera is one of the most virulent and infectious diseases of poultry. Diagnosis depends upon identification of the causative bacterium, *Pasteurella multocida*, following isolation from birds with signs and lesions consistent with this disease. Presumptive diagnosis may be based on the occurrence of typical signs and lesions and/or on the microscopic demonstration of bipolarly stained bacteria in smears of tissues such as blood, liver, or spleen.

All avian species are susceptible to *P. multocida*, although turkeys may be particularly severely affected. Often the first sign of disease is dead birds. Other signs include: depression, diarrhoea, ruffled feathers, increased respiratory rate, and cyanosis at the time of death. Lesions which are often observed include: passive hyperaemia, haemorrhages, swollen liver, focal necrotic areas in the liver and/or spleen, pneumonia, and slightly increased amounts of peritoneal and pericardial fluids. Birds which 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, tendon sheaths, sternal bursa, conjunctivae, wattles, pharynx, lungs, middle ears, bone marrow, and meninges. Lesions resulting from these infections are usually characterised by fibrinosuppurative exudate, necrosis and varying degrees of fibroplasia.

Other bacterial diseases, including listeriosis in chickens and erysipelas in turkeys, often have signs and lesions similar to those of fowl cholera. Differential diagnosis depends upon isolation and identification of the causative organisms.

1. Identification of the agent

*Pasteurella multocida* is a facultative anaerobic bacterium which grows best at 35-37°C. Primary isolation is usually accomplished using media such as dextrose starch agar, blood agar or trypticase soy agar, but 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 to 24 hours incubation. They usually appear discrete, circular, convex, and butyrous. Capsulated organisms usually produce larger colonies than those of unencapsulated organisms. The watery-mucoid colonies often observed with mammalian isolates are very rare with avian isolates. The cells are coccobacillary or short rod shaped, usually 0.2-0.4 x 0.6-2.5 μm in size, stain Gram-negative, and generally occur singly or in pairs. Those organisms recently isolated or in tissue smears stain bipolarly with Wright’s or Giemsa stains, and are usually encapsulated.

Isolation of the organism from visceral organs such as liver, spleen or heart blood of birds which 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 which 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 obtained by inserting a sterile cotton swab or wire loop through the heat-sterilised surface. The specimen is then used to inoculate an appropriate agar medium and incubated, or is used to inoculate tryptose or another broth medium which is incubated for a few hours, transferred to agar medium and incubated.

Identification is based primarily on the results of biochemical tests. Carbohydrate fermentation reactions are essential. Those carbohydrates which 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. *P. multocida* does not cause haemolysis, is not motile, and does not grow on MacConkey agar. Ox-ferm medium is fermented. It produces catalase, oxidase, and ornithine decarboxylase, but does not produce urease, lysine decarboxylase, β-galactosidase, or arginine dihydrolase. Phosphatase production is variable. Nitrate is reduced; indole and hydrogen sulfide are produced, and methyl red and Voges-Proskauer tests are negative.

Differentiation of *P. multocida* from other avian *Pasteurellae* can usually be accomplished using the biochemical tests and results indicated in Table 1. (The classification of the genus *Pasteurella* is currently under review and may be changed.)

**Table 1**

<table>
<thead>
<tr>
<th>Test*</th>
<th><em>multocida</em></th>
<th><em>anatipestifer</em></th>
<th><em>haemolytica</em></th>
<th><em>gallinarum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemolysis on blood agar</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Growth on MacConkey agar</td>
<td>–</td>
<td>–</td>
<td>+ u</td>
<td>–</td>
</tr>
<tr>
<td>Indol production</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Gelatin liquefaction</td>
<td>–</td>
<td>+ u</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Catalase production</td>
<td>+</td>
<td>+</td>
<td>+ u</td>
<td>–</td>
</tr>
<tr>
<td>Urease production</td>
<td>–</td>
<td>v</td>
<td>–</td>
<td>–</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>–</td>
<td>+ u</td>
<td>–</td>
</tr>
<tr>
<td>Sucrose fermentation</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Maltose fermentation</td>
<td>– u</td>
<td>–</td>
<td>– u</td>
<td>+</td>
</tr>
</tbody>
</table>

* Test reaction results:
  - no reaction  
  - u usually no reaction
  + reaction  
  + u usually a reaction  
  v variable reactions

Antigenic characterisation of *P. multocida* strains is accomplished through somatic serotyping and/or capsular serogrouping. Somatic serotypes are usually determined by a gel diffusion precipitin test (2). Serotypes 1-16 have been reported; all but serotypes 8 and 13 have been isolated from avian hosts. Capsular serogroups are
determined by a passive hemagglutination test. Serogroups A, B, D, E, and F have been reported; all but serogroup E have been isolated from avian hosts. The most effective characterisation involves determination of both serotype and serogroup. These determinations require a specialised laboratory with appropriate diagnostic reagents.

2. Serological tests

Serological tests for the presence of specific antibodies 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. Serological tests, such as agglutination, agar gel immunodiffusion 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 predict immunity in vaccinated poultry, but not for diagnosis.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Avian pasteurellosis or fowl cholera may be caused by any one of sixteen serotypes of *Pasteurella multocida*, although certain serotypes appear to be more often associated with disease. The *P. multocida* vaccines in general use are bacterins, containing aluminium hydroxide as adjuvant, prepared from inactivated cells of serotypes selected on the basis of epidemiological information. Vaccination plays a significant role in the control of this disease. Live vaccines containing modified *P. multocida* are not generally used except in North America. This monograph is concerned only with killed vaccines.

Vaccine use

Vaccine is normally administrated by intramuscular injection in the leg or breast muscles, or subcutaneously at the back of the neck. Two doses are required with an interval of two to four weeks. As with most killed vaccines, full immunity cannot be expected until approximately two weeks after the second dose of a primary vaccination course. Vaccination of birds in the presence of disease or poor nutritional status should be avoided since a satisfactory immune response would not be expected in such cases.

Vaccines are stored at 2-8°C and protected from freezing. Partly used packs should be discarded at the end of a day's operations.

1. Seed management

a) Characteristics

The strains of *P. multocida* to be incorporated in a vaccine should be virulent, of avian origin and of proven antigenicity.
b) Culture

Each strain is cultivated separately in a suitable liquid medium, then lyophilised. Ampoules are preferably kept at -70°C for long term storage. Seed cultures are normally produced in a "grandmother, mother and daughter" system. One ampoule of the "grandmother" stock culture is used to produce a number of "mother" ampoules (e.g. 100) and each one of these is in turn used to produce a number of "daughter" or working ampoules. One "daughter" ampoule is used to produce the seed culture for each batch of *P. multocida*.

Subcultures are made in a suitable liquid medium (e.g. brain heart infusion broth) which are checked for purity before inoculation of a larger volume of medium, for the final seed. Seed cultures are checked microscopically before use and by cultural techniques for retrospective confirmation of purity.

c) Validation as a vaccine

i) Efficacy

Candidate vaccine strains should be shown to be effective as monovalent vaccines against homologous challenge infection in a suitable animal model. Final vaccines may be tested according to the methods outlined under potency testing.

ii) Safety

Five healthy turkey poults aged 1-2 weeks are injected intramuscularly with twice the recommended dose and observed for at least seven days. The birds should show no systematic reaction and only a slight local reaction.

In addition, safety may be tested by injecting subcutaneously ten healthy mice, each weighing 18-20 g, with 0.5 ml of the vaccine under test. The mice should show no significant local or systematic reactions during the subsequent ten-day observation period.

iii) Stability

Stability of a vaccine may be tested by repeated potency testing after appropriate periods of storage.

2. Manufacture

Each strain to be included in the vaccine is blended to include a specified number of cells of each strain. Cells should be grown on a production scale in such a way as to produce a safe, effective vaccine. This may be by growth on agar surface or in liquid broth medium.
3. **In-process control**

a) **Antigen content**

Cells are harvested aseptically using suitable methods (e.g., washing from agar surface with sterile buffer) and the cell yield determined. This may be done by direct counting using a microscope and counting chamber, or by using optical density measurement with a standard curve against cell numbers. The cell concentration at this stage should be sufficiently high to facilitate vaccine blending but not so high as to unduly affect the rate of formalin inactivation. Vaccines normally contain approximately $10^9$ cells per dose and a harvested cell concentration in the region of $10^{11}$ per ml would be appropriate.

b) **Inactivation**

Cell suspensions are usually inactivated with formalin at 37°C. The pH may be adjusted to 6.7-6.9 to ensure the optimum rate of inactivation. The amount of formalin added should be the lowest level that ensures sterility. Samples are taken periodically to check sterility. Inactivation should take less than one week. After inactivation, cell concentrates are stored in the dark at 2-8°C either as single strains or as a final vaccine-cell concentrate containing appropriate volumes of each component strain. Thiomersal may be added as a preservative.

4. **Batch control**

a) **Sterility**

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

b) **Safety**

Safety testing of the final vaccine may be done according to the method outlined in section B.1.c.ii.

c) **Potency**

Potency testing of avian *P. multocida* vaccines is not described in either the British or European Veterinary Pharmacopoeias. A suitable potency test would be to infect vaccinated and non-vaccinated turkeys with one or more of the serotypes of *P. multocida* most commonly associated with the disease. The vaccination schedule would be that recommended for the vaccine in the field, and the challenge infection may be achieved by any suitable means, e.g. intramuscular injection, subcutaneous injection, swabbing of nasal cleft. Challenge infection should be done no less than two weeks after the second vaccination. A satisfactory vaccine is one which protects a specified number or more of turkeys against fatal infection. For the test to be valid a minimum number of non-vaccinates must die following infection. In the USA Code of
Federal Regulations, the specifications are that at least 12/20 vaccinates should survive and at least 16/20 non-vaccinates must die.

It may be acceptable to perform potency tests in other small animal models based on vaccination and challenge infection. Mice may be used in passive protection tests but tests involving vaccination of mice should be avoided since evidence suggests that their immune response to certain *Pasteurella* antigens is poor.

**REFERENCES**


Fowl pox 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.

Fowl pox causes a transient drop in egg production and a reduced growth rate in young chickens.

**Identification of the agent:** Fowl pox 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 skin.

Virus isolation is done by inoculation of chorioallantoic membranes of 9- to 12-day-old developing chicken embryos or avian cell cultures.

**Serological tests:** Immune responses to fowl pox virus may be demonstrated by the use of virus neutralisation, agar gel immunodiffusion, immunofluorescence, or passive haemagglutination tests, and by enzyme-linked immunosorbent assay (ELISA).

**Requirements for biological products:** Modified live fowl pox or pigeon pox virus vaccines of chick embryo or avian cell culture origin are available commercially. The use of fowl pox vaccines is indicated in areas where the disease is endemic, or on premises where infection has been diagnosed.

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**A. DIAGNOSTIC TECHNIQUES**

Fowl pox has a world-wide distribution and is caused by a DNA virus of the genus *Avipoxvirus* of the family *Poxviridae*. Its incidence is variable in different areas because of differences in management and hygiene or the practice of regular vaccination. It can cause drops in egg production or retarded growth in younger birds.
Fowl pox is a slow-spreading virus disease of chickens and turkeys, characterised in the cutaneous form 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), cankers or diphtheritic yellowish 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.

1. **Identification of the agent**

Fowl pox virus multiplies in the cytoplasm of epithelial cells with the formation of large intracytoplasmic inclusion bodies (Bollinger bodies) which 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, acridine orange or Giemsa stains. The elementary bodies can be detected in smears from lesions by the Gimenez method (3). Electron microscopy can be used to demonstrate typical poxvirus morphology by negative staining or in ultrathin sections of infected tissues.

Fowl pox virus can be isolated by the inoculation of suspect material into embryonated chicken eggs. Approximately 0.1 ml of tissue suspension of skin or diphtheritic lesion, with the appropriate concentration of antibiotics, is inoculated onto the chorioallantoic membranes (CAM) of 9- to 12-day-old developing chicken embryos. These are incubated at 37°C for 4-7 days, and then examined for focal white pock lesions or a generalised thickening of the CAMs. Histopathological examination of the CAMs will reveal eosinophilic intracytoplasmic inclusion bodies following staining with haematoxylin and eosin.

Primary chicken embryo fibroblasts, chicken embryo kidney, chicken embryo dermis cells, or the permanent quail cell line QT-35 can also be used to propagate fowl pox virus. The adaptation of virus strains to cell cultures is an important requirement for plaque formation, since not all strains will form plaques spontaneously.

Restriction endonuclease analysis of viral DNA can be used for strain comparisons.

2. **Serological tests**

Virus neutralisation (VN), passive haemagglutination (PHA), fluorescent antibody, or agar gel immunodiffusion (AGID) tests, as well as enzyme-linked immunosorbent assay (ELISA) can be used to measure specific antibody responses.

VN: After virus-serum interaction, the residual virus activity may be assayed in embryonating chicken eggs or in cell cultures. Neutralising antibodies develop within 1-2 weeks of infection.

AGID: Precipitating antibodies can be detected by reacting sera against partially purified viral antigen prepared by sonication and homogenisation of infected lesion material. The partial purification of the antigen is done by homogenisation with a
fluorocarbon such as Genetron 113. Precipitation lines develop in 24-48 hours after incubation of the antigen with antibody to homologous or closely related strains.

**PHA:** Sheep or horse red cells are sensitised with a partially purified fowl pox viral antigen. The antigen is prepared from infected CAMs by treatment with fluorocarbon. PHA is more sensitive than ID.

**Fluorescent antibody tests:** Direct or indirect immunofluorescence tests will reveal specific intracytoplasmic fluorescence in infected cells.

**ELISA:** Antigen is adsorbed onto a polystyrene surface and reacted with the test serum, followed by a horse radish peroxidase or alkaline phosphatase-labelled anti-avian immunoglobulin antibody. The test is sufficiently sensitive to measure antibodies to fowl pox virus within 7-10 days after infection. Infected tissue cells contain dark brown intracytoplasmic inclusions when reacted against specific antibody labelled with horse radish peroxidase.

Minor antigenic variations that occur between strains can be evaluated by means of immunoblotting.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

Vaccination is indicated in areas where fowl pox is endemic or on premises where infection has been diagnosed previously. Modified live fowl or pigeon pox virus vaccines derived from chicken embryos or avian cell cultures and of variable degrees of attenuation are available commercially.

Passively acquired immunity should be taken into consideration during vaccination of progeny from flocks which have either had a recent natural infection or been recently vaccinated. Since passive immunity may interfere with vaccine virus multiplication, such progeny should be vaccinated only after the decline of passively acquired antibody. Fowl pox vaccine is applied by a wing web stab method.

1. **Seed management**

   a) **Characteristics**

   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 attenuated fowl pox virus or pigeon pox virus. The MSV must be propagated in suitable premises with materials which meet approved standards, and must be tested for freedom from contamination as well as for identity and purity.

   b) **Culture**

   The MSV may be propagated in SPF chicken embryos, using the chorio-
allantoic membranes, or in avian cell cultures, such as primary chicken fibroblast, chicken embryo kidney or chicken embryo dermis. Quail cell cultures (QT-35) will support growth of fowl pox viruses after adaptation.

c) Validation as a vaccine

Purity: The MSV should be neutralised with a specific hyperimmune serum and used in tests to demonstrate freedom from extraneous agents. These tests should be done in: a) embryonating eggs or avian cell cultures, to demonstrate absence of virus replication, and b) specific pathogen free (SPF) chicks, to demonstrate freedom from antibodies to extraneous agents. The purpose is to exclude the presence of avian pathogens.

Safety: Vaccines should be prepared only from virus which 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 chicks 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. This test should be repeated after at least six serial passages of the virus to show that there has been no reversion to virulence.

Efficacy: Data should be obtained using the highest passage level and the lowest titre of virus to be used in the final product. Twenty SPF chicks of the minimum age stated for vaccination should be vaccinated with one dose of vaccine by the recommended method. Three weeks later the chicks, together with 20 unvaccinated chicks of the same age and source, should be challenged by scarification with a virulent strain of fowl pox virus. The birds should be observed for three weeks. All the control birds should develop lesions due to the challenge virus and at least 90% of the vaccinated birds should remain free of such lesions.

2. 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 may be compared with the virus content of the vaccine. A suitable potency can thus be established. The vaccine will 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 of contamination of biological materials may be found in the chapter on General Information.

b) **Safety**

The test described in 1c) above 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 used 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 fowl pox vaccine is likely to be in the region of $10^5 \text{ EID}_{50}$ (embryo infective dose) per ml.

d) **Duration of immunity**

The efficacy test given in 1c) may be used to determine the duration of immunity 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 three months beyond the requested shelf life on at least six batches of vaccine kept under recommended storage conditions.

**REFERENCES**


SUMMARY

Pullorum disease of chickens is caused by Salmonella pullorum and, in its acute form, is almost exclusively a disease of chicks.

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. The clinical signs resemble those of septicaemia, with inappetence and, eventually, death. Diagnosis depends on the demonstration of the organism or specific antibodies.

Samples should not originate from birds or eggs that have recently been treated with antimicrobial drugs. They can be swabs or bacteriological loops made from affected tissues, or intestinal and cloacal contents. Other sites to sample include eggs, egg shell surfaces, embryos, faecal droppings and hatcher debris, especially fluff and dust. The nature and quantity of the sample will depend on whether it is taken from live poultry or carcasses, and whether lesions or faecal contamination are present.

Identification of the agent: Samples are inoculated onto and into non-selective and selective enrichment broth and agar medium as soon as possible. In case of delay, samples should be stored at 0-4°C. Typical colonies can be identified by serological and biochemical means.

Serological tests: The serum agglutination test is satisfactory for individual birds for establishing the presence and prevalence of infection within a flock. This is usually a rapid whole blood plate agglutination test or a tube test. It can be applied as macro- or micro-agglutination tests. Any positive reactors should be confirmed as being infected by culture at post-mortem examination.

Requirements for biological products: Live and inactivated vaccines are available for fowl typhoid. The most commonly used vaccine is a live vaccine derived from the stable rough strain of S. gallinarum known as "9R".

The whole blood agglutination test requires a stained polyvalent antigen prepared from S. pullorum.

SPECIAL NOTE ON NOMENCLATURE OF GENUS SALMONELLA

See the note on nomenclature in Chapter 44 (B31/47: S. abortus ovis and S. abortus equi) for the principles followed concerning the nomenclature of Salmonella.
A. DIAGNOSTIC TECHNIQUES

Pullorum disease of chickens is caused by Salmonella pullorum. Fowl typhoid in chickens is caused by S. gallinarum.

In its acute form pullorum disease is almost exclusively a disease of chicks, and the agent can be recovered from almost all chick organs, tissues and faeces. In older birds that have become carriers, S. pullorum is most frequently recovered from the ova, and only exceptionally from other organs and tissues, including the alimentary tract. In contrast, fowl typhoid is more often observed in the later growing period and in mature stock. However, it may occur in young chicks where it is indistinguishable clinically from pullorum disease. In carrier birds, the organism is most frequently found in the liver and faeces.

To recover the organisms, the birds should not have been treated with antimicrobial drugs for about 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, hatchers or transport boxes. Swabs may be taken from the cloaca of live birds. Carcasses can be swabbed or bacteriological loops taken from spleen, liver, gall-bladder, kidneys, lungs, heart, ova, testes, alimentary tract or joint lesions, or conjunctiva. The demonstration of infection in serological reactor birds that are apparently normal may require the culture of large volumes of homogenised tissues with or without direct swabbing. Tissue pools may be made of a number of birds.

Eggs should be incubated before being opened. The shells are first swabbed with 70% alcohol or submerged in 2% iodophor. The egg contents may be homogenised. When floor litter is being sampled, it is important to remember that salmonellae survive better in dry material than in wet.

Culture media

Both S. pullorum and S. gallinarum grow well on simple media, but selective and enrichment media have been described which contain substances to inhibit the growth of extraneous organisms. The efficiency of recovery of these bacteria 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 simple and enrichment media for isolation. Both solid media and broth 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 (1, 2, 4, 6).

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 nutrient and meat infusion broth.
Selective and enrichment media include:

MacConkey agar. Inhibitory to non-enteric organisms, differentiates lactose fermenters (pink colonies) from non-lactose fermenters (colourless colonies). NaCl is omitted to limit the spread of *Proteus* colonies. Salmonella colonies are smooth and colourless.

Desoxycholate citrate agar. Inhibitory to non-enteric organisms. *S. pullorum* grows sparsely as very small colourless colonies; *S. gallinarum* colonies are 2-3 mm in diameter, dome-shaped, with a central black spot. *Proteus* grow readily, as does *Pseudomonas*.

Brilliant green agar. 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.

Brilliant green sulphapyridine agar. Inhibitory to coliforms and *Proteus* strains. The sulphapyridine is added to stabilise selectivity in the presence of egg materials.

Selenite F broth. Inhibitory to coliforms but not *Proteus*, improved by addition of brilliant green.

Tetrathionate/brilliant green broth. Inhibitory to coliforms and *Proteus*.

Selenite/brilliant green/sulphapyridine broth. Inhibitory to coliforms and *Proteus*.

**Recovery of salmonellae**

The methods for recovering *S. pullorum* and *S. gallinarum* vary according to the origin of the samples. They are as follows:

**Cloacal swabs and fresh faeces from live birds.** Swabs dipped in broth are suitable, small ones being used for chicks. The swabs are streaked onto simple and selective media, and placed into enrichment broth. The plates are incubated at 37°C and the broth at 42°C. At this temperature *Proteus* and *Pseudomonas* organisms tend to be inhibited. subcultures are made onto selective media after 24 and 48 hours.

**Gall-bladder contents.** Swabs of gall-bladder contents are streaked onto simple and enriched agars and placed into inhibitory and non-inhibitory broths, followed by incubation at 37°C and subculture onto inhibitory agar after 24 and 48 hours.

**Organs and tissues.** Swabs are taken from individual tissues and lesions and cultured onto simple and selective media, and into similar broths. These are incubated at 37°C and subcultured onto enrichment agar after 24 and 48 hours. Intestinal material in inhibitory broths should be incubated at 42°C; *S. gallinarum* grows well but there may be some inhibition of *S. pullorum* at this temperature.
Carrier birds. Here larger amounts of material are required. The ova should be tested for S. pullorum, the liver and gall-bladder for S. gallinarum. Tissues are homogenised in a small amount of broth and directly plated out. About 10 ml are put into 100 ml of enrichment broth and incubated at 37°C; this is subcultured onto simple and selective agar after 24 and 48 hours.

Alimentary canal, including the intestinal contents. After grinding or homogenisation in a little broth, 10 ml of the homegenate are incubated in 100 ml of enrichment broth. This is incubated at 42°C and subcultured onto selective agar after 24 and 48 hours.

Egg-shell surfaces. The eggs are placed in plastic bags containing 25-50 ml of enrichment broth and their surfaces massaged through the walls of the bags. The broth is incubated at 37°C and subcultured onto selective agar after 24 and 48 hours.

Egg contents. Homogenised contents of fresh eggs are mixed with about 100-200 ml of nutrient broth, incubated at 37°C, and subcultured onto simple and selective agar after 24-48 hours. Incubated eggs, whether infertile or containing small embryos, can be similarly treated; or 10 ml of homogenised contents can be mixed with 100 ml of enrichment broth, incubated at 37°C, and subcultured onto selective agar after 24 and 48 hours.

Embryos. Swabs should be taken from the yolk sacs of well developed embryos and streaked onto simple and selective agar, one swab being placed into 10 ml of simple and enrichment broth. Incubation is carried out at 37°C and subcultures made onto simple and selective agars after 24 and 48 hours.

Hatcher fluff and dust. A few grams are mixed with 50-100 ml of simple and enriched broth, incubated at 37°C, and subcultures made onto simple and selective agars after 24 and 48 hours.

Floor and nest litter. 2-3 g are mixed with 50-100 ml of inhibitory broth, incubated at 42°C, and subcultured onto selective agar after 24 and 48 hours.

1. Identification of the agent

Typical salmonella colonies on non-inhibitory media are round, glistening, domed, smooth, and 1-2 mm in diameter after 24-48 hours incubation. On selective media their appearance varies with the medium. Suspect colonies can be investigated serologically and biochemically, and observed for motility.

For serological investigations, a typical colony (preferably from a non-inhibitory medium) is transferred to a slide, and mixed with a little sterile saline. A drop of polyvalent salmonella "O" (A-G) antiserum is mixed with the emulsion. The slide is rocked for one minute and observed for agglutination. A control for auto-agglutination is performed on another slide, using saline instead of antiserum. If a colony is taken from a selective medium, a straight loop is applied to the mid-point of the domed colony and an emulsion made as before. If positive with polyvalent
salmonella antiserum, the culture may be tested with group-specific sera. S. pullorum and S. gallinarum are members of Group D.

Colonies causing agglutination are then investigated biochemically by testing in composite media or into individual reagents. Composite media are triple sugar iron (TSI) agar and lysine iron (LI) agar slants. Suspect colonies are touched at the mid high point with a straight wire and for each medium the centre of the butt is stabbed down to the base of the medium and the wire streaked along the surface of the slope. The inoculated medium is then incubated at 37°C for 24 hours. With TSI medium, both salmonella species show a yellow (acid) butt and a red (alkaline) slant. Gas bubbles in the medium are not produced by S. gallinarum, and are variable with S. pullorum; blackening due to hydrogen sulphide production is variable with both organisms. With LI medium, both salmonella species produce a purple (alkaline) or colourless (neutral) butt and a purple slant; in contrast, Proteus spp. produce a yellow (acid) butt and a red slant (deamination). Citrobacter spp. produce a yellow (acid) butt and a yellow or purple slant.

For tests with individual reagents, part of the non-lactose fermenting colony giving a positive reaction with the polyvalent "O" serum is picked off into peptone water and incubated at 37°C for 3-6 hours. A drop of culture is then pipetted into the peptone water for the indole test, into glucose with an inverted Durham tube for acid and gas production, and for fermentation into lactose, sucrose, salicin, mannitol, maltose and dulcitol. A drop is also examined for motility of organisms, and a blood agar plate should be inoculated as a test for the purity of the culture. The reactions shown in Table 1 occur.

Another method of identification is to examine a pure culture of the suspect organism using the Analytical Profile Index (API) system for Enterobacteriaceae.

Table I

<table>
<thead>
<tr>
<th>Biochemical investigation of S. pullorum and S. gallinarum</th>
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<tr>
<td>Indole production</td>
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<tr>
<td>Acid (A), gas (G) in glucose</td>
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<tr>
<td>Fermentation of lactose</td>
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<td>Fermentation of sucrose</td>
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<td>Fermentation of salicin</td>
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<td>Fermentation of mannitol</td>
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<td>Fermentation of maltose</td>
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<tr>
<td>Fermentation of dulcitol</td>
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<tr>
<td>Decarboxylation of ornithine</td>
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<tr>
<td>Urease production</td>
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<tr>
<td>Motility</td>
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</tbody>
</table>

- = negative    + = positive
2. Serological tests

The tests that are most readily applied include rapid whole blood agglutination, rapid serum agglutination, tube agglutination and micro-agglutination (5).

Both S. pullorum and S. gallinarum possess "O" antigens 1, 9 and 12. However, in the case of S. pullorum, there is a variation in the ratio of $12_1$, $12_2$ and $12_3$; the standard strain contains more $12_3$ than $12_2$, while the reverse is true for the variant form. Intermediate forms also exist. (No such variation occurs in the case of S. gallinarum.) Since this variation occurs, it is necessary to use a polyvalent antigen in the agglutination tests. The same antigen is used to detect both salmonella species.

a) Rapid whole blood agglutination test

Detecting both S. pullorum and S. gallinarum, the rapid whole blood agglutination test can be used under field conditions and the reactors identified immediately. However, it is not reliable in turkeys. Chickens can be tested at any age, although some authorities specify a minimum age of four months (5).

A drop of crystal violet stained antigen is put on a slide or white tile, and a similar drop of fresh whole blood added to it. The plate is gently rocked and the results read within two minutes. Visible clumping of antigen within one minute is a positive reaction, but if there is no clumping within two minutes the sample is negative. The antigen should be tested against known positive and negative control sera. 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 rapid serum agglutination test is performed in the same manner, except that serum is substituted for whole blood.

c) Tube agglutination test

Fresh serum from chickens, turkeys or other birds is used at a dilution of 1:25, obtained by mixing 0.04 ml of serum with 1.0 ml of antigen. Positive and negative control sera are included in each test. The antigen is prepared from unstained S. pullorum or S. gallinarum cultures diluted to a concentration of no. 1 on the McFarland scale. The mixture is incubated at 37°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.

d) Micro-agglutination test

This resembles the tube agglutination test, but requires much smaller amounts
of reactants. The test is performed in microtest plates. Sera are diluted 1:20 by adding 10 µl of serum to 100 µl of normal saline and then adding 100 µl of previously standardised stained microtest antigen. The plates are sealed and incubated at 37°C for 24-48 hours. A positive reaction consists of a fine diffuse precipitation, whereas a negative reaction shows a button-like precipitate.

Other serological tests include micro-antiglobulin (Coombs), immunodiffusion, haemagglutination and ELISA.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Although both live and inactivated vaccines have been prepared for use against S. gallinarum, the one most widely used is made from the rough 9R strain (3). It has only been employed on chickens. The number of viable organisms/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 (7) 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.

Current vaccines, however, have only a minor role to play in the control of fowl typhoid. This can best be achieved by hygiene in management, monitoring and removal of infected stock, and the careful use of antimicrobials.

A stained polyvalent antigen is produced for use in the rapid whole blood agglutination test. This is made from S. pullorum cultures and is also suitable for the detection of infections with S. gallinarum.

1. Seed management

a) Characteristics

Vaccine: 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.

Antigen: A standard strain of S. pullorum of antigenic structure 9, 121, 123 and a variant strain 9, 124, 122, are cultured separately. The bacteria should conform to the characteristics of S. pullorum; they must be smooth and not agglutinate in the presence of 1:500 acriflavine, or auto-agglutinate in normal saline and normal serum.
b) **Culture**

S. gallinarum is grown on or in a suitable medium, such as nutrient agar or broth, for 24 hours at 37°C.

S. pullorum is grown on agar; adequate growth is obtained in about 48-60 hours.

c) **Validation**

**Vaccine:** There is no satisfactory method of assessing the protection afforded by the vaccine in the field.

**Antigen:** To assess safety, a culture test is made on agar for non-viability of the unwashed antigen before standardisation. Each bottle of antigen must be tested after alcoholic precipitation and before standardisation against standard titre antisera for S. pullorum and S. gallinarum, and against a negative serum.

The antigen is kept at 0-4°C and is usually given a 6-month expiry date from the bottling of the final product.

2. **Manufacture**

**Vaccine:** 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 a nutrient agar. In either case the suspension is diluted in phosphate buffer solution (pH 7.0) and may be freeze-dried. The dose used per bird is between 5 and 50 million organisms.

**Antigen:** The antigen is prepared from smooth, standard and variant strains of S. pullorum colonies shown to be agglutinated by specific antisera and not by acriflavine or normal saline and normal serum. Typical colonies of each strain are seeded separately onto agar slopes, incubated at 37°C for about 24 hours, and the growth emulsified in sterile normal saline to provide about 2 ml of seed inoculum on agar for each Roux flask. Equal numbers of flasks are seeded with each strain of S. pullorum, which is evenly distributed by gently rocking the flasks. Adequate growth should occur in 48-60 hours.

The antigen is harvested by the addition of sterile glass beads and 10 ml sterile buffered formol saline to each Roux flask. The flasks are rocked until all growth is in suspension, and then left in the vertical position for 15 minutes. The suspension is pipetted into a large flask and absolute alcohol added to the suspension in the ratio of 2:1 by volume. These are mixed and allowed to stand until the precipitation is complete. This can take 24-48 hours but the more densely packed the precipitate the better the yield; there is no disadvantage in leaving the mixture for as long as a week. The clear supernatant fluid is drawn off and the cells syphoned into
stopped bottles for storage at 0-4°C until required for standardisation. For this the cells are centrifuged to remove remaining alcohol. For both standard and variant strains a small quantity of buffered formal saline is added and the resuspended cells are mechanically blended to give fine, even suspensions. One-millilitre volumes of each are then diluted in saline to determine the concentration of cells present and to compute their concentration in the original suspension.

At this stage the preparation of stained antigen and unstained antigen (for the thermostable antigen test) is different. For stained antigen, amounts of standard and variant strain suspensions are diluted in buffered formol saline (pH 6.5) to give in the final product the required concentration of each strain of S. pullorum; then 10% (v/v) glycerol and 1% (v/v) of 3% alcoholic crystal violet solution are added. For unstained antigen, the suspensions are diluted in 0.5% (v/v) phenol-saline, mixed and calibrated before final mixing.

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 with biological materials may be found in the chapter on General Information.

b) Safety

Vaccine: Six healthy, susceptible chickens, 8 to 16 weeks of age, are each injected subcutaneously with a quantity of vaccine containing 500 million viable organisms, and are observed for seven days; no local or systemic reaction should develop.

Antigen: A culture test is performed on agar for the non-viability of the unwashed antigen before standardisation.

c) Potency

Vaccine: Fifteen healthy chickens, 8 to 16 weeks of age, of the Light Sussex or Rhode Island Red breeds, or crosses of these, and taken from a stock which is free from S. pullorum infection, are each injected subcutaneously with a quantity of vaccine containing 50 million viable organisms. After an interval of 21 to 28 days the vaccinated chickens and an equal number of similar unvaccinated chickens are deprived of food for about 18 hours. They are then challenged by oral administration of 1 ml of a broth suspension containing 50 million 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 to 21 days. The vaccine passes the test if at the end of this period the number of surviving vaccinated chickens which show no macroscopic lesions of fowl typhoid at post-mortem exceeds by eight or more the number of similarly defined control chickens.

**Antigen:** The finished antigen is tested with standard antisera for both S. pullorum and S. gallinarum, in parallel with a previous batch for comparison. The titre of the positive sera may be determined as an indication of the potency and specificity of the antigen. It is also valuable to test the antigen with the blood of uninfected chickens and those infected with the strains of either species, for the speed and nature of the agglutination.

**REFERENCES**


INFECTIOUS BURSAL DISEASE
(B63)

SUMMARY

Infectious bursal disease (IBD) is caused by a virus which has been difficult to classify, but is now considered to be a birnavirus. Although turkeys and ducks may be infected, disease only occurs in chickens. Only young birds are affected. Severe acute disease is associated with high mortality but a less acute, or subclinical, disease is common. This can cause secondary problems due to the effect of the virus on the bursa of Fabricius. It causes lymphoid depletion of the bursa, and if this happens in the first week of life, significant depression of the humoral antibody response may result. Two serotypes of IBD virus are recognised. To date, vaccines have been made only against IBD type I. Recently it has been shown that serological variants of IBD type I occur. These may require special vaccines for maximum protection.

Clinical disease due to infection with the IBD virus, also known as Gumboro disease, can usually be diagnosed by a combination of characteristic signs and post-mortem lesions. Laboratory confirmation, or detection of subclinical disease, can be carried out by demonstration of a humoral immune response or by detecting the presence of viral antigen in tissues. In the absence of such tests, histological examination of bursae may be helpful.

Identification of the agent: Isolation of IBD virus is not usually carried out as a routine diagnostic procedure. When this is required, some difficulty must be anticipated. Tissue cultures, chickens or embryonating eggs may be used for attempted virus isolation. The virus can be identified 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 and homogenised, and used as antigen in a test against known specific positive antiserum. This is particularly useful in the early stages of the infection, before the development of an antibody response.

Sero logical tests: Either an AGID or VN test 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 need be tested to detect the presence of antibodies. If positive reactions are found, then the whole flock must be regarded as infected.

Requirements for biological products: Both live attenuated and inactivated (killed) vaccines are available to control the disease. It is important that live vaccines be well attenuated and stable, with no tendency to cause bursal damage. To be effective, the inactivated vaccines need to have a high antigen content.
Live vaccines are used to produce an active immunity in young chicks. A preferred alternative to this is to provide chicks with passive protection by vaccination of their parents with a combination of live and killed vaccines.

Live vaccine: An attenuated strain of IBD virus is used and may be administered by intramuscular injection or in the drinking water. In the absence of maternal antibodies the vaccine is given at one day old. When maternal antibodies are present, vaccination should be delayed, usually until about 28 days of age. The best schedule can be determined by serological testing of the birds, to detect the time at which maternal antibodies have fallen to a low level.

Killed vaccines: These are used to produce high and uniform levels of antibody in parent chickens so that the progeny will have high and uniform levels of maternally derived antibodies. The killed vaccine is manufactured in oil emulsion and is given by injection. It 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 maternally-derived antibody can be obtained in breeder birds by giving, for example, live vaccine at about 8 weeks of age, followed by inactivated vaccine at about 18 weeks of age.

A. DIAGNOSTIC TECHNIQUES

Laboratory diagnosis of infectious bursal disease (IBD) depends on detection of specific antibodies to the virus, or on serological detection of the virus in tissues. Isolation and identification of the agent are not usually attempted for routine diagnostic purposes (2).

1. Identification of the agent

Clinical IBD has very characteristic signs and post-mortem lesions; confirmation or detection of subclinical disease is best done by using serological methods. The virus of IBD is difficult to isolate, and this is not usually attempted in routine diagnosis. When there are special reasons for attempting virus isolation the following methods should be followed.

a) Sample preparation

Remove the bursae of Fabricius aseptically from about five affected chickens in the early stages of the disease. Chop the bursae using two scalpels, add a small amount of peptone broth containing 1,000 units/ml penicillin and 1,000 µg/ml streptomycin and homogenise in a tissue blender. Centrifuge the homogenate at 3,000 g for 10 minutes. The supernatant fluid is harvested and used for the following investigations.

b) Isolation of virus in tissue culture

Inoculate 0.5 ml of sample onto each of four confluent chick embryo fibroblast
cultures in 25 cm² flasks. Absorb at 37°C for 30 minutes, wash twice with Earle's balanced salt solution and add maintenance medium to each flask. Observe daily for evidence of cytopathic effect (CPE). This is characterised by small round refractive cells. If no CPE is observed after six days, freeze and thaw the cultures and inoculate the resulting lysate onto fresh cultures. This procedure may need to be repeated three times. If CPE is observed the virus should be tested against known IBD antiserum in a tissue culture virus neutralisation test (see below).

c) Isolation of virus in embryos

Inoculate 0.3 ml of sample into the yolk sacs of ten 6- to 8-day-old, specific antibody negative (SAN) chicken embryos or onto the chorioallantoic membranes of ten 9- to 11-day-old SAN chicken embryos. Candle daily and discard deaths up to 48 hours post-inoculation. Embryos which die after this time are examined for lesions. IBD produces dwarfing of the embryo, subcutaneous oedema, congestion and haemorrhages. The livers are usually swollen, with patchy congestion producing a mottled effect. In later deaths the livers may be swollen and greenish, with areas of necrosis. Spleens are enlarged and the kidneys are swollen and congested, with a mottled effect.

IBDV usually causes death in at least some of the embryos on primary isolation.

d) Isolation of virus in chickens

Inoculate five susceptible chickens and five which are IBD immune (3 to 7 weeks of age) with 0.05 ml of sample intraocularly. Sacrifice the chickens on the third day after inoculation and examine their bursae of Fabricius. The bursae of IBDV-infected chickens 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 with absence in immune chickens is diagnostic.

2. Serological tests

a) Agar gel immunodiffusion test

The agar gel immunodiffusion test (AGID) is the most useful of the serological tests for detection of specific antibodies in serum, or for detecting viral antigen in bursal tissue.

Blood samples should be taken early in the disease, and repeat samples taken three weeks later. Because the virus spreads rapidly, only a small proportion of the flock needs to be sampled. Usually about 20 blood samples are enough. For detection of antigen in the bursa of Fabricius, the bursae should be removed aseptically from about ten chickens. The bursae are homogenised using a tissue
blender, centrifuged for 10 minutes, and the supernatant cell extract is placed in the wells of the AGID plate, against known positive antiserum.

The AGID test is carried out as follows:

i) Preparation of plates

The medium is 1.25% agar (Oxoid no. 1 Oxoid, UK) with 8.0% sodium chloride and 0.5% phenol.

Dissolve the sodium chloride and phenol in distilled water. Adjust to pH 7.5 using 1 M sodium hydroxide. Add the agar and steam to dissolve. While the mixture is still very hot filter through a pad of cellulose wadding covered with a few layers of muslin.

The medium is dispensed, while still hot, into 15 ml volumes in universal bottles and stored at 4°C until required for use. Plates are poured 24-72 hours before use by dissolving the agar in a steamer and pouring 15 ml into a 9 cm plastic petri dish. This gives an agar depth of 3 mm. When the agar is set, plates are stored at 4°C.

The linear pattern of well arrangement is preferred for the testing of numerous samples. Three vertical rows of wells are cut and the agar removed using a pen and nib. For the IBD system, wells of 6 mm diameter are used, with 3 mm interspace. A positive reaction system and a known negative antiserum or antigen are incorporated in each test. Figures 1 and 2 show model reagent protocols which are suitable for routine use, although lines of identity will not be seen for all reactions.

Wells are filled by pipetting fluid reagents or by carefully placing small pieces of chopped tissue using pointed forceps. Plates are incubated at

\[
\begin{array}{|c|c|c|}
\hline
& AG & AB \\
\hline
T & AG = \text{Positive antigen} & T \\
A & AB = \text{Positive antiserum} & A \\
B & AB- = \text{Negative antiserum} & B \\
G & AG- = \text{Negative antigen} & G \\
\hline
\end{array}
\]

\[
\begin{array}{|c|c|c|}
\hline
& AC & A2 \\
\hline
T & T \\
A & A2 \\
B & A2 \\
C & A2 \\
\hline
\end{array}
\]

\[
\begin{array}{|c|c|c|}
\hline
& T = \text{Test tissues} & T = \text{Test sera} \\
\hline
T & T \\
T & T \\
T & T \\
T & T \\
\hline
\end{array}
\]

Fig. 1
Protocol for tests for antigen

Fig. 2
Protocol for tests for antibody
22°C. Specific lines develop within 24-48 hours. Care should be taken in interpreting lines which develop after these times, as non-specific reactions often occur later.

ii) Preparation of specific antisera and antigens

**Antiserum:** Inoculate 4- to 5-week-old susceptible chickens intraocularly with 0.05 ml of a clarified 10% w/v bursal homogenate known to contain viable IBD virus. Exsanguinate 28 days post-inoculation and store serum in aliquots at -20°C.

**Antigen:** Inoculate 3- to 5-week-old susceptible chickens intraocularly with a clarified 10% w/v bursal homogenate known to contain viable IBD virus. Sacrifice the birds 3 days post-inoculation and harvest bursae aseptically. Discard haemorrhagic bursae and pool the remainder, weigh and add an equivalent volume of cold distilled water and an equivalent volume of Arcton 113 (ICI Chemicals, UK). Thoroughly homogenise the mixture in a tissue blender and centrifuge at 2,000 g for 30 minutes. Harvest the supernatant fluid and dispense in aliquots for storage at -40°C.

A suitable strain of IBD virus (type 1) is the strain 52/70, obtainable from the Central Veterinary Laboratory, Weybridge, England.

iii) Evaluation of results

The AGID test is surprisingly sensitive, though not as sensitive as the virus neutralisation test which 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. The AGID test can also be used to measure antibody level, by using dilutions of serum in the test wells, and taking the titre as the highest dilution to produce a precipitin line. This can be very useful for measuring maternal or vaccinal antibodies and deciding on the best time for revaccination.

b) Virus neutralisation tests

Virus neutralisation tests (VN) may be carried out in tissue culture or in young chickens. The test is more laborious and expensive than the AGID test, but is more sensitive in detecting antibody. This sensitivity is not required for routine diagnostic purposes but may be useful for evaluating vaccine responses.

The VN tests are carried out as follows:

i) Tissue culture (TCVN test)

0.05 ml of virus dilution containing 100 TCID$_{50}$ (tissue culture infective doses) is plated in each well of a microtitre plate. The test sera are heat
Infectious bursal disease (B63) 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 chick embryo fibroblast cell suspension is dispensed into each well. Tests are incubated at 37°C for 4 to 5 days, after which the monolayers are observed microscopically for typical CPE. End-points are determined to be the reciprocal (log$_2$) of the final dilution which did not show CPE.

ii) In chicks

The test sera are pooled and inactivated at 56°C for 30 minutes. One ml of pooled serum is mixed with 1 ml of known IBD-positive antigen containing 200 TCID$_{50}$/0.05 ml (a clarified 10 per cent w/v bursal homogenate – Cheville strain – is used) and incubated at 37°C for 30 minutes. 0.05 ml of the mixture is dropped into the eyes of seven susceptible chickens which are sacrificed three days later, followed by examination of their bursae. A control test is made using IBD-negative serum.

Absence of bursal lesions in positive serum birds with presence of lesions in the controls indicates exposure of the test birds to infection.

c) Enzyme-linked immunosorbent assay (ELISA)

ELISA tests have been developed for the detection of antibodies to IBD. Coating of the plates requires a purified, or at least semi-purified, preparation of virus. However, the ELISA does not correlate well with AGID or with VN test titres.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Two types of vaccine are available for the control of infectious bursal disease (IBD), also known as Gumboro disease. These are live, attenuated vaccines or inactivated oil emulsion adjuvanted vaccines (5). To date, IBD vaccines have been made only from type I IBD virus, although a type II virus has been detected in poultry. The type II virus has not been seen to be associated with disease, although its presence will stimulate antibodies. Type II antibodies do not confer protection against type I infection, neither do they interfere with the response to type I vaccine. Recently there have been descriptions of serological variants of type I virus. Cross protection studies have shown that "classical" type I virus vaccine does not give good protection against some of these variants. Consideration is therefore being given to making IBD vaccines which contain both classical and variant IBD type I viruses.

Live vaccines: method of use

Live IBD vaccines are used to produce a primary immune response in birds not previously vaccinated (3). Sometimes second or third vaccinations may be given, but generally these are not as effective as secondary vaccination with inactivated oil emulsion vaccines.
Live IBD vaccines are susceptible to the effect of maternally derived antibody (MDA). Chicks from antibody-negative parents may be vaccinated at one day old. Chicks from parents with antibody should not be vaccinated until the MDA has reduced to a low level, usually about 30 days of age, if inactivated vaccines of high potency have been used in the parents. This will depend on the antibody titres of the parent birds at the time the eggs were laid. Live IBD vaccines should be made from strains of virus which are shown to cause no bursal lesions in young birds. In some countries live IBD vaccines are used which are not entirely apathogenic. They are said to give a better antibody response in the presence of MDA. However, there is always the risk that they may cause bursal damage in chicks with below-average MDA, and therefore they cannot be recommended. Live IBD vaccines may be administered either by individual injection, by the intramuscular route, or more usually by mass medication, either in the drinking water or by use of a spray. Clean water must be used, free of smell or taste of chlorine or metals. Liquid skim milk may be added at a rate of 5% v/v. 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. It is preferable to divide the medicated water into two parts, giving the second part half an hour after the first.

Live IBD vaccines are generally regarded as compatible with other avian vaccines. However, IBD vaccines which cause bursal damage could interfere with the response to other vaccines. Only healthy birds should be vaccinated. 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 which have previously been primed by live vaccine or by natural exposure to field virus during rearing (1). The usual programme is to administer the live vaccine at about eight 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 route is intramuscular, into the leg muscle, avoiding proximity to joints, tendons or major blood vessels. A multi-dose 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 +2°C to +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 IBD virus, should be vaccinated. Used in this way the vaccine should produce such a good antibody response that chicks hatched from those parents will have passive protection against IBD for up to about 30 days of age (6). 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 been seeded out into the peripheral lymphoid tissues. Using sensitive tests such as serum neutralisation, it may be shown that chicks retain MDA for some time after they become susceptible to
Infectious bursal disease (B63)

If such chicks are being retained for breeding they should be given live IBD vaccine at about eight weeks of age. The precise level and duration of immunity conferred by inactivated IBD vaccines will depend mainly on the quantity of antigen present per dose. It should be the objective in manufacture to obtain a high antigen concentration and hence a highly potent vaccine.

1. **Seed management**

a) **Characteristics**

i) **Live vaccine**

An important characteristic is that the virus should not cause damage to the bursa of Fabricius in susceptible birds.

The seed virus must be shown to be free of extraneous viruses, bacteria, mycoplasma and fungi, particularly avian pathogens. This includes freedom from contamination with other strains of IBD virus. The seed virus must be shown to be stable, with no tendency to revert to virulence. This can be done by carrying out at least six consecutive chick to chick passages at 3- to 4-day intervals, using bursal suspension as inoculum. 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 initial and final passage material.

ii) **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 of extraneous viruses, bacteria, mycoplasma and fungi, particularly avian pathogens (4).

b) **Culture**

Seed virus may be propagated in various culture systems, such as chick 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, which go on to lay, so that their progeny can be challenged, to determine resistance due to maternally derived antibody at the beginning and end of lay.
i) Live vaccine

**Efficacy test:** The method described under live vaccine potency test may be used.

**Test for immunosuppression:** In the case of live vaccines it is necessary to carry out a test for immunosuppression to validate the suitability of the virus strain.

The vaccine is administered by injection or eyedrop, one field dose per bird, to each of 20 SPF chicks, at one day of age. A further group of birds of the same age and source are housed separately as controls. At two weeks of age, each bird in both groups is given one field dose of live Newcastle disease vaccine by eyedrop. Two weeks later the haemagglutination inhibition (HI) response of each bird to Newcastle disease vaccine is measured, and the protection is measured against challenge with $10^{6.5}$ ELD$_{50}$ (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 ($P < 0.01$) in the group given IBD vaccine than in the control group.

ii) Killed vaccine efficacy test

Approximately 20 unprimed SPF birds are given one dose of vaccine at the recommended age (near to point of lay) and the antibody response is measured with reference to a standard antiserum* over a period from 4 to 6 weeks after vaccination. If the agar gel immunodiffusion test is used, a level of at least 800 UK units per ml should be obtained. Eggs are then collected for hatching and 25 progeny chicks are then challenged at 4 weeks of age by eyedrop with $10^2$ CID$_{50}$ (chick infective doses) of a recognised virulent strain of IBD virus such as strain CVL 52/70*. Ten control chicks of the same breed but from unvaccinated parents are also challenged. Protection is assessed three 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 chicks from vaccinated parents should show evidence of IBD infection whereas all those from unvaccinated parents should be affected. The test need be performed once only using a typical batch of vaccine.

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* For quantitative agar gel immunodiffusion tests, the British Standard serum is available from the Central Veterinary Laboratory, Weybridge, England, UK. IBD strain CVL 52/70 is also obtainable from this address.
2. **Manufacture**

The vaccine must be manufactured in suitable clean and secure accommodation, well separated from diagnostic facilities or commercial poultry.

Production of the vaccine should be on a seed lot system, using a suitable strain of virus of known origin and passage history. Specific pathogen free (SPF) eggs must be used for all materials used 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 IBD virus grown in cell culture or embryonating eggs. A high virus concentration is required. These vaccines are made as water-in-oil emulsions. A typical formulation is to use 80% mineral oil to 20% 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 chicks 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 which has been shown to be effective in laboratory and field trials.

**Inactivation of killed vaccines:** This is frequently done with either β-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 potential contaminants which may arise from the starting materials, particularly Newcastle disease and avian leucosis virus.

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 virus 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 two passages in susceptible cell cultures, embryos or chicks, 10 replicates per passage. No evidence for the presence of any live virus or microorganism should be observed.

**Sterility of killed vaccines:** Oil used in the vaccine must be sterilised by heating at 160°C for one 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 the chapter on General Information.
b) Safety

i) Live vaccine safety test

One field dose of vaccine is administered by eyedrop to 10 SPF chicks, seven days of age. One hundred field doses are inoculated by the intramuscular route, and 10 field doses by eyedrop, into each of 10 chicks at one day of age. These birds are observed for three weeks. The vaccine fails the test if any lesions are seen in the bursa on histological examination. A further group of 10 birds of the same age and source are separately housed as controls. The vaccine fails the test if the mean bursa/body weight ratio of the vaccinated birds differs significantly (P<0.01) from that of the controls.

ii) Killed vaccine safety test

Ten SPF birds, 14 to 28 days of age, are inoculated by the recommended routes with twice the field dose. The birds are observed for two weeks. No abnormal local or systemic reaction should develop. The test is performed on each batch of final vaccine.

c) Potency

i) Live vaccine potency test

The vaccine is administered as it will be used in the field to 25 SPF chicks at seven days of age so that each chick receives one field dose of vaccine. A further group of 25 unvaccinated birds of the same age and source is separately housed as controls. Ten to 14 days later, the vaccinates, together with the group of unvaccinated controls, are challenged by eyedrop with $10^{2.0} \text{CID}_{50}$ of virulent IBD virus such as strain CVL 52/70. Strain CVL 52/70 is a virulent challenge strain of IBD virus, which may be obtained from the Central Veterinary Laboratory, Weybridge, Surrey, UK. The birds are observed for 10 days. At least half of the control birds must die or show symptoms of IBD and all surviving controls show severe bursal lesions determined by histological examination at the conclusion of the test period. The vaccine fails the test if more than three of the vaccinates die, show symptoms of IBD, or show severe bursal lesions determined by histological examination at the conclusion of the test period.

ii) Killed vaccine potency test

Twenty SPF chicks, approximately four weeks of age, are each vaccinated with one dose of vaccine given by the recommended route. An additional 10 control birds of the same source and age are housed together with the vaccinates. The antibody response of each bird is determined with reference to a standard antiserum four to six weeks after vaccination. The mean antibody level of the vaccinated birds should not be significantly less
than the level recorded in the test of protection. No antibody should be detected in the control birds. This test must be carried out on each batch of final vaccine.

d) Duration of immunity (killed vaccine)

Evidence should be provided to show that progeny hatched from eggs taken at the end of the laying cycle are as adequately protected as those taken soon after vaccination. Information should also be provided on the duration of antibody levels in the breeders throughout the laying cycle. The test is performed once, using a typical batch of vaccine. The evidence may be obtained from primed birds vaccinated by the recommended schedule, but the final dose of vaccine is given at the earliest recommended age and the final observations of progeny protection and antibody levels are made when the vaccinated birds are not less than 60 weeks of age.

e) Stability

Evidence should be provided on three batches of vaccine to show that the vaccine passes the batch potency test at three 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 persistence throughout shelf life should be checked. A suitable preservative already established for such purposes should be used.

g) Special precautions

Oil emulsion vaccines cause serious injury to the vaccinator if accidentally injected into the hand or other tissues. In the event of such an accident the person should go at once to a hospital, taking the vaccine package with him. Each vaccine bottle and package should be clearly marked with a warning of the serious consequences of accidental self-injection. Such wounds should be treated by the casualty doctor as a "grease gun injury".

REFERENCES


MAREK'S DISEASE
(B64)

SUMMARY

Diagnosis of Marek's disease (MD) in domestic fowl is made on clinical signs and gross or microscopic lesions. Infection by MD virus (MDV), not necessarily accompanied by clinical disease, is detected by virus isolation and demonstration of viral antigen and antibodies.

MD is prevented by vaccination of day-old chicks with monovalent or bivalent live virus vaccines of various types.

Identification of the agent: MD occurs in chickens, most frequently from 12-24 weeks of age, but can affect chickens from six weeks of age to adulthood. 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, lung, heart, proventriculus and skin. Histologically, the nerve infiltration and lymphomas usually consist of lymphoid cells of various types. Differentiation of MD from lymphoid leukosis is important.

Under field conditions, most chickens become infected by 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 onto monolayer cultures of chick kidney cells or duck embryo fibroblasts, in which characteristic viral plaques develop within a few days. Two serotypes, 1 and 2, of MDV are recognised, and a third serotype, 3, is represented by the related herpesvirus of turkeys (HVT). 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 a week or two of infection and are commonly recognised by the agar gel immunodiffusion test, and sometimes by other serological tests.

Requirements for biological products: MD is prevented by the vaccination of day-old chicks with a live virus vaccine. 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 strain 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 are also used. The bivalent vaccines have been introduced to combat the very virulent strains of MDV which are not well controlled by the usual monovalent vaccines, such as HVT.
Vaccination greatly reduces clinical disease, but not persistent infection by MDV. The vaccine viruses are also carried throughout the life of the fowl.

A. DIAGNOSTIC TECHNIQUES

Marek’s disease (MD) (1, 7, 8) is caused by a herpesvirus and affects chickens from about six weeks of age. It occurs most frequently between 12 and 24 weeks of age, but sometimes later. In the classical form of the disease, with 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, an incidence of 10-30% of 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 continue at a steady or slowly falling rate for several months.

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 autopsy 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.

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 lesion consists of infiltration by proliferating lymphoblasts and large, medium and small lymphocytes, and macrophages, and appears 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 which 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 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-Grunwald Giemsa, is an important feature in differentiating the disease from lymphoid leukosis, in which the lymphomatous infiltrations are comprised 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 main features in the differential diagnosis of MD and lymphoid leukosis are shown in Table I. 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 the detection by immunofluorescence of T-cell antigens or the MD tumour-associated surface antigen (MATSA) on MD tumour cells, or of B-cell antigens or IgM on the tumour cells of lymphoid leukosis.

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.

1. Identification of the agent

Infection by MD virus (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. 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 chick kidney cells or duck embryo fibroblasts (chick embryo fibroblasts are less sensitive for primary virus isolation). Serotype 2 and 3 viruses (see section B.1.a) are more easily isolated in chick embryo fibroblasts than in chick kidney cells. Usually a 0.2 ml suspension containing $10^6$ to $10^7$ live cells is inoculated onto duplicate monolayers grown in 60 mm diameter plastic cell culture dishes. Inoculated and uninoculated control cultures are incubated at 38.5°C in a humidified incubator containing 5% CO$_2$. Alternatively, closed culture vessels may be used. Culture medium is replaced at intervals of three days. Areas of cytopathic effects, termed plaques, appear within three or four days and can be enumerated at about seven days.
<table>
<thead>
<tr>
<th>Feature</th>
<th>Marek’s disease</th>
<th>Lymphoid leukosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>6 weeks or older</td>
<td>Not less than 16 weeks</td>
</tr>
<tr>
<td><strong>Symptoms</strong></td>
<td>Frequently paralysis</td>
<td>Non-specific</td>
</tr>
<tr>
<td><strong>Incidence</strong></td>
<td>Frequently above 5% in unvaccinated flocks</td>
<td>Rarely above 5%</td>
</tr>
<tr>
<td><strong>Macrosopic lesions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neural enlargement</td>
<td>Frequent</td>
<td>Absent</td>
</tr>
<tr>
<td>Bursa of Fabricius</td>
<td>Diffuse enlargement or atrophy</td>
<td>Nodular tumours</td>
</tr>
<tr>
<td>Tumours in skin, muscle and proventriculus</td>
<td>May be present</td>
<td>Usually absent</td>
</tr>
<tr>
<td><strong>Microscopic lesions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neural involvement</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Liver tumours</td>
<td>Often perivascular</td>
<td>Focal or diffuse</td>
</tr>
<tr>
<td>Spleen</td>
<td>Diffuse</td>
<td>Often focal</td>
</tr>
<tr>
<td>Bursa of Fabricius</td>
<td>Interfollicular tumour and/or atrophy of follicles</td>
<td>Intrafollicular tumour</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Lymphoid proliferation in skin and feather follicles</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>Cytology of tumours</strong></td>
<td>Pleomorphic lymphoid cells, including lymphoblasts; small, medium and large lymphocytes and reticulum cells. Rarely can be only lymphoblasts.</td>
<td>Lymphoblasts</td>
</tr>
<tr>
<td><strong>Category of neoplastic lymphoid cell</strong></td>
<td>T-cell</td>
<td>B-cell</td>
</tr>
</tbody>
</table>
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 a buffer containing sucrose, phosphate, glutamine, bovine serum albumen and sodium ethylenediamine tetra-acetic acid (SPGA-EDTA). This suspension is ultrasonicated and then filtered through a 0.45 μm millipore filter for inoculation onto 24-hour-old drained chick kidney cell monolayers. After absorption for 40 minutes, the medium is added and cultures incubated as above for seven days.

Using these methods, MDV of serotypes 1 and 2 may be isolated, together with the herpesvirus of turkeys (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 chickens from about four 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.

Agar gel immunodiffusion tests are employed most commonly to detect antibody. The test is conducted using glass slides coated with 1% Noble agar in phosphate buffered saline containing 8% sodium chloride. Adjacent wells are filled with antigen or serum and these are diffused in a humidified atmosphere at room temperature or 37°C for 24 to 72 hours; 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 of skin containing feather tracts obtained from MDV-infected chickens.

A variant of the immunodiffusion 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 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.

Other tests for MDV antibody are direct and indirect immunofluorescence tests for the ability of a test serum to stain MDV plaques in cell cultures. A virus neutralisation test of the ability of a serum to neutralise the plaque-forming property of cell-free MDV can also be employed. However, both these tests are
more suitable for research purposes than for routine diagnostic use. An enzyme-linked immunosorbent assay (ELISA) for detecting MDV antibodies is available but not yet in routine use.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Commercial biological products used in the control of MD are the cell-associated or cell-free (lyophilised) live virus vaccines based on a variety of strains of MDV or HVT, respectively. The requirements for producing vaccines are outlined as follows, but other sources (2-6, 9, 10) should be consulted for more detailed information on the procedures.

1. **Seed management**

   a) **Characteristics**

   Viruses of the MDV group as classified under three serotypes: 1, 2 and 3, on the basis of their antigenic relatedness.

   **Serotype 1:** This includes all the pathogenic strains of virus, ranging from strains that are very virulent (e.g. Md/5, Md/11, Ala-8, RB-1B), to virulent (e.g. HPRS-16, JM, GA), to 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 strains have been used in experimental vaccines to protect against the variant form of acute MD caused by the very virulent strains but have not yet been developed commercially. 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, HN-1), and several of these have been shown to provide protection against virulent strains. The SB-1 strain has been developed commercially and used, particularly with HVT, in bivalent vaccines for protection against the very virulent strains. Serotype 2 vaccines exist only in a 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 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) **Culture**

   The substrates used for commercial vaccine production are primary chick embryo fibroblasts (CEF) derived from specific pathogen free (SPF) flocks or
Marek's disease (B64)

duck embryo fibroblasts. CEF from SPF flocks are preferred to duck cells because more is known about chick-embryo-transmitted pathogens and methods for their detection.

c) Validation as a vaccine

Methods for testing SPF flocks for freedom from infection are available (5, 9). SPF chicken flocks should be free from avian adenoviruses, including egg-drop syndrome 76 virus, avian encephalomyelitis virus, avian leukosis virus (subgroups A and B), avian nephritis virus, avian reoviruses, avian rotaviruses, chicken anaemia agent, fowl pox virus, infectious bronchitis virus, infectious bursal disease virus, infectious laryngotracheitis virus, influenza type A virus, Marek's disease virus, 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 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 non-pathogenic for chickens by inoculating 10 times the field dose into 1-day-old SPF chicks of a strain susceptible to MD, to ensure that it does not cause gross lesions or significant microscopical 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 microscopical 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 chicks. Ten times the field dose of vaccine are inoculated initially and then passaged by inoculation of heparinised blood at 3-week 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 at least 12 weeks and must be free of MD lesions. 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 chicks at one day of age and challenge with sufficient virulent MDV 8 days later to cause at least a 70% incidence of MD in unvaccinated chicks. Two types of tests are used. In the protection index test, a single field dose (1,000 PFU) (plaque-forming units) of vaccine is given and the incidence of MD in vaccinates 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, as compared with unvaccinated controls.

A PD$_{50}$ (protective dose) test is also used, involving the inoculation of five 4-fold serial dilutions of vaccine virus selected to provide protection above and below the 50% level, followed by challenge eight 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 chicks. 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 various 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.

Stability tests should be conducted under the condition of storage of the vaccine to ensure proper shelf life. 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 the cell-associated and freeze-dried vaccines. The stability of reconstituted vaccine over a 2-hour period should be tested.

2. 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 minimum essential medium, or 199 medium, buffered with sodium bicarbonate and supplemented with 5% calf serum. Incubation is at 38-39°C.

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 a further 48 hours. For cell-associated vaccine, cultures are incubated for a further 48 hours, then the infected cells are harvested by treating the washed cell sheet with EDTA/trypsin solution to allow 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 (DMSO), and held at 4°C or dispensed immediately into the final vaccine containers, usually glass ampoules, which are flame sealed and
frozen in liquid nitrogen.

Cell-free lyophilised vaccine may be prepared from HVT, but not from MDV strains. For the production of this form of vaccine, HVT-infected cultures are incubated for 72 hours, infected cells are detached from the vessel as described above, or scraped from the walls of the vessel. The cells are suspended in a small volume of growth medium, centrifuged, and resuspended in a buffered stabiliser solution containing 8% sucrose, but free of protein to prevent frothing. The cell suspension is sonicated to release virus, cell debris removed, diluted with a complete stabiliser — such as sucrose, phosphate, glutamate and albumen solution (SPGA) — filled into the final containers, and lyophilised.

The dilution rate for both cell-associated and cell-free vaccines is based on previous experience and the number of doses required per container, because the virus content of the harvested material cannot be assayed prior to filling out 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 ampouling. 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**

The final product should be tested for identity, safety and potency.

a) **Identity**

By use of monospecific neutralising serum, checks should be carried out to show that the final product is of the same specificity as the seed virus.

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 free, in particular, from vertically transmitted agents. Substances of animal origin used in the preparation of vaccines, such as serum, trypsin and bovine serum albumen, must be free of 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 (5, 6, 9) and in the chapter on General Information.
Ten doses of vaccine or two doses of diluent should be inoculated into groups of ten 1-day-old SPF chicks. No adverse reactions should occur during a 21-day period.

c) Potency

The standard dose of each type of vaccine is 1,000 PFU per chick. Virus content assays are conducted on batches of vaccine to ensure that the appropriate dose of vaccine is inoculated.

REFERENCES


Mycoplasma gallisepticum (MG) infects chickens and turkeys, causing acute or chronic disease. The diagnosis of infection of chickens or turkeys with MG consists of the demonstration of the organism or the detection of specific humoral antibodies.

Identification of the agent: MG is usually isolated in mycoplasma media although, if there is any difficulty, an initial passage of the material through mycoplasma-free chick embryos or mycoplasma-free chickens may be required.

Samples for isolation consist of swabs of organs or tissues, or diluted tissue homogenates. Culture may also be attempted from aspirates from the infraorbital sinuses or joint cavities and from yolk or embryos. The sample selected will be influenced by the clinical signs observed or by any lesions present. Broth and agar are used for isolation, but it is necessary to obtain mycoplasma colonies on agar before attempting identification.

Although biochemical tests can be helpful in preliminary classification, a final identification must be by serological tests. The most satisfactory one for this purpose is the indirect fluorescent antibody test.

Sero logical tests: A number of serological tests have been used to detect MG antibodies, but specificity and sensitivity are lacking to some degree in all of them. They are more satisfactory for flock screening than for testing individual birds.

The techniques most commonly applied are the rapid serum agglutination (RSA) and the haemagglutination inhibition (HI) tests. In the RSA test, sera from individual birds are tested for agglutination using commercially-produced stained MG antigen. Chicken sera that yield agglutination reactions with the antigen within two minutes, or turkey sera within three minutes, should be heated at 56°C for 30 minutes and retested. Sera that still react, especially when diluted, are considered positive. These may then be confirmed by an HI test. Doubtful results should be checked by performing rapid serum agglutination tests with M. synoviae antigen since infection with this organism sometimes causes cross-reactions.

Requirements for biological products: Although the preferred method of control is maintenance of MG-free flocks, both live and killed vaccines have been used. Vaccination should be considered only on multi-age sites where it is inevitable that flocks will become infected. The usual use of vaccination is to prevent egg production losses in commercial layers, although it may also be
used to reduce the level of egg transmission. It is important for either live or killed vaccine to be administered before the flock is exposed to field MG.

**Live vaccines:** Available products generally are produced from the F strain of *Mycoplasma gallisepticum*, although other strains may be considered. Administration by the intranasal or intraocular route is preferred, but aerosol or drinking water administration may be used. Pullets are generally vaccinated between 12 and 16 weeks of age. A single dose is sufficient. Vaccinated birds are permanent carriers. Long-term use of the F strain on a multi-age site may result in displacement of the field strain with the vaccine strain. F strain is virulent for turkeys.

**Killed vaccines (bacterins):** Bacterins consist of a concentrated suspension of killed MG organisms in an oil emulsion. These are ordinarily administered to growing pullets at 12-16 weeks of age to prevent egg production losses. They are administered parenterally, usually subcutaneously in the neck. Although two doses are desirable, a single dose is usually given because of cost and labour considerations. Bacterins are effective in preventing egg production losses and respiratory disease, but they do not prevent infection.

### A. DIAGNOSTIC TECHNIQUES

*Mycoplasma gallisepticum* (MG) infects poultry, and is particularly important in chickens and turkeys. Under certain circumstances it may be associated with acute respiratory disease, especially in young birds. A more chronic form of the disease occurs and causes reduced egg production in breeders and layers. The infection may be spread by vertical transmission through infected eggs. The severity of the disease is greatly affected by the degree of secondary infection with certain viruses and bacteria, such as *E. coli*. MG can be isolated by using a cell-free medium, but where there is any lack of success, chick embryos or chickens are inoculated. Serological tests are also widely used for diagnosis.

#### 1. Identification of the agent

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 chicks or pouls which have broken the shell but failed to hatch. From live birds, swabs may be taken from the 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 be taken from embryonated eggs, e.g. from the inner surface of the vitelline membrane, and from the oropharynx and airsacs.

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 vigorously agitated in 1-2 ml of mycoplasma broth and then discarded. Alternatively, the swabs can be dipped in mycoplasma broth before specimens are taken and then replaced in the swab holders for transportation. Serial dilutions of specimens may be of value since the presence of specific antibodies or antibiotics or inhibitory substances in tissues, may inhibit mycoplasma growth unless they are diluted out.

A number of culture media have been formulated (6), all of which contain a protein digest and an infusion base, supplemented with serum or a serum fraction, yeast factors, glucose and bacterial inhibitors. Ideally, new batches of media should be tested with known mycoplasma cultures because of the variation in the ability of some components to support mycoplasma growth and because of the inhibitory effect of some others.

For the growth of *M. gallisepticum* the following is satisfactory:

- Part A: 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 30 minutes), 150 ml; fresh yeast extract (25% w/v), 100 ml; glucose solution (10% w/v), 10 ml; thallous acetate (5% w/v) 10 ml; penicillin G (200,000 IU/ml) 5 ml; and phenol red solution (0.1% w/v) 20 ml. The pig serum may be replaced by horse serum, but it is important to ascertain that it does not inhibit 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.

For the corresponding solid medium, 10 g of purified agar, known to be non-inhibitory to mycoplasmas, 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 2 weeks.

Fresh yeast extract is available commercially, although it may be prepared 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 3,000 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), sterilised and also stored at -20°C. Penicillin solution (10⁶ IU benzyl penicillin in 5 ml distilled water) is stored at 4°C and has a shelf life of one week. Phenol red (0.1 g) is ground in 2.8 ml 0.1 M
NaOH and 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 some workers prefer to replace it and penicillin with ampicillin.)

Specimens are inoculated onto mycoplasma agar and into broth. It may be necessary to make serial dilutions up to $10^{-3}$ for successful isolation (q.v.). Inoculated plates are incubated at 37°C in sealed containers. Increased humidity and carbon dioxide 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% carbon dioxide in nitrogen or by placing a lighted candle in the container.

The caps on liquid medium containers should be tightly sealed before incubation at 37°C to avoid spurious changes in pH. Plates are examined daily for colonies with a stereoscopic microscope for the first few days and then 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 onto solid medium immediately. Even if no colour change occurs, subculture onto solid medium should be made after 7-10 days since the presence of an arginine-hydrolysing (alkali-producing) mycoplasma species may mask the acid colour change produced by \textit{M. gallisepticum}.

The specimens required for the inoculation of chick 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 0.05-0.1 ml inoculated into the yolk sacs of 6- to 8-day-old chick embryos. Embryos should be from MG-free flocks. The eggs are candled daily and embryos which die within 24 hours of inoculation are discarded. Any further dead embryos are kept refrigerated until cultured; any embryos surviving after five days are placed at 4°C for at least four hours to kill them and reduce haemorrhages on opening. The yolk is subcultured into broth and onto agar; since yolk lipid tends to obscure colonies, it is essential to streak the yolk thinly or, preferably, to dilute it first in mycoplasma broth.

Bioassays may be performed by the inoculation of a homogenate of suspect material into at least four 8- to 16-week-old susceptible mycoplasma-free chickens. Diagnosis is confirmed by the recovery of MG from these birds and/or the demonstration of specific antibodies (7).

Samples such as nasal turbinate, infraorbital sinus, trachea, lungs, air sacs, oviduct, or joint fluid are homogenised in 5-10 volumes of broth, and immediately inoculated into at least four test birds intranasally, supra-conjunctivally, intratracheally, into the infraorbital sinus and the air sacs. The birds are kept in isolation. Two additional control birds are inoculated by the same routes with broth only and kept separately in isolation.
At 14, 21, 28 and 35 days after inoculation, the sera of all birds are tested for MG antibodies by the rapid serum agglutination (RSA) test. After the final bleed, 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 positive for MG if the organism is recovered from the birds inoculated with the homogenate, or if they develop an RSA titre of 1:4 or more. Titres less than this are interpreted as being doubtful. An HI test should be performed. The organism should not be recovered from the control birds, nor should they develop any RSA titres. There should be no RSA reaction to *M. synoviae* in either group of birds.

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 passage on mycoplasma media.

Biochemical tests (e.g. fermentation of glucose, failure to hydrolyse arginine or to produce film and spots, partial or complete lack of phosphatase activity) assist identification but are not specific. Serological identification is therefore essential, and a number of tests have been used. These include: the indirect fluorescent antibody (IFA) test, which is simple, sensitive, specific and rapid to perform; growth inhibition (GI); and metabolism inhibition (MI). Purified cultures are required for the GI and MI tests but not for the IFA test. The latter will detect whether more than one species of mycoplasma is present, since the colonies specific for the antiserum will fluoresce while the others will not.

**a) Indirect fluorescent antibody test**

The recommended technique for the IFA test (9) requires an agar culture of the unknown isolate, consisting of numerous small discrete colonies; a known *M. gallisepticum* culture as a positive control; and a culture of a further mycoplasma species, such as *M. synoviae* or *M. gallinarum*, as a negative control. Rabbit anti-MG serum, a normal rabbit serum and an anti-rabbit immunoglobulin fluorochrome-conjugated serum are also required. Sera may be prepared in species other than rabbits. Suitable working dilutions in sterile phosphate buffered saline (PBS) of the anti-MG serum and the conjugate are first determined by cross-titration and are selected for use at 2- to 4-fold dilutions less than the actual endpoints. These are applied to the colonies of mycoplasmas to be identified that have been previously grown on agar plates as indicated below.

Blocks of about 1.0 x 0.5 cm are cut from the plates and placed, with the colonies uppermost, onto microscope slides. To make subsequent orientation possible the lower right hand corner of the block is cut off. On one slide are placed a block with the unknown isolate, a block with the known MG culture, and a block with a different but known mycoplasma culture. On another slide is put a block of the unknown isolate. A drop of suitably diluted MG antiserum is added to each block of the first slide and the normal rabbit serum is added to
the single block on the second slide. All blocks are incubated for 30 minutes at room temperature in a humid atmosphere. Each block is placed in a labelled tube containing PBS pH 7.2, washed for 10 minutes in a rotary mixer, then similarly rewashed, and finally returned to the original microscope slides. Excess moisture is blotted from the sides of the blocks. One drop of the diluted conjugate is placed on each block, incubated and washed as before, the blocks finally returned to their original slides, and the colonies examined 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.

b) Growth inhibition test

In the GI test (4), growth of mycoplasma is inhibited by specific antiserum, enabling species to be identified. It is relatively insensitive. Sera must be high-titred, monospecific and prepared in mammalian hosts; poultry sera do not inhibit mycoplasma efficiently. The organism under test must be in pure culture (cloned) and several dilutions should be tested: a concentration of $10^4$ colony-forming units (CFU) 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 given in ref. 4.

2. Serological tests

The serological tests in common use lack specificity and/or sensitivity; they may be used for monitoring flocks rather than for testing individual birds.

There are a number of serological tests other than those described below; tests being developed include ELISA (3), radio-immunoassay, micro-immunofluorescence and indirect immunoperoxidase assay.

a) Rapid serum agglutination test

Sera are collected from a sample of a flock and, if not tested immediately, stored at 4°C and not frozen. The test is carried out at room temperature. One volume (about 0.02 ml) of serum is dropped onto a white tile and then one volume of stained antigen. The tile is rotated to ensure mixing. Agglutination is indicated by clumping: in two minutes for chicken serum and in three minutes for turkey serum. Known positive and negative controls should be incorporated in the test. The antigens are available commercially but may vary in specificity and sensitivity from different manufacturers and from batch to batch. Any sera that agglutinate should be heated at 56°C for 30 minutes and retested. If they still react strongly, they are considered positive, especially if they do so on dilution. A high proportion of positive sera in a flock indicates MG infection, especially if confirmed by HI. To confirm this, sera should be retested within a month. Inconclusive results make it necessary to attempt to
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isolate the organism. Doubtful results should be checked by performing tests with *M. synoviae* antigen since infection with this organism sometimes causes cross-reactions.

Tests can be conducted on yolk as well as sera. The yolk is extracted or diluted.

### b) Haemagglutination inhibition test

MG is capable of haemagglutinating avian red cells, and sera can cause specific inhibition. The HI test requires a satisfactory haemagglutinating MG antigen, washed fresh chicken or turkey red cells, and the 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; at the same time, the concentrated suspension of antigen increases the likelihood of non-specific reactions, unless the sera are pre-treated with receptor-destroying enzyme (RDE).

For RDE treatment, one volume of serum is mixed with 4 volumes of RDE; this includes the control sera. The mixtures are incubated overnight at 37°C and then at 56°C for one hour to destroy the RDE.

The HI test follows well known procedures (1). The haemagglutination (HA) titre of the antigen is first determined in doubling dilutions, the HA unit being defined as the least amount of antigen giving complete haemagglutination in the test system employed. The HI test should be performed 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 multi-well plastic plates with V-shaped wells and using constant volumes of 50 µl. A positive and negative control serum is incorporated in each test. One row of eight wells is required for each serum to be tested. To the first well in each row 50 µl of PBS is added. To well number 2 in each row 8 HA units of antigen in 50 µl volumes is added and to each of wells 3 to 8, 50 µl of 4 HA units of antigen. A 1:5 dilution of serum under test, already prepared, is added in a volume of 50 µl to the first well, mixed and 50 µl transferred to the second well and so on, with 50 µl being discarded from the last well. The first well is the serum control well.

For the antigen control, six wells are required. To wells 2 to 6 inclusive add 50 µl of PBS and to wells 1 and 2, 50 µl of the 8 HA unit antigen. Mix the contents of well 2 and transfer 50 µl to well 3, mix and repeat to well 6 and discard 50 µl. For erythrocyte control two wells are required, into each of which 50 µl of PBS is added. To all wells is added 50 µl of a 0.5% suspension of red blood cells (chicken cells for chicken serum and turkey for turkey serum).

The plate is lightly shaken to ensure thorough mixing of the well contents and read after about 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 red cells 'stream' at the same rate as those from wells with red cells and diluent only, should be considered inhibited. The serum control should show a clear button of red cells and the other controls should react as expected. The HI titre is the highest serum dilution exhibiting complete inhibition of haemagglutination.

Sera giving non-specific haemagglutination must be adsorbed to remove all non-specific 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 to 8 drops of packed washed chicken red blood cells. The cells are removed after incubation at 37°C for 10 minutes, and the supernatant tested for haemagglutinating activity.

Some strains of MG induce only a weak HI response in some birds, and in some no response. Such strains are also difficult to isolate by the usual methods. In these cases, the in vivo bioassay approach should be used.

Note on the preparation of *Mycoplasma gallisepticum* and *synoviae* antigens

i) *Mycoplasma gallisepticum* antigens

Antigens are usually prepared from S6 strains (11). Antigens prepared from other strains may be used in addition when necessary.

*M. gallisepticum* antigen for the RSA test

The methods of quality control described below apply solely to suspensions of *M. gallisepticum* stained with a suitable dye and containing preservative and intended for use in the rapid plate agglutination test with blood or serum. Such antigens are available commercially.

On microscopic examination, the antigen should appear as a homogeneous suspension without floccules or precipitates. 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. It must 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.

*M. gallisepticum* 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.
ii) *Mycoplasma synoviae* antigens

Antigens prepared from the WVU 1853 (8) strain or other suitable strains should be used.

*M. synoviae* antigen for the RSA test

The specifications under 'M. gallisepticum antigen for the RSA test' apply.

*M. synoviae* antigen for the HI test

The same specifications apply as for *M. gallisepticum* HI antigen.

iii) Additional comments

Sera giving non-specific 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 an HI test with sera taken after the first two or three 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.

Samples of serum should not be frozen and should be free from haemolysis and contamination to avoid non-specific reactions. Samples should be tested as soon as possible because mycoplasma antibodies deteriorate on storage. Sera may be inactivated at 56°C for 30 minutes.

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B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

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. Although there is antigenic variability among MG strains, it is thought that vaccination with a single strain is sufficient.

**Live vaccines: method 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, air sacculitis, and egg production drops caused by MG. Vaccination also results in reduced levels of egg transmission in breeders.
The F strain of MG is the most commonly used strain (2). 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 air sacculitis may result. Vaccinated chickens are permanent carriers, so it is felt that 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.

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 occur before the flock is naturally infected. Vaccination in cases of probable early field exposure can be carried out in birds as young as two to four weeks of age. Intranasal or intraocular 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 five to seven days after vaccination if aerosol administration is used. Vaccinated flocks should be tested with the agglutination test approximately three to four 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.

Commercial live vaccines should be used within one to two 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.

Other vaccine strains may also be considered. These are generally avirulent mutants or naturally occurring avirulent strains. Some of these strains are avirulent for turkeys and may be incapable of spreading from bird to bird. Such strains are inherently safer than F strain 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.

Inactivated vaccines: method of use

MG bacterins are prepared from a concentrated suspension of whole cells which 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 which occur after MG exposure on multi-age layer sites (5). They may also be used to reduce the level of egg transmission in breeder pullets (10). 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, air sacculitis, 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 of 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 usually dictate the use of a single dose, usually between 16 to 18 weeks of age for commercial pullets. A multi-dose 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**

   **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 level of immunity.

   The seed culture should be free of 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 10 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 of all extraneous organisms.

   b) **Culture**

   The seed culture may be propagated in a medium similar to that described...
above. 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 in the way the vaccine 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 mycoplasma-free chickens two weeks of age or older are vaccinated by eye drop 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. Two to three weeks post-vaccination all chickens should be challenged with a 24 hour broth culture of a virulent strain of MG. 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 to 10 days post-challenge and airsac lesions scored. An alternative method is to challenge with 0.1 ml into the infraorbital sinus and examine the birds for nasal discharge from 7 to 14 days post-challenge.

2. **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 to prevent contamination of any mycoplasmal organisms that may be present, and filter sterilised. A source of SPF serum is desirable.

Broth medium is inoculated with a rapidly growing inoculum at a rate of approximately 5% (v/v). Incubation is at 37°C. Production can be in batches using large flasks or in a fermenter. In batch cultures, harvest is approximately 24 hours after inoculation. Live vaccines are preserved by lyophilisation or by freezing at -70°C, in liquid nitrogen, or on dry ice.

For bacterin production, the antigen must be concentrated, usually by centrifugation, ultrafiltration, or other suitable method. Bacterins are made as water-in-oil emulsions, typically 80% mineral oil, 20% aqueous, with suitable emulsifying agents.
3. **In-process control**

**Antigen content:** At harvest the titre should be $10^8$ to $10^9$ colony-forming units per ml. 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 β-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 one 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 the chapter on General Information.

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 untoward local or systemic effects.

c) **Potency**

Potency tests for both live and killed vaccine can be conducted by the procedures given above for the efficacy test. The titre of live vaccines should be sufficient to induce infection by the route recommended, $10^5$ CFU/ideal is sufficient for intraocular administration of live F strain vaccine.
d) Duration of immunity (killed vaccine)

Because flocks are generally exposed within one to two months after vaccination, duration of immunity is not a primary consideration. After field challenge resistance is considered permanent.

e) Stability

Evidence should be provided on three batches of vaccine to show that the vaccine passes the batch potency test at three months beyond the requested shelf life.

f) Preservatives

A preservative is normally required for vaccine in multi-dose containers. The concentration of the preservative in the final vaccine and its persistence throughout the shelf life should be checked. A suitable preservative already established for such purposes should be used. Mycoplasmas are susceptible to many antibacterials except for penicillins; such antibiotics should not be included as preservatives.

g) Special precautions

Oil emulsion vaccines cause serious injury to the vaccinator if accidentally injected into the hand or other tissues. In the event of such an accident the person should go at once to a hospital, taking the vaccine package with him. Each vaccine bottle and package should be clearly marked with a warning of the serious consequences of accidental self-injection. Such wounds should be treated by the casualty doctor as a "grease gun injury".

REFERENCES


AVIAN CHLAMYDIOSIS
(B66)

SUMMARY

Avian chlamydiosis (AC) is caused by the organism Chlamydia psittaci. The disease in man and psittacine birds was originally called psittacosis, but later the term ornithosis was introduced to differentiate the disease in domestic and wildfowl from the disease in psittacine birds. These two diseases are similar, but chlamydiosis as it occurs naturally in mammalian species is caused by a distinctly different strain of the organism. It must be noted that avian chlamydial strains can cause serious illness and possibly death in man.

The laboratory diagnosis of AC requires the isolation and identification of the organism, the demonstration of chlamydiae in tissues, or the demonstration of a 4-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 laboratory animals or tissue cultures, and the testing for chlamydiae by cytochemical stains or immuno-histochemical methods. The isolation of avian chlamydiae has been done by the yolk sac inoculation of 6- to 7-day-old developing chick embryos; the yolk sac membranes from dead embryos are examined for chlamydiae. This is often done either on impression smears and staining with cytochemical stains, or by repassage in tissue culture and subsequent testing for chlamydial inclusions by immunofluorescence or immunoperoxidase techniques. Samples must be blind passaged three times in eggs before being considered negative.

The direct inoculation of samples into tissue cultures of VERO, McCoy, or L cells is preferable. Cell cultures are as sensitive for the isolation of chlamydiae as chicken embryos. To enhance the infectivity of samples, a preferred method is the centrifugation of the inoculum onto monolayers and the addition of inhibitors of cell division, such as cycloheximide. Cultures can be stained to demonstrate inclusions by immunofluorescence or by other appropriate stains, and other cultures used for blind passage. One to two blind passages are usually done before considering the sample negative.

The use of monoclonal antibodies in immunofluorescence or immunoperoxidase techniques provides increased specificity in identifying the organism, and is rapidly becoming the method of choice for demonstrating chlamydiae. Monoclonal antibodies against the group antigen are now commercially available and strain-specific monoclonal antibodies can identify the strains of chlamydiae present.

Serological tests: The standard serological test for chlamydial antibodies is complement fixation (CF). The antigen is a group reactive lipopolysaccharide
antigen present in all strains. The CF test is often used to examine a flock for chlamydial infection. The occurrence of high CF titres in the majority of a flock with clinical signs is presumptive evidence of active infection. The demonstration of a 4-fold increase in titre in an individual bird is considered diagnostic of current infection.

Other serological tests, such as the enzyme-linked immunosorbent assay (ELISA), latex agglutination (LA), micro-immunofluorescence (micro-IF), and the agar gel immunodiffusion (AGID) tests, are available. These tests, especially ELISA, will replace the CF test but comparisons of reliability and reproducibility are not yet available.

Responsibilities for biological products: There are no commercial vaccines available for chlamydiosis control in poultry. Antibiotics are the only current means of control. C. psittaci is susceptible to chloramphenicol, tetracycline, and, to a lesser degree, penicillin. Tetracycline is the only antibiotic which is economical to use in animals.

A. DIAGNOSTIC TECHNIQUES

Avian chlamydiosis (AC) is caused by the organism Chlamydia psittaci. The disease in psittacine birds was originally called psittacosis, but the term ornithosis was introduced later to differentiate it from that in domestic and wild fowl. The two syndromes are currently considered to be the same (23). Their earlier separation was based on the assumption that in man ornithosis was a milder disease than psittacosis. However, it should be noted that the disease in man contracted from turkeys is often as severe as that from psittacine birds.

C. 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 bird and the strain of chlamydia. Avian chlamydiosis can produce lethargy, hyperthermia, abnormal excretions, nasal and eye discharges, and reduced egg production. Mortality rates will range up to 30%. In pet birds the most frequent clinical signs are anorexia and weight loss, diarrhoea, yellowish droppings, sinusitis and respiratory distress (18). Many birds, especially older psittacine birds, may show no clinical signs; however, they will often shed the agent for extended periods. Necropsy of infected birds will often reveal spleen and liver enlargement, fibrinous air sacculitis, pericarditis and peritonitis (23).

1. Identification of the agent

The laboratory diagnosis of avian chlamydiosis is usually made by the isolation and identification of the organism. As this requires living cells in which to multiply, it is necessary to inoculate laboratory animals or cell cultures.

Collection and treatment of samples

The samples 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 the kidney, lung, spleen, and liver. In cases with diarrhoea, colon contents or excrement should be cultured. In live birds, the following samples can be taken: whole blood, intestinal excrement, tracheal swabs, conjunctival scrapings, and peritoneal exudate.

In a recent study on the pathogenesis of the disease in turkeys, we found that the lateral nasal glands became infected early and remained infected for at least 50 days. This prompted us to compare isolation successes from oral swabs, faecal samples, and cloacal swabs from a group of 134 cockatiels. Of these birds, 36.7% were positive by one or more methods and of those that were positive, 83.7% were detected by oral swabs, 46.9% by faecal samples and 38.8% by cloacal swabs. Similar results have been obtained from turkeys. This demonstrates the importance of oral swabs for isolation of the organism, and also the importance of the upper respiratory tract and aerosol potential in the transmission of the disease.

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 (5) and has proven satisfactory for transport of chlamydial field samples. The medium as recommended for chlamydiae (29) 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. Added to this are fetal calf serum (10%), vancomycin and streptomycin (100 µg/ml), and 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 pre-treated before being used to inoculate animals or cell cultures. There are three basic methods: treatment with antibiotics (3), treatment with antibiotics together with low speed centrifugation (23), and treatment with antibiotics with filtration (3, 8). A number of antibiotics that do not inhibit chlamydia can be used. Samples are homogenised in phosphate buffered saline (PBS) (pH 7.2) containing streptomycin (1 mg/ml), vancomycin (1 mg/ml) and kanamycin (1 mg/ml). Gentamicin (200 µg/ml) can be used. Amphotericin B can be added to control yeast and fungal growth. Other antibiotic solutions are often used. Penicillin, tetracycline and chloromycetin should be avoided, as these inhibit the growth of chlamydia.

When contamination is light, samples should be homogenised in an antibiotic solution prior to inoculation into chick embryos, guinea pigs, mice or tissue cultures. Samples are often left to stand in the antibiotic solution for 24 hours before inoculation. Heavily contaminated samples, such as faecal samples, should be homogenised in antibiotics and then centrifuged at 1,000-2,000 g for 30 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.

**Isolation from cell culture**

Cell cultures are the most convenient method for the isolation of *C. psittaci*. Cell lines are satisfactory, the more common ones being McCoy, HeLa, VERO and L cells. The cells are grown as monolayers, using standard tissue culture media containing 5-10% foetal calf serum and antibiotics.

When selecting tissue culture equipment it is important to remember that:

(i) chlamydiae can be identified by direct immunofluorescence or some other appropriate staining technique;
(ii) the inoculum is usually centrifuged onto the monolayer to enhance its infectivity;
(iii) the sample may need to be blind passaged at 6-7 days to increase sensitivity of isolation;
(iv) the sample will need to be examined 2-3 times during any one passage; and
(v) chlamydia can be infectious to man.

Small flat-bottomed vials, such as 1 dram (3.7 ml, 15 x 45 mm) shell vials or bottles containing 12 mm diameter coverslips, will meet these requirements (3, 4, 8). A number of vials, often 4-6, are inoculated with each sample to permit fixing and staining at various intervals, and to permit repassaging of apparently negative samples six days after inoculation (see Figure 1).

Chlamydiae can be isolated from cells which are replicating normally, but the use of non-replicating cells is preferable since 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-deoxyiodine, cyto-cholasin B, cycloheximide, and emetine hydrochloride (24). Cycloheximide 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 (3, 24). This can be an advantage. The monolayer is first treated for 5 minutes with 0.5 μg/ml of emetine, after which the emetine is removed and replaced with culture medium, when the monolayer is then ready for use. The effect of these drugs on replication of chlamydiae appears to be strain dependent. The growth of most strains will be enhanced by the treatment of the monolayer by one of these drugs; however, the treatment will have no effect on the growth of other strains.

There are various methods to enhance the infection rate of chlamydiae for cell cultures. Attachment of chlamydia to cells is increased by centrifuging the inoculum onto the monolayer at 1,000-3,000 g for 30-90 minutes at 37°C. The inoculum is removed and replaced with tissue culture medium containing an inhibitor of cell division, 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 the second or third days, as well as on the fifth or sixth days (see Figure 1). Cultures that appear to be negative at the sixth day are harvested and passaged.
Before staining the cultures, the medium is first removed, the cultures washed with PBS, and fixed with acetone for 2-10 minutes. The fixation time will depend on the tissue culture vessel used. Since 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 (3, 19). A chlamydial fluorescein-conjugated antiserum is applied to the infected cells and incubated in a moist chamber for 30 minutes at 37°C. The coverslips are then washed three times with PBS, air-dried, mounted and examined. Chlamydial inclusions fluoresce a light green color. Commercial conjugate preparations using monoclonal antibodies are available and are highly specific. Conjugates may also be prepared from polyclonal sera, but it is important to obtain specific, high-titred antisera. Polyclonal antisera can be prepared in rabbits, guinea pigs, sheep or goats. Sheep and goats are excellent sources because of the volume and high titres that are readily obtained following infection. Conjugates are then prepared using standard techniques (3, 23).
Chlamydial inclusions can also be demonstrated by indirect fluorescent antibody and immunoperoxidase techniques (19). Direct staining can be done with Gimenez, Giemsa, or Macchiavello's stains. Except for immunofluorescence, all these stains have the advantage that standard light microscopes can be used.

**Differentiating between strains/species**

The *C. psittaci* group contains a number of specific strains (26). The syndromes caused by the various strains are quite specific; the natural host range of a particular strain may also be fairly specific (1). The use of monoclonal antisera for identifying chlamydial inclusions could be advantageous in typing the isolates (2, 30). A monoclonal antiserum that is specific for ovine abortion will not react with the arthritis strains (11). Monoclonal antisera have been prepared that are highly specific for the virulent turkey strains, a parrot strain, and an ovine abortion strain (2). The ability to type *C. psittaci* strains by immunofluorescence awaits only the characterisation of different strains and the production of the requisite specific monoclonal antisera.

Chick embryos are still employed 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 6- to 7-day-old embryo (23). The eggs are then incubated 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 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, and can be frozen to preserve the strain, or inoculated into further eggs or onto tissue cultures.

The organism can be identified by preparing an antigen from an infected yolk sac. This antigen can detect the chlamydial group antibody by complement fixation (CF), or by some other appropriate serological test. Monolayer tissue cultures 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. This is often seen with the virulent turkey isolates.

Tests for the group antigen will merely identify the agent as being chlamydiae, and will not differentiate between *C. trachomatis* and *C. psittaci*. At present the species are differentiated by biochemical tests (23). These tests will either detect the presence of glycogen or sensitivity to sodium sulfadiazine. Carbohydrate or glycogen is demonstrated in fixed smears of infected cells by staining for several hours with 5% potassium iodide-iodine. *C. trachomatis* forms carbohydrate-containing microcolonies which stain dark brown against a light tan background.

The multiplication of *C. trachomatis* in chicken embryos is inhibited by inoculating 1.0 mg sodium sulfadiazine into the yolk sac of each embryo immediately prior to
the inoculation of the organism. Sodium sulfadiazine will lower the titre of *C. trachomatis* by at least $10^2$ compared to a parallel control titration. Sodium sulfadiazine will not affect *C. psittaci*.

When available, monoclonal antisera against the various strains of *C. psittaci* could be used not only to type strains but to distinguish *C. psittaci* from *C. trachomatis*. A number of laboratories now produce such antisera.

Microcolonies of *C. psittaci* are readily identified in fresh exudate with phase contrast optics (23) at a magnification of x 800 or more. However, mycoplasmas may produce similar spherical bodies. The Gimenez procedure stains single or aggregated elementary bodies bright red, but bacteria are also readily stained.

Histological examinations may be used to identify chlamydiae-infected cells. Tissue sections embedded in plastic give the best results; specimens should be sectioned at a thickness of 4 μm or less.

Direct immunofluorescence and immunoperoxidase techniques provide greater specificity than the cytological and histological procedures (13, 19). Until recently, fluorescein conjugates were not available commercially and few laboratories prepared their own. With the availability of conjugated monoclonal antisera these techniques may provide rapid detection of chlamydiae in tissue scrapings, exudates and other clinical and histological specimens.

2. **Serological tests**

   a) **Complement fixation**

The complement fixation (CF) test detects antibodies to the chlamydial group antigen. The test varies greatly, and as sera from many avian species are anti-complementary, modified methods have to be employed. An indirect CF test has been described for the detection of fowl anti-chlamydial antibodies in the presence of precise quantities of mammalian anti-chlamydial antibody (27). It has also been shown that a direct CF test could be used for testing avian sera if normal rooster serum is added to assist the fixation of guinea pig complement (10). Standard heat inactivation of sera results in failure of avian antibody-antigen complexes to activate guinea pig C1 complement (9). The addition of normal fowl serum is still used in tests on avian sera.

A direct micro-CF test can yield satisfactory results (6, 7, 14). This employs antigens that are easily prepared in tissue culture (q.v.) and is rapidly becoming adopted as the CF test of choice for numbers of samples.

A number of procedures are used to prepare CF antigen. They include the growth of chlamydiae in tissue culture, inactivation, partial purification of the antigen, mechanical disruption, and dilution in an appropriate buffer. Both chlamydiae and cell debris are harvested when cytopathic effects are noted in infected cultures (14, 16). The harvest is inactivated by the addition of phenol to a final concentration of
1.0% and incubated for 24 hours at 37°C. The chlamydiae are concentrated by centrifugation at 10,000 g for one hour. The deposit is reconstituted to 10% of the original volume, using veronal-buffered saline (pH 7.2) containing 1.0% phenol and 1.0% glycerol. This is homogenised for 3 x 1 minute periods at 4°C. The homogenate is centrifuged for 5 minutes at 100 g to remove debris. The supernatant fluid is diluted to an appropriate concentration.

In a second method (6), CF antigen is prepared from L cells infected with a parrot strain. The culture medium is discarded and the cells 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 CF test diluent. The antigen is tested against a standard sheep convalescent serum and used at 2 units in the direct micro-CF test.

CF antigen may also be prepared from infected yolk sacs (23). The procedure is more elaborate than for the production of antigen in tissue culture. This antigen is very efficient in tests on mammalian and pigeon sera; for other avian sera it is used in either the indirect CF test or the modified direct CF test. On account of the problems in tests on most avian sera, this method is introduced as a potential method for production of antigen when a laboratory is not equipped to perform the other procedures.

Complement-fixing antibodies usually appear within 7-10 days of infection. For a positive diagnosis, a 4-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 or more.

b) Other tests

Other serological tests have been developed, but their specificity has not yet been sufficiently evaluated. The enzyme-linked immunosorbent assay (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* (12, 17, 25, 28) indicate that it can substitute for the CF test in most cases. However, it has yet to be tested extensively, and standards for its use have not been established.

Other tests include the agar gel immunodiffusion test (20) and a latex agglutination (LA) test. Immunodiffusion is less sensitive than the CF test. The LA test will detect antibodies to *C. psittaci*, being easy and rapid to perform (15). 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 non-specific 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 (15). This degree of specificity suggests that it could detect active or recent infections.
B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

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 (21, 22), whereas vaccine manufacture has not been directed towards reactions of this type.

REFERENCES


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 in a portion of them by immunodiffusion tests. 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.

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 complement fixation or by virus neutralisation tests. 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.

Qualitative agar gel immunodiffusion tests have the advantage of detecting humoral antigen and antibodies simultaneously.

Requirements for biological products: Modified live virus vaccines prepared from fibroma virus or modified myxoma virus strains are available for immunisation of rabbits.

A. DIAGNOSTIC TECHNIQUES

Myxomatosis is a virus disease of the European rabbit 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 non-myxomatous 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 through inanition. The virus is transmitted to susceptible rabbits by biting insects, such as fleas, mosquitoes, etc. 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 as a sign of generalisation, affection of the anogenital region, and the formation of secondary skin lesions in various other sites. In the oculo-respiratory form, the primary lesion is usually an inflammatory pink macule followed by oculonasal catarrh. Virulent strains can kill a rabbit within 10-15 days.

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 with 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) and homogenised with ground glass at a dilution rate of 1 g tissue/4.5-9.0 ml PBS. Cells are disrupted by two cycles of freezing and thawing, or by ultrasonication to liberate virions and viral antigens. This suspension is centrifuged for 5-10 minutes at 1,500 g. The supernatant fluid is used for the tests.

Immunodiffusion tests in agar gel are simple and rapid (24 hours) to perform. Agar plates are prepared with 0.6 g Difco Noble agar, 2.5 g ethylenediamine tetra-acetic acid (EDTA), 4.5 g NaCl, and 500 ml distilled water containing thiomersal (merthiolate) at 1:100,000 dilution. In opposing wells of 6 mm diameter, 5 mm apart, are placed reference antiserum (q.v.), and the test sample. 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. Several 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.

Isolation of the virus in cell culture is accomplished using primary cultures of rabbit kidney (RK) cells, or with established cell lines such as RK13 and RL in minimum essential medium (MEM) containing 5% calf serum, penicillin (100 IU/ml), streptomycin (100 µg/ml), gentamycin (100 µg/ml), nystatin (Mycostatin, 50 IU/ml) and amphotericin (Fungizone, 5 µg/ml). The inoculum consists of the supernatant fluid from a homogenised lesion in MEM medium with antibiotics. This is removed from the cell layer after two hours. The cell layer is washed with a few ml of medium and then replenished with maintenance medium.

A cytopathic effect (CPE) (6) typical of poxviruses develops after 24-48 hours. Groups of cells with a confluent cytoplasm form syncytia which vary in size from 2-50 or even 100 nuclei together, according to the strain of virus. The nuclei of some cells change, the chromatin forming basophil aggregations varying in size and number, and giving the culture a leopard-skin appearance. Eosinophilic intracytoplasmic inclusions remain discrete, if present at all. Affected cells round up and contract and become pyknotic. They then lyse and become detached from
Shope's fibroma virus at first produces well-defined voluminous masses of rounded cells, which proliferate and pile up. 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.

Immunofluorescence can be applied to cultures from 24 hours onwards. It reveals intracytoplasmic multiplication of virus, without being able to distinguish myxoma virus from fibroma virus.

The inoculation of chick embryo cells (trypsinised at day 11 of incubation) does not result in CPE, but it is useful for detecting the viral antigens by immunofluorescence.

Rabbit 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 has the advantage of being a gauge of virulence, from the type of inflammation in lesions, generalisation and survival time, and can distinguish fibroma virus (with its simple fibromatous local lesion) from myxoma virus (capable of infecting adults). Rabbits should be of a domestic breed, weighing about 2 kg, unvaccinated and previously tested for absence of antibodies.

The inoculum may be the supernatant fluid from a homogenised lesion (plus antibiotics) or the product of a cell culture. Between 0.1-0.2 ml is given intradermally behind the ear or into the dorso-lumbar region previously depilated. The inoculum may be assayed by injecting serial dilutions in saline buffer solution at one site for each dilution. A primary lesion will appear at the sites within 2-5 days, followed by conjunctivitis. If the animal survives, the disease can be confirmed serologically after 15 days.

### 2. Serological tests

Antibodies develop within 8-13 days. In the non-lethal forms and in vaccinated rabbits, the titre which is an indicator of virulence is highest after 20-60 days; it declines thereafter, disappearing after 6-8 months in the absence of reinfection.

Various serological tests require reference antigens and antisera. The antigen can be prepared from the Lausanne strain, or some antigenically related strain, propagated in rabbits or cell cultures. 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 following centrifugation. Any anticomplementary activity is abolished by adding 0.6% chloroform. This is titrated against a reference antiserum, and frozen at -30°C or -70°C for stock purposes. This can be used directly in complement fixation (CF) tests.

Antigen is made from cell cultures using the RL or RK13 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 or ultrasonicated, resuspended and recentrifuged, and the supernatant is added to the former fluid. The final supernatant fluid is the antigen which is stored at -30°C or -70°C. It is titrated in cell cultures before use in virus neutralisation (VN) tests.

For the reference 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 it is inoculated with myxoma lesion material derived from the Lausanne strain of virus. Serum is obtained three weeks later and titrated by CF test. If the titre is 1:640 or more the animal is bled out and the serum stored at -70°C.

CF tests (7) can be done in tubes or on microtitre plates by conventional methods, recording 100% or 50% haemolysis. This is the reference method. VN tests (7) are done using the constant virus-varying serum method with 100 TCID₅₀ virus. These may be conducted in tubes or on microtitre plates.

Immunofluorescence (4) is done with chick embryo cell cultures in flat-bottomed wells of microtitre plates. A cell suspension 1:1,000 in medium is distributed and a confluent cell sheet is formed within 24 hours. The medium is discarded and 100 μl viral suspension containing 10⁴ TCID₅₀ is put into each well. After two hours, 100 μl 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. They are then dried at 37°C for 15 minutes. The plates can be stored at -30°C or -70°C for three months. Sera are tested by indirect immunofluorescence using rabbit anti-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.

For epidemiological surveys, tests can also be done using sera dried onto filter paper discs (paper punch size) and putting two discs per well, to which are added 100 μl PBS to extract the serum. The dilution is about 1:30 and this can be used in another well for testing.

Agar gel immunodiffusion (9) is qualitative and can detect antigen or antibody. Agar is prepared as described previously, 6 mL/10 cm petri plate. Strips of filter paper containing the reference antigen and antiserum, and discs containing test sera are arranged on the surface of the agar. The plates are incubated in a moist atmosphere at 37°C and read after 24-48 hours. There are three lines of precipitation. If the test sera contain antibody, at least one of the three lines is distorted towards the antigen band; otherwise it remains straight. If it contains antigen, at least one of the lines is distorted towards the reference serum strip.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

Two types of live vaccine are used for vaccination against myxomatosis: a heterologous vaccine prepared from Shope's fibroma virus (3, 5), and a homologous vaccine prepared from an attenuated strain of myxoma virus (1, 2, 3, 8, 10, 11). They are given subcutaneously, intradermally, or intramuscularly.
1. Seed management

a) Characteristics

The viruses employed are fibroma virus or myxoma virus.

The strains of fibroma virus are usually the original OA, Boerlage's strain or various closely related strains.

The strains of myxoma virus are modified by passaging in embryonated chicken eggs, rabbit kidney cells at decreasing temperatures, or chick embryo cells. They usually result from several clonings.

b) Culture

Fibroma strains are maintained by passage in unvaccinated rabbits from a stock known to be free from myxomatosis. The skin is shaved on the backs of healthy adult rabbits, 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, the tumours 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.

Myxoma virus can be maintained in the form of infected cells in suitable conditions. Because of the risk of latent viruses in cell line, it is best to use chick embryo cells as these are susceptible to all myxoma virus strains. Modified virus obtained after 2-3 passages in cells obtained by trypsinisation from 11-day-old SPF chick embryos is lyophilised and stored at -30°C or -70°C.

c) Validation as a vaccine

Fibroma virus

Fibroma virus strains are characterised by titration of virulence to define the ID₅₀ (infective dose): this is done by inoculating rabbits with serial dilutions of supernatant fluids obtained by centrifugation of tumour preparations. Specific and paraspecific antigenic characteristics are verified by immunodiffusion using sera against fibroma and myxomatosis.

Macroscopic and histopathological features and the course of development of fibromas are tested in rabbits periodically. Numerous serial passages in rabbits may induce mutation to the inflammatory IA strain which produces severe lesions that are more inflammatory than neoplastic.

To test duration of immunity, several batches of 10 susceptible rabbits are vaccinated. One batch is tested by challenge infection at 1, 2, 3 (etc.) months later. The duration of immunity is deduced from the time during which at least 7 of the 10 prove to be resistant to infection.
For stability tests, samples of batches of vaccine are stored at 3 to 8°C in the dark. The activity of each batch is tested in rabbits at intervals of three months. Comparison of the titres obtained provides information on the stability of the product and the optimum storage life.

Myxoma virus

The identity of myxoma virus is confirmed by neutralisation tests in RK13 cells using a monospecific antiserum. The titrations are done in microtitre plates using varying virus-constant serum: the results are expressed as TCID$_{50}$. Residual pathogenicity is tested by intradermal inoculation of 10$^3$ TCID$_{50}$ into three unvaccinated rabbits free of myxomatosis. They should not develop more than a local reaction with perhaps small secondary lesions on the head which disappear within a few days.

Duration of immunity to myxoma vaccine is determined by vaccinating several batches of 10 susceptible rabbits. Challenge infection is performed after 1, 3, 6, 9 months. The duration of immunity is deduced from the period during which at least 7 of every 10 rabbits survive challenge infection.

Samples of myxoma virus vaccine batches are stored in the dark at 3 to 8°C. The activity of a portion of each sample is tested at 3-monthly intervals by titration in cell culture. The evolution of the infective titre (TCID$_{50}$) provides evidence of the stability of the product and the storage life, which is the time during which the virus titre of a dose of vaccine remains above 10$^2$ TCID$_{50}$.

2. Manufacture

Fibroma virus is produced by multiple intradermal inoculations of seed virus into the skin of the back of a number of rabbits. 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 chick embryo cells, limiting the passage number to 5 maximum. 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.

3. In-process control

The fibroma virus titre is tested by calculating the ID$_{50}$ after intradermal
inoculation into several sites (e.g. five) of up to six rabbits of serial dilutions of the clarified supernatant fluid. A dilution of a standard preparation of fibroma virus is inoculated into each rabbit to confirm their correct response to inoculation. The preparation should provide at least $5 \times 10^2 \text{ID}_{50}$ in each dose of vaccine packed.

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 $37^\circ C$. The mixture is filtered through a 0.22 μm millipore filter and 1 ml portions inoculated into 5 x 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 cycles of freezing and thawing, 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 chick red cells.

The identity of myxoma virus is checked in rabbit kidney cells. All chick embryos should be derived from eggs from specific pathogen free flocks.

4. **Batch control**

a) **Sterility**

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

b) **Safety**

After rehydration, 10 doses of the lyophilised fibroma vaccine are injected into each of three susceptible rabbits which are observed for 21 days. Local reactions should be slight, without generalisation and with no effect on general health.

Myxoma vaccine is tested similarly, and rabbits are observed for 21 days. The primary myxoma lesion should remain mild.

c) **Potency**

Ten adult rabbits are inoculated with a dose of fibroma vaccine, while two more serve as unvaccinated controls. After 14 days, all rabbits are inoculated intradermally into the eyelids with 0.1 ml inoculum containing $10^3 \text{ID}_{50}$ of a pathogenic strain of myxoma virus (grade II virulence). During the ensuing 21 days the controls will die from myxomatosis, and at least 7 of the 10 vaccinated rabbits must present no signs of generalised infection.

Similarly, myxoma vaccine is tested in 10 rabbits with two controls. After 14 days, all 12 are challenged with 0.1 ml Lausanne virus strain containing $10^3 \text{ID}_{50}$. After 21 days 7 of 10 rabbits must have survived, while the controls die from myxomatosis.
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TULARAEMIA
(B69)

SUMMARY

Tularaemia is 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 and over twenty species of birds have been affected.

Two types of F. tularensis are recognised on the basis of cultural characteristics, epidemiology, and virulence in some hosts. F. tularensis tularensis (Type A) is associated with lagomorphs in North America. It is primarily transmitted by ticks and biting flies, is highly virulent for people and rabbits, and ferments glycerol. F. tularensis palearctica (Type B) occurs mainly in voles and aquatic rodents in northern North America, and in rodents in northern Eurasia. It may be water- or arthropod-borne, is less virulent for people and rabbits, and does not ferment glycerol. In addition to vector transmission, tularaemia may be spread by direct contact with contaminated material, by inhalation, or by ingestion of poorly cooked flesh of infected animals or contaminated water.

The disease is characterised by fever, apathy and septicaemia. There may be ulcers or abscesses at the site of inoculation with 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 and liver. The spleen is hypertrophied.

It is important to understand that there is a high risk of direct infection to man by simple contact with this organism. Special precautions including the use of gloves, masks and eyeshields are therefore required when handling infective materials.

Identification of the agent: The bacteria can be demonstrated in impression smears of organs, such as liver, spleen, kidney and lung, as well as in blood smears. After Gram staining they appear as very small punctiform Gram-negative organisms, often difficult to distinguish as bacteria. They can also be stained with May-Grunwald-Giemsa or phenol thionin, or by fluorescent antibody methods.

The organism cannot be cultured on ordinary media, and it is necessary to use Francis medium, MacCoy and Chapin medium, or GCA agar with added blood. The colonies are small, round and transparent, and do not appear before 48 hours at 37°C. On Francis medium the colonies may be confluent with 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 tularaemia. There are also fluorescent antibody and capillary tube precipitation tests to demonstrate F. tularensis in pathological specimens. Infection is fatal within 8-10 days.

Serological tests: Serological tests are useful diagnostic aids in human infection, but are of limited value in rodents since these species usually die before the development of antibodies. Epidemiological surveys may be conducted in domestic animals, such as sheep, cattle, pigs or dogs, since they are relatively resistant to infection but do develop antibodies.

Requirements for biological products: Tularin is produced for use in diagnostic skin tests in man.

Live attenuated vaccines are used in the Soviet Union. An avirulent live strain of F. tularensis biovar palaearctica is available in the USA for vaccine purposes.

A. DIAGNOSTIC TECHNIQUES

Tularaemia 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 and over twenty species of birds have been affected.

Two types of F. tularensis are recognised on the basis of cultural characteristics, epidemiology, and virulence in some hosts. F. tularensis tularensis (Type A) is associated with lagomorphs in North America. It is primarily transmitted by ticks and biting flies, is highly virulent for people and rabbits, and ferments glycerol. F. tularensis palaearctica (Type B) occurs mainly in voles and aquatic rodents in northern North America, and in rodents in northern Eurasia. It may be water- or arthropod-borne, is less virulent for people and rabbits, and does not ferment glycerol (1, 2, 6, 7).

Clinically there are signs of intense apathy; these are accompanied by a septicaemia which is rapidly fatal. The course of the disease lasts about 2-10 days in susceptible species, and animals are usually presented dead for diagnosis. Most domestic species do not usually manifest signs of tularaemia infection, but they do develop specific antibodies to the organism following infection. Outbreaks with high mortality rates have occurred in sheep (1, 2).

At necropsy, animals are usually in good body condition, since the disease is generally acute. There are signs of septicaemia characterised by whitish foci of necrosis randomly distributed in the liver and spleen, which is usually hypertrophic. 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
nodes in the abdominal or pleural cavities or draining the extremities.

There is a high risk of human infection with *F. tularensis*: infection can occur by simple contact. Extreme care must be taken using suitable precautions, such as the wearing of gloves, masks and eyeshields during any manipulations of pathological specimens or cultures. Experimentally inoculated animals and their excreta are especially hazardous to man.

1. **Identification of the agent**

   The organism can be demonstrated in smear preparations and identified by culture or by animal inoculation.

   a) **Smear preparations**

   These are made on microscope slides as impression smears of organs such as liver, spleen, kidney and lung tissues, and blood. The bacteria are abundant in such smears but may be overlooked because of their very small size, 0.2-0.7 µm.

   Gram staining reveals a scattering of small, punctiform Gram-negative bacteria near the limit of visibility; the bacteria may be difficult to distinguish from precipitates of stain. They are non-motile, non-sporulating, bipolar staining bacteria of uniform appearance in 24 hour cultures, but pleomorphic in older cultures. Other suitable stains include May-Grunwald-Giemsa and phenol thionin.

   Fluorescent antibody staining, either direct or indirect, is a rapid and specific diagnostic tool that can be used on smears or formalin-fixed, paraffin-embedded tissue sections (9).

   b) **Culture**

   *F. tularensis* will not grow on ordinary media, although an occasional strain can sometimes grow on ordinary blood agar on initial isolation. Incubation is done at 37°C. Heart blood, liver, spleen and 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 blood of rabbit, horse or man.

   ii) MacCoy 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.

   Both media can be stored for up to 8-10 days at 4°C. Colonies that form on MacCoy medium are small, prominent, round and transparent. A more abundant growth is obtained on Francis medium, with confluent colonies having a milky appearance with a mucoid consistency. On either medium, colonies do not appear until after 48 hours' incubation at 37°C.
iii) 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, non-commercial medium described by Downs et al. (4). Suspend 58 g of the dry material in a litre of distilled or demineralised water and mix thoroughly. Heat with frequent agitation and boil for 1 min. Dispense into tubes and sterilise by autoclaving at 118-121°C for 15 min. For litre quantities, autoclave at the same temperatures for 39 min. 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 h before use to decrease surface moisture and to test for sterility (4).

Differential criteria for the identification of *F. tularensis* include absence of growth on special media, distinctive cellular morphology, and specific fluorescent antibody and slide agglutination reactions. 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 it ferments glycerol.

The bacteria are identified in stained smears, by agglutination with tularaemia hyperimmune antiserum, or by animal inoculation.

e) Animal inoculation

This is extremely hazardous and is not recommended for routine identification. It should only be undertaken where proper biosafety rooms and cages are available. Humane considerations relating to the use of animals for diagnostic purposes must also be heeded.

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 since the lesions are more definitive than in mice, which usually die before lesions can form.

Intraperitoneal injection is sufficient for passage of pure cultures. All routes of administration, such as subcutaneous, percutaneous, or intravenous, will lead to an infection which is invariably fatal within 8-10 days. Inoculation may be made into a foot-pad to provoke a lymphadenitis that is detectable after 3-4 days. If this reaction is pronounced, the animal may be killed at 5-6 days. The percutaneous route, by smearing a shaved area of skin with the material, allows the selective isolation of *F. tularensis* from material which contains a mixture of organisms.

Post-mortem examination reveals hypertrophy and periadenitis of regional lymph nodes, oedema at the inoculation site which is sometimes haemorrhagic, and hypertrophy of the spleen which contains a scattering of minute nodules. The liver is also hypertrophied, but foci of necrosis may not be evident. Smears of blood, spleen and liver are extremely rich in the bacterium.
In infected animals, the only clinical evidence at the onset of infection is a slight anaemia with a lymphocytosis and monocytosis. A very discrete polynucleosis appears after the lymph nodes become suppurative. The regional lymph nodes show a lymphadenitis, oedema which distends the node capsule, and a lymphocytic infiltration and peripheral micro-abscessation. Blood vessels are congested and thrombotic. Later, areas of necrosis become confluent to form multiple abscesses which, if the animal survives long enough, may become surrounded by an epithelioid reaction with giant cells, plasma cells and macrophages.

2. **Serological and other tests**

Serology is currently practised in man for diagnosis, but serological tests are of limited value in animals, which usually die before specific antibodies can develop. However, serology may be employed in epidemiological surveys on animals that are constitutionally more resistant to infection, such as sheep, cattle, pigs or dogs (5, 9, 10).

The antigen is a culture of *F. tularensis* on Francis medium. This is harvested after 5-6 days: younger cultures yield a poorer antigen. The colonies are suspended in 96% alcohol giving a thick suspension which 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 incubation at 37°C for at least 24 hours and not more than 7 days. After the supernatant fluid has been discarded, the deposit is suspended in normal saline with or without thiomersal (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 which gives readily visible stained agglutination reactions on slides against a clear fluid background.

The test is performed in Kahn 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 one 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, better, by using a hand lens. The positive tubes are those which have a clear supernatant fluid. Possible cross-reactions with *Brucella abortus* and *B. melitensis* have to be taken into consideration.

An early diagnosis is now possible using an ELISA test (3).

Fluorescent antibody tests have also been developed for infected tissue samples and to confirm the identity of an isolate in culture.

**Capillary tube precipitation test on pathological samples**

Tissues, such as spleen or liver, are ground with sterile sand in 3-5 times their volume of normal saline. The suspension is transferred to a tube and 2 volumes of
ethyl ether 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 2,000 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.

Tularin intradermal test

This is used in man (8), but there have been reports of its use in animals.

Tularin is a diluted autolysate of *F. tularensis* prepared from a three-day culture. A dense suspension of the culture is prepared with normal saline, and this is stored for 20 days at 37°C. The opalescent fluid which forms above the deposit is carefully decanted, and diluted to an optical density of 0.272 in a Bonet-Maury photocolorimeter with filter "W no. 38" at a wavelength of 460 nm. The stock solution is diluted further to 1:25 with normal saline. The clear product is put into ampoules and heated at 75°C for one hour. The final product is tested for sterility, innocuity by the intraperitoneal inoculation of mice with 0.1 ml, and efficacy by the intradermal reaction in an infected animal in comparison with a tularin preparation of known potency. If positive, a papule 2-4 cm in diameter will develop, accompanied by a peripheral erythema and oedema. A small vesicle may also occur.

Testing is also carried out on healthy seronegative animals with no prior clinical evidence of tularemia. For this, 0.1 ml of tularin is injected intradermally, and the result read after 48 hours. Normal animals should not react to tularin.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

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 in the Soviet Union since 1946, either as monocultures or as a mixture of strains. An avirulent live strain vaccine of *F. tularensis*, biovar *palaearctica* is available in the USA.

REFERENCES


INTRODUCTORY NOTE ON THE DISEASES OF BEES

The honey bee colony is a family of social insects. There are many species, sub-species, races and sub-races of bees adapted to their environment. The following races are of interest:

- **Apis cerana (indica)**: South and South-East Asia
- **A. mellifica L. (= A. mellifera L.)**: Worldwide
- **A. mellifica mellifica**: European honeybee
- **A. mellifica adansonii**: Central Africa
- **A. mellifica capensis**: Cape, South Africa
- **A. mellifica meda**: Near East
- **A. mellifica intermissa**: North Africa

No cross-breeding occurs between *A. cerana* and *A. mellifica L.*, however, it does occur between the sub-species of *A. mellifica L.* The Africanised bee in Brazil is a cross between the European bee and the Central African bee. *A. 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 European bee (*A. mellifica mellifica*) is preferred for honey collection and docility.

A colony of *A. mellifica mellifica* consists of:

**The queen:** This is a productive fully developed adult female from a fertilised egg. She develops in 16 days in special queen cells and has a lifespan of 3-4 years. She lays eggs from spring to autumn and during summertime; she may lay up to 3,000 eggs per day. She secretes pheromones that help to co-ordinate the colony.

**Drones:** These are fully developed adult males from unfertilised eggs and there may be 500-1,500 individuals in each colony. They exist only during the summer months. They take 24 days to develop and have a lifespan of 2-3 months. Their sole purpose is to mate with virgin queens.

**Worker bees:** These are sexually incompletely developed adult females from fertilised eggs. In winter they number 6,000-18,000, and in summer 40,000-70,000 individuals per colony. They develop in 21 days. Their lifespan in summer is 5-7 weeks; in winter, 6-8 months. Their activities include the collection of nectar, honeydew and pollen; they build, clean, repair and defend their nest of honeycombs. They transform raw food to wax, honey and hypopharyngeal protein food (royal jelly) for the larvae. They feed and tend the brood as well as the queen, store honey and pollen, and maintain the brood-nest temperature of 30-35°C by ventilation.

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 more susceptible to acarasis, varroasis and American foulbrood, whereas it resists nosematosis. When sampling a colony of bees for diagnosis of diseases, live bees must first be killed with diethyl ether or chloroform.
SUMMARY

Acariasis or acarine disease is a disease of the adult honey bee Apis mellifera L and other Apis species. It is caused by the Tarsenemid mite, known as the tracheal mite, Acarapis woodi (Rennie). Its size is approximately 150 µm. It is an internal parasite of the respiratory system, living and reproducing mainly in the large prothoracic trachea of the bee. The mites feed on the haemolymph of their host. Sometimes they are also found in the head, thoracic and abdominal air sacs.

The pathogenic effects 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, the depletion of haemolymph and to toxic effects. 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 infestation normally go unnoticed, and only when infestation is heavy does it become apparent. This is generally in the early spring. The infestation spreads by direct contact. Generally only newly-hatched bees, less than 10 days old, are susceptible. The life-span of the mites in dead bees is approximately one week, but may be only one day when there are no contact bees.

Identification of the agent: The parasites are demonstrated only by laboratory methods. These include dissection, grinding and staining.

The thoraces of suspect bees are dissected to expose the trachea. Each trachea is examined under a dissecting microscope (x 18-20), where the mites will be seen as small oval bodies through the transparent wall.

After grinding or homogenising larger samples of suspect bees 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 microscopical examination. The efficacy of this method is questionable.

The parasites may be stained by histological techniques to observe them within the bee trachea. The tracheas are separated out, cleared with 8% potassium hydroxide and stained with 1% methylene blue. This is the best method of choice for large numbers of samples.

Serological tests: Serological tests are not available, although an enzyme immunoassay (ELISA) is under development.
Requirements for biological products: There are no biological products available.

A. DIAGNOSTIC TECHNIQUES

Acarine disease is a disease of the adult honey bee *Apis mellifera* L and other *Apis* species, caused by the microscopic Tarsonemid mite *Acarapis woodi* (Rennie). Its size is approximately 150 μm and it is an internal parasite of the respiratory system (Fig. 1). These tracheal mites enter, live, and reproduce mainly in the large prothoracic tracheae of all bees, feeding on the haemolymph of their host. Sometimes they are also found in the head, thoracic and abdominal air sacs (5).

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 manifestations of infestation normally go unnoticed, except for a slow dwindling in the colony size. Only when infestation is heavy does it become apparent. This is generally in the early spring, after the winter clustering period when the mites breed and multiply undisturbed in the longer-living worker bees.

The infestation spreads from one bee to another by direct contact. Generally only newly-hatched bees, less than 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 honeybee itself has been only partially successful (7). The life-span of the mites in dead bees is approximately one week, but this is reduced to one day in the absence of any contact bees.

There are no reliable clinical signs for the diagnosis of acarine disease since the signs of infestation 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. Sometimes the hindwings appear to be dislocated, a phenomenon known as "K wing".

1. **Identification of the agent**

Acarine disease can be detected only in the laboratory.

a) **Dissection** (8)

A sample of 50 bees is collected at random from the suspected colony. These may be living, dying, or dead. Live bees must first be killed with ethyl ether or chloroform; bees must not have been dead for longer than 2-3 days. They may
be preserved indefinitely in a preservative such as Oudemann solution (glacial acetic acid, 80 ml; glycerol, 50 ml; ethanol 70%, 870 ml).

Bees are laid and secured on their backs; the heads and forelegs are removed with small forceps. A transverse cut is made across the anterior part of the thorax, immediately anterior to the base of the forewings. At this point both tracheae can be located. These are severed by an antero-posterior vertical incision. Both pieces of thorax, each containing its trachea, are placed on a slide together with a few drops of lactic acid; this clears the preparation and makes it easier to remove the muscles. From the sample of 50 bees the resulting 100 pieces of thorax and their trachea are placed on 2 slides of 50 pieces each. They can now be examined under a dissecting microscope at a magnification of x18-20. By means of a pair of very thin needles, the surrounding muscles are removed and the trachea drawn out, taking care to keep the spiracular end intact and if possible the distal end also.

The mites are easily seen as small, oval bodies through the transparent tracheal wall. Diagnosis is easier if the trachea is blotchy.

The magnification used for the dissecting microscope is sufficient for routine observations, but where necessary, higher magnifications can be employed. In those cases where diagnosis is uncertain or if higher magnifications are required, the isolated trachea should be transferred to another slide and mounted in Meyer's medium.

This is the simplest and most reliable technique for the laboratory diagnosis of acarine disease, allowing the detection of early infestations and enabling the infestation rate to be established. Even light infestations 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.

b) Grinding (3)

A sample of about 20-200 bees is collected at random from the suspect colony. The wings and legs of each bee are removed from the thorax, and all the latter pooled in a 100 ml container a quarter-filled with water. This suspension is homogenised 3 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 rinsed with water to a final volume of about 50 ml. The filtrate is centrifuged at 1,500 g for 5 minutes and the supernatant fluid discarded. A few drops of absolute lactic acid solution are added to the debris of the deposit which will contain any mites. This is left for 10 minutes to allow the muscle fibres to be dissolved, and mounted under a coverslip for microscopical examination. This technique is more rapid than dissection but its sensitivity is doubtful and its accuracy more questionable. External mites *Acarapis externus* and *A. dorsalis*, both of which are morphologically similar to *A. woodi*, are often found on the thorax of healthy bees and can very easily be mistaken for the pathogen (Fig. 2).
Fig. 1. Acarapis woodi *Rennie*. Left: adult male, Centre: female, Right: egg and larva.

Fig. 2. *Location of the external Acarapis*

c) **Staining (10)**

Both pathogen and trachea can be stained specifically, rendering them easily visible by microscopy. Very thin sections of the bee thorax (1-1.5 mm thick) are obtained by making two transverse cuts, the first through the membranous areas behind the forelegs in order to remove the head and the forelegs, and the second in front of the middle pair of legs at the base of the forewings. The sections are macerated by gentle stirring in an 8% solution of potassium hydroxide heated near to boiling until the soft internal tissue are dissolved and
cleared, leaving the chitinous tissues intact. This takes approximately 10 minutes. The sections are retrieved by filtration, washed with tap water and stained and mounted after processing. They are finally examined 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. Sections are differentiated in distilled water for 2-5 minutes and then rinsed in 70% ethanol. 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. The method is capable of processing a large number of samples rapidly and conveniently.

2. **Serological tests**

There are no tests applicable to date, but an immunoenzyme assay (ELISA) is being developed.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

There are no biological products available.

**Acknowledgments**


**REFERENCES**


AMERICAN FOULBROOD
(B75)

SUMMARY

American foulbrood affects the larval stage of the honey bee Apis mellifera and other Apis spp. occurring throughout the world. Bacillus larvae (White), the causative organism, is a bacterium which can produce over a 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 a sole characteristic of American foulbrood. Cell cappings which contain 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 a dark brown with a ropy appearance when drawn out. A very unpleasant 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 signs of the disease and precedes the formation of the scales.

American foulbrood can usually be diagnosed in the field, but laboratory tests are available for its confirmation. Most cases are diagnosed by light microscopy. A simple stain such as Gram is used for diagnosis. This differentiates the bacteria by its morphology. The modified hanging drop technique will differentiate American foulbrood from other brood diseases. This technique and the characteristic morphology of the spores permits almost immediate diagnosis.

Immunofluorescence may be used, but it should be noted that cross-reactions can occur with B. alvei. B. larvae can be distinguished biochemically from other spore-forming bacilli. It reduces nitrate to nitrite and is negative for catalase production. This and its growth requirements will give a positive identification.

B. larvae spores can be recovered from honey by centrifugation or by dialysis. Spores similar to those of B. larvae have also been recovered from contaminated beeswax.

Serological tests: There are no serological tests available.

Requirements for biological products: There are no biological products available.
A. DIAGNOSTIC TECHNIQUES

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. *Bacillus larvae* (White), the causative organism, is a bacterium which may produce over a billion spores in each infected larva. The bacterium is a slender rod with ends slightly rounded and a tendency to grow in chains. It varies greatly in size from 2.5-5.0 μm long by 0.5 μm wide. The spores are oval, approximately twice as long as they are wide, about 0.6 μm by 1.3 μm. They 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 from nurse bees or by spores remaining at the base of a brood cell. Although the larval stages of worker bees, drones or queen are susceptible to infection, under natural conditions infected queen and drone larvae are rarely seen. The susceptibility of larvae to American foulbrood disease decreases with increasing age (12); larvae cannot be infected later than 53 hours after the egg has hatched. The mean infective dose (LD~50~) needed to initiate infection is 35 spores in one-day-old bee larvae (2). Exchanging combs containing the remains of diseased larvae is the most effective means 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.

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 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 which contains a diseased larva appears moist and darkened and becomes concave and punctured as the infection progresses. Also, the larva or pupa changes in 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 very unpleasant foul odour develops at this stage, resembling that of animal glue. Finally, after a 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. 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. The tongue may persist also on the dried scale.

In most cases, American foulbrood can be diagnosed in the field by visual inspection. When diagnosis requires confirmation, several laboratory tests are available.
a) Sample preparation

The correct diagnosis of any bee disease depends on the sample submitted. The sample of comb should be about 4 inches square and contain 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, but wrappings such as plastic bags, aluminium foil, waxed paper, tin or glass should be avoided since these materials allow samples to go mouldy which makes 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 cells may have enough material on it for any tests. This too can be wrapped in paper and sent to the laboratory in an envelope.

The sample is now checked for typical signs of disease. 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 3,100-4,000 Å will cause any scale material to fluoresce. Some discretion must be used when employing this technique since both honey and pollen will also fluoresce.

b) Microscopy

In most cases American foulbrood disease can be diagnosed by observing *B. larvae* by light microscopy. The modified hanging drop technique (8) can be used to differentiate between American foulbrood and other brood diseases. Suspect material is mixed with water, and a drop of this suspension is placed on a coverslip, 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 coverslip is inverted with a very thin layer of immersion oil. The slide is gently blotted dry and examined by high-power microscopy. By examining fields where pockets of water are formed in the oil, the spores of *B. larvae* will be seen exhibiting Brownian movement. This is a valuable diagnostic technique since the spores formed by other members of the genus *Bacillus* associated with known bee diseases remain fixed. This taken together with the characteristic morphology of the spores permits a diagnosis. If the infection is less than 10 days old, long vegetative forms of the bacterium are present and some newly-formed spores may be seen. The observation of Brownian movement is however less specific than the detection of giant flagellae (8).

In some laboratories, a simple stain such as Gram is used for diagnosis. This relies solely on differentiating the bacterium by its morphology. A further refinement is the immunofluorescence technique.

c) Immunofluorescence

This technique is preferable and requires the preparation of specific antibodies labelled with a fluorescent dye. Rabbits are injected with pure cultures of
B. larvae and the antiserum conjugated with a fluorochrome dye. The resulting fluorescent antibody is reacted with a bacterial smear on a slide. Any excess antiserum is washed off and the smear examined by fluorescence microscopy. B. larvae stains specifically as brightly fluorescing bacteria on a dark background (9, 11, 13).

d) The Holst milk test

The Holst Milk test (5) is based on the fact that a high level of proteolytic enzymes is produced by sporulating B. larvae. The test is performed by suspending a suspect scale, or a smear of a diseased larva, in a tube containing 3-4 ml of 1% powdered skim milk in water. The tube is then incubated at 37°C. If B. larvae are present, the suspension will clear in 10-20 minutes.

e) Culture techniques

Absolute identification and further biochemical tests require that B. larvae be grown in the laboratory. It requires complex media for growth, for which Difco Brain-Heart Infusion® (BHI) can be used, the medium fortified with 0.2 mg/l thiamin hydrochloride. Another medium contains yeast extract, soluble starch, glucose and potassium phosphate buffer, pH 6.6 (1). The medium can be liquid, semi-solidified by the addition of 0.3% agar, or solidified with 2% agar.

To culture B. larvae, spore suspensions are prepared by mixing diseased material in 9 ml sterile water in test tubes. The suspension is heated at 80°C for 10 minutes to kill non-sporulating bacteria. A sterile cotton swab is used to transfer a portion of the suspension onto the surface of solid BHI plates which are incubated for 2-3 days at 34°C. Individual colonies are small and opaque. If the inoculum contains large numbers of spores, a solid layer of growth will cover the plate. Difficulties can occur when the spore suspensions contain other spore-forming bacteria which may completely overgrow the culture.

f) Nitrate reduction

B. larvae reduces nitrate to nitrite (7). It can be grown on a medium such as BHI containing potassium nitrate (1-2 mg/l of medium). When growth has occurred, the addition of a drop of sulfanilic acid-alpha naphthyl reagent produces a red colour if any nitrate has been reduced to nitrite. This test should be used for confirmation and not be employed alone.

g) Catalase

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 B. larvae is almost always negative for this reaction (4).
Figure 1. Progression of the disease:

a) Point of infection.
b) Larval development to the prepupal stage.
c) Cell contents reduce and capping is drawn inwards or is punctured.
d) Cell contents become glutinous.
e) Residual scale tightly adherent to bottom of cell.

Figure 2. External appearance of affected comb: cappings with moist and sunken appearance, some punctured with larval remains.

Figure 3. Diagram of Bacillus larva:

a) Bacillus with flagella
   (2.5-5 μm by 0.7-0.8 μm)
b) Sporulating bacillus.
c) Multiple aggregates.
h) Detection of spores in honey

Occasionally, it may be necessary to examine honey for the presence of *B. larvae*. The classical method (Sturtevant, 1932) is to dilute the honey 1:5 with water and centrifuge at high speed to deposit the spores. The following method has been developed (10) to detect *B. larvae* spores in honey: Samples of honey to be examined for spores are heated to 45°C and shaken to distribute any spores that may be present. Honey (25 ml) from each sample is diluted with 25 ml of water to permit easier handling. The diluted honey is transferred into 1.75 inch (44 mm) lay-flat width dialysis tubing 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 in the same period. After dialysis, the contents are centrifuged at 2,000 g for 15 minutes. The supernatant liquid is discarded and the deposit resuspended in 9 ml of water. The suspension is heated at 80°C for 10 minutes to kill non-spore-forming bacteria and plated onto BHI as previously described.

Spores similar to *B. larvae* have also been recovered from contaminated beeswax by chloroform extraction (6) and from pollen by filtration of a suspension through filter paper (3).

2. Serological tests

Apart from the fluorescent antibody technique described above for detection of the organism there are no available serological tests.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

There are no requirements for biological products.

Acknowledgments


**REFERENCES**


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 caused for reasons other than European foulbrood. Most infected colonies display few visible signs which then 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: The examination of suitable preparations of larval remains by high-power microscopy for the presence of numerous lanceolate coci 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 biological products: There are no biological products available.

A. DIAGNOSTIC TECHNIQUES

Bee larvae usually die of European foulbrood within 1 or 2 days of being due to be 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 early summer 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 becoming increasingly brown, at the same time melting into a semi-liquid mass. They then dry out and form a dark brown
scale, and can easily be removed from the cells. Severely affected brood may have a very stale or sour odour, 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 (1, 5).

1. Identification of the agent

Before any decomposition occurs, diseased larvae or those that have recently died can be dissected on a microscope slide by pinching the cuticle about the centre of the body with 2 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 a few cm², 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. 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 appear more confusing, especially when secondary organisms predominate.

During the early stages of an infection, most bacteria will be *Melissococcus pluton* (type strain NCDO 2443) (2, 3). It can be cultivated on a medium (expressed in g/l or ml/l) comprised of yeast extract or certain peptones (2) 10; cysteine or cystine, 0.2-2.0; glucose or fructose, 10; soluble starch 10; 1 mol/l KH₂PO₄, 100 at pH 6.6; 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. This treatment 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 which 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 about 5 to 10% CO₂ at 34°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. *M. pluton*
grows poorly or not at all if any component is omitted from the medium or when substantial quantitative changes are made to it. Anaerobic conditions and the presence of CO\textsubscript{2} are essential. Slopes of the medium, inoculated with isolated colonies of *M. pluton*, 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 an medium of 10% sucrose, 5% yeast extract and 0.1 mol/l KH\textsubscript{2}PO\textsubscript{4} at pH 6.6, and then lyophilised.

A number of other bacteria are frequently 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*. Its incidence in healthy bees is very low in winter and early spring but it increases in summer. It forms thin square-ended rods which can be either single or in chains. It sometimes resembles streptococci when grown in certain media, and has been confused with *M. pluton*. However, its cultural characteristics closely resemble those of *Corynebacterium pyogenes* (6) and it multiplies poorly in the form of thin rods, under the conditions necessary for the cultivation of *M. pluton*.

*Streptococcus faecalis* closely resembles *M. pluton* morphologically and has frequently 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 the foraging adult bees. It is reponsible for the sour smell sometimes encountered with European foulbrood.

*S. faecalis* grows well under the conditions suitable for *M. pluton in vitro*. 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 CO\textsubscript{2}. The final pH in the presence of glucose is 4.0. *S. faecalis* rarely exceeds the numbers 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 multiplication of *M. pluton in vitro*.

*S. 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.

*Bacillus alvei* is generally more common than *S. faecalis* in bee colonies affected with European foulbrood; but it is not invariably associated with the disease, and so may not 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. It forms very resistant spores and becomes well established in bee colonies with enzootic European foulbrood. It causes a characteristic stale odour. *B. alvei* multiplies poorly under the conditions necessary for the growth of *M. pluton in vitro*. 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.
Figure 1. Bacteria associated with European foulbrood.


b) Bacillus alvei: vegetative rods 2.0-7.0 x 0.8-1.2 μm with flagella; sporulating with spores lying adjacently. Both rods and spores are larger than those of Bacillus larvae (see American foulbrood).

c) Bacterium eurydice: slender, square-ended rods in vivo but can form chains of cocci in vitro in certain media.

For the identification of *M. pluton*, antisera can be prepared in rabbits against washed cultures of *M. pluton* either by intravenous injections (4) 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.

2. Serological tests

No tests for detecting antibodies in bees are available.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

There are no biological products available.

Acknowledgments

REFERENCES


BEE NOSEMATOSIS
(B77)

SUMMARY

The microsporidium Nosema apis (Zander) is a parasite of the honeybee that invades the epithelial cells of the mid-gut. In heavy infestations other organs can be affected. Infestations are acquired by the uptake of spores during feeding or grooming.

The parasite hyphae invade the posterior region of the mid-gut giving rise to large numbers of spores within a short time. The parasite is ubiquitous and occurs in greatest numbers in the spring when there is an increase in the brood. Spores can persist for up to 2 years in faecal droppings and up to 1 year in honey and in bee carcasses. They are inactivated by acetic acid or by heating to 60°C for 15 minutes.

Identification of the agent: In the acute form, brown faecal marks are seen on the comb with sick or dead bees in the vicinity of the hive. There may be an increase in the mortality rate during winter. In milder cases, there may be no special signs, perhaps only poor colonies with large quantities of brood and few adult bees. In affected bees the mid-gut, which is normally brown, is white and very fragile. Microscopical examinations of homogenates of the abdominal contents of affected bees will reveal the oval spores of Nosema, about 5-7 μm by 3-4 μm with a dark edge. Their internal contents cannot be distinguished. Staining is usually not necessary.

The appearance of Nosema infestations can be confused with yeast cells, fungal spores, fat and calciferous bodies or cysts of Malpighamoeba mellificae. The latter are larger than Nosema spores, being 6-7 μm around.

Only positive identifications by observation of typical spores in the mid-gut or faeces can be regarded as significant. Very mild infestations may not be demonstrable. The extent of infestation is determined by counting the spores in 10 areas of a slide and calculating the average number of spores per area.

Serological tests: There are no applicable serological tests.

Requirements for biological products: There are no biological products available.

A. DIAGNOSTIC TECHNIQUES

The microsporidium Nosema apis (Zander) is a parasite exclusive to the epithelial cells of the mid-gut of adult bees. In extreme cases other organs may be affected as well. Infection occurs by the ingestion of the spores in the feed or after the grooming of the body hairs of the bee.
Spore hyphae penetrate the peritrophal membrane of the intestine, particularly in the posterior region of the mid-gut. Primary bodies pass down the hyphae and enter the epithelial cell, where they reproduce. 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 the spring, which coincides with the increase in the brood. In winter spores are rarely to be found, or are perhaps only found in bees which are heavily infested. The development stages necessary for hibernation are difficult to find microscopically because of their very small size.

Any inherent natural defense by a bee colony against a heavy infestation 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. If these conditions are unfavourable, the overall life expectancy of the colony is reduced due to hormonal imbalance. This may lead to the premature death of bees during the winter at a time when they are responsible for the brood. In a typical case of a colony being depleted on account of a *Nosema* infestation, the queen can be observed surrounded by a few bees, confusedly attending to brood that is already sealed.

The spores have a variable viability. In faecal droppings they may persist for up to two years, and in honey or in the carcasses of dead bees they may last for one year. The comb is mainly infested. Spores may be killed by heating to a temperature of at least 60°C for 15 minutes. Fumes from a solution of acetic acid of at least 60% will inactivate any spores, depending on the concentration, within a few hours; higher concentrations are even more effective and will kill spores within a few minutes. Disinfection can be carried out by putting acetic acid solution into bowls or onto sponges which can soak up the liquid. Following disinfection after an outbreak, all combs should be well ventilated for at least 14 days prior to use.

1. **Identification of the agent**

In acute forms of infestation, especially in early spring, brown faecal marks will be noted on the comb. At the entrance of the hive both sick and dead bees will be seen, and during winter there will be an increased mortality rate in the colony. In milder infestations there may be no special signs except for colonies of weak appearance with large numbers of brood but only a few adult bees.

To diagnose a *Nosema* infestation, a number of bees are killed by compression of the thorax. The posterior pair of abdominal segments are removed with forceps to reveal the mid-gut, complete with the malpighian tubules, the small intestine and rectum. The mid-gut is normally brown but following a *Nosema* infestation becomes white and very fragile. For a reliable diagnosis a number of bees in a sample should be examined.
It is advisable to distinguish between a *Nosema* infestation and that of *Malpighamoeba mellificae*. In the former infection there is seldom an indication of dysentery, whereas in the latter there may be a sulphur-yellow diarrhoea with a distinct odour. Secondary mixed infestations may occur. A sample of about 20 dead bees is collected from a suspect colony. The abdomens are separated and ground up in 2-3 ml of water. Three drops of the suspension are dropped onto a slide under a coverslip and examined microscopically at x 400 magnification. 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, capsule, and filament, cannot be seen. Dyes are usually not necessary.

*Nosema* spores are not to be confused with yeast cells, fungal spores, fat and calc bodies, nor with cysts of *Malpighamoeba mellificae*. The latter are larger than *Nosema* spores; they are round and 6-7 μm in diameter.

As a variation of this method, a relatively fresh preparation of mid-gut may be examined. The intestine is ground up and a sample put directly onto a slide, diluted with a little water, covered with a coverslip and examined microscopically. The result may be negative even if a low-grade infestation is present. The degree of infestation is determined by counting the spores in 10 known areas of a slide and calculating the average number of spores per unit area. These results may be evaluated as:

<table>
<thead>
<tr>
<th>Number of spores per unit area</th>
<th>Description</th>
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<tbody>
<tr>
<td>≤ 20 spores</td>
<td>Weak infection</td>
</tr>
<tr>
<td>20 - 100 spores</td>
<td>Increased infection</td>
</tr>
<tr>
<td>&gt; 100 spores</td>
<td>Strong infection</td>
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A laboratory method for the simultaneous detection of *Nosema* spores and *Amoeba* cysts consists of the individual examination of the colonies (30-60 bees per colony). A suspension of the abdomens of dead bees is prepared by grinding with 5-10 ml water; the amount of water depends on the number and condition of the bees. One drop of the suspension is placed on a slide and examined microscopically at x 200 magnification. The results are assessed as ≤20 spores per area as negative; >20 spores per area, positive.

The suspension is filtered first through a 100 μm and then a 40 μm filter. *Nosema* spores, pollen and 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 and examined at x 200-400 magnification. Only a few tubules are filled with cysts after an amoeba 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 *Amoeba* cysts are often confused with fungal spores and yeast cells.

There are no cultural methods of growing these organisms.

2. **Serological tests**

There are no serological tests available.
B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

No biological products are available.

Acknowledgments

The authors would like to thank Dr F. Gnädinger for his valuable advice.

REFERENCES


VARROASIS
(B78)

SUMMARY

The mite 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, thorax and abdomen. The number of parasites steadily increases with increasing brood activity and the growth of the bee population, especially in late summer and autumn, when clinical signs of infestation can first be recognised. The life span of the mites depends upon temperature and humidity, but in practice it can be said to last from 5 to 12 days.

Identification of the agent: The clinical signs of varroasis can only be recognised at a late stage of infestation so that diagnosis entails the examination of bee debris. This is possible as long as two years after the first infestation. The debris produced during the summer is especially useful for diagnosis. The earliest and most precise diagnosis can be made only after an application of a medication that obliges the mites to leave the bees.

Larger amounts of debris, especially that produced in winter, can be examined using a flotation procedure. Bees and brood samples are washed in petroleum spirit or water respectively. 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 biological products: No biological products are available.

A. DIAGNOSTIC TECHNIQUES

The mite Varroa jacobsoni (Oudemans) is a parasite of adult bees and their brood (Fig. 1) and is responsible for the condition of varroasis or varroatosis. The mite inserts itself between the abdominal sclera in adult bees (6) where it penetrates the intersegmental skin in order to ingest haemolymph. Sometimes it can also be found between the head and thorax. The mites usually reproduce slowly at the beginning of the year, but their number increases with the bee population. Maximum numbers are reached in the late summer and autumn (Fig. 2), when the first clinical signs of infestation can be recognised. The course of this parasitism is usually lethal in all cases (10). The lifespan of mites on larval or adult bees depends upon temperature and humidity. Under practical conditions the lifespan may vary from 5-12 days to a few months.

In heavily infested bee colonies clinical signs of varroasis can often first be seen in the latter end of the year (7), but heavy infestations are usually reached 3-4 years after the primary invasion. Bees show disfiguration of the wings, legs or abdomens.
(Fig. 3) and heavily infested bees are often short-lived (4). This is due to an increased susceptibility to acute paralysis virus, as well as to the infection of wounds and loss of haemolymph (1, 2). In addition, the brood may be affected by secondary infections. Apart from sacbrood, European foulbrood can occur (7).

Figure 1. Infestation of pupa and adult bee. Left: pupa infested with 1 male Varroa mite (white) and 4 female mites. Right: worker bee with 2 female mites.

Figure 2. Graph of populations of bees and mites over 1 year: Brood numbers (solid line); mite numbers (broken line).

Figure 3. Effect of Varroa infestation on bee morphology. Left: normal bee appearance. Right: bee with heavy infestation of mites, showing newly emerged but crippled bee.
1. **Identification of the agent**

An easy method of diagnosis of varroasis is by the examination of the debris generated by bees themselves. An insert covered with a gauze material is placed on the floor of the hive. Unless this insert is covered with such a gauze, or else smeared with grease, the bees will dispose of the mites outside the hive. The debris produced within a few days in late summer usually contains little other than visible mites (5, 8). The debris collected in winter however must be examined in the laboratory. An insert is placed in the hive as before but a medication is used to cause the mites to fall off the bees, so that after a given time, numbers of mites may be observed on the floor insert.

Large amounts of debris can be examined in the laboratory using a flotation procedure (3). Debris is first dried for 24 hours, then flooded with industrial alcohol and stirred continuously for 10-20 minutes. The debris forms a deposit while the mites float to the surface.

In a second method, preferably 18-day-old drones and 13-day-old worker bees are selected. The brood, which in this case can have been stored at -20°C, is partially thawed and the cell cappings removed. The pupae are washed out of the cells with...
lukewarm water onto a sieve series of coarse and fine meshes. The sieves are rinsed thoroughly several times until the mites are located exclusively on the finest sieve. These sievings are emptied out onto a light-coloured surface and examined for mites (9).

In a third method, about 200-250 bees are removed from open brood combs during the summer when the mite infestation will be heaviest. The bees are killed in a special container, covered either with petroleum spirit or alcohol, and the whole stirred for 10 minutes. The mites are then separated from the bees by means of a sieve having a mesh size of approximately 2-3 mm (9).

Under some circumstances the Varroa mite may be confused with the bee louse, Braula coeca (Fig. 4). The latter is round, not oval, and being an insect, has only 3 pairs of legs. A number of different species of mite may be associated with Varroa mites on bees, but are easily distinguished.

2. **Serological tests**

No serological tests are available for diagnosis.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

There are no biological products available.

**Acknowledgments**


**REFERENCES**


SUMMARY

Leishmaniasis is not a single entity but comprises a variety of syndromes due primarily to at least sixteen species and sub-species of Leishmania. Dogs are commonly affected by L. infantum and L. chagasi, but infections with L. donovani, L. major and L. braziliensis braziliensis are reported. In human beings the clinical spectrum ranges from asymptomatic infections to those with high mortality, three distinct forms being classically described: visceral (VL), cutaneous (CL) and mucocutaneous (MCL). The vectors of these diseases are phlebotomine sandflies belonging to the genera Phlebotomus and Lutzomyia.

Identification of the agent: When clinical features and characteristic lesions are present in the affected humans and animals, the demonstration of the parasites in stained smears of splenic, bone marrow and lymph node aspirates and of skin scrapings gives a positive diagnosis. If the infection is low grade, detection of parasites is possible only by attempting in vitro or in vivo isolation. Since there are very few morphological differences among various species, any isolated Leishmania organism must be identified by biochemical and/or immunological methods. Several centres throughout the world are presently using isoenzyme, DNA and antigen characterisation to identify the agent.

Serological tests: Serology is the preferred method for diagnosis of VL and canine leishmaniasis (CanL) even during the early stages of the disease. In sub-clinical forms, seropositive cases are confirmed by parasitic diagnosis. Serology is of less value for CL and MCL. Of the several techniques available the indirect fluorescent antibody (IFA) test and the enzyme-linked immunosorbent assay (ELISA) are the most suitable.

Delayed hypersensitivity test: The leishmanin skin test is useful for determining the distribution of human infections, distinguishing immune from non-immune cases. The test is positive in CL, MCL and cured VL but negative in active VL.

Requirements for biological products: There is no effective vaccine available at present for use in humans and dogs. Leishmanin, no longer available commercially, needs to be standardised. Serodiagnostic antigens need to be prepared in the laboratory, though some commercial products are now under evaluation.
A. DIAGNOSTIC TECHNIQUES

Leishmaniasis is caused by the vector-borne protozoan parasite, *Leishmania*. Various forms of clinical manifestations of human leishmaniasis have been described (14) and divided into three entities: visceral leishmaniasis (VL, kala azar), cutaneous leishmaniasis (CL, oriental sore, uta, pian bois, chicleros's ulcer) and mucocutaneous leishmaniasis (MCL, espundia). In the New World leishmaniases are caused by *L. braziliensis* complex (MCL and CL), *L. mexicana* complex (CL), *L. peruviana* (CL) and *L. chagasi* (VL); in the Old World by *L. donovani* (VL), *L. infantum* (VL and CL), *L. tropica* (CL), *L. major* (CL) and *L. aethiopica* (CL). The diseases are mainly zoonoses with a few exceptions. Canine leishmaniasis (CanL) is a chronic viscero-cutaneous disease caused by *L. infantum* and *L. chagasi*, of which the dog plays the role of reservoir. In some instances parasites belonging to *L. braziliensis* complex, *L. major* and *L. donovani* have been isolated from this host (7). The vectors of leishmaniases are phlebotomine sandflies belonging to the genera *Lutzomyia* (New World) and *Phlebotomus* (Old World).

1. Identification of the agent

Clinical examination of suspected cases, parasitological diagnosis and immunodiagnosis are the routine methods available for the diagnosis of leishmaniasis. However, the demonstration of the parasite is the only way to confirm the disease conclusively (6). In VL, isolation and identification of the parasite from biopsies (bone marrow, spleen aspirate) coupled with immunodiagnostic tests are recommended. Parasitological diagnosis is necessary for confirmation of CL (through lesion scraping) as neither clinical examination nor serology are adequate. Smears of biopsy material are stained with Giemsa stain and examined microscopically at 600-1,000 magnifications. Material should also be cultured in appropriate media.

Morphological characteristics of amastigotes (in man and mammalian hosts) and promastigotes (in invertebrate hosts and in cultures) are the following:

- Amastigote: small intracellular rounded or oval body, 1.5-3 μm x 2.5-6.5 μm in size, found in the cytoplasm of the macrophages. There is no free flagellum. The organism has a relatively large nucleus and a kinetoplast consisting of a rod-like body and a dot-like basal body;

- Promastigote: elongated extracellular organism, body size 15-20 μm x 1.5-3.5 μm with a single flagellum 15-28 μm long, arising close to the kinetoplast at the anterior. The nucleus is situated centrally.

The choice of the isolation and culture methods will depend on the immediate circumstances and on the technical capability and experience of the staff (11). *In vitro* isolation offers certain advantages over the *in vivo* methods: cultures become positive more rapidly (5-30 days), in contrast to months for lesions to appear on an animal; and the materials are less expensive. However, for the *in vitro* isolation the techniques used should be strictly sterile; hence, it is not always feasible in field conditions. Since *Leishmania* species differ in their nutritional requirements it is
unlikely that a single medium will be able to support their growth. Individual laboratories have to find the most suitable medium by experimenting with a number of different media for the parasite of their particular region (2). When attempting cultivation a blood agar-based medium should be used – preferably NNN medium, otherwise USMARU (Difco Blood agar medium) or EMTM (Evans' modified Tobie's medium). The organisms from patients with VL and MCL can be very difficult to cultivate. Sometimes, even when the initial isolation is successful, the parasites may die when subcultured. This seems especially common when the initial isolation has been into a rich medium. Often this can be overcome if subcultures are made into less nutritionally rich media such as NNN, or one of the semi-solid media such as "sloppy Evans" or semi-solid Locke-blood agar.

Hamster (*Mesocricetus auratus*) is the most commonly used animal for the in vivo isolation (8). Tissue suspension or aspirate are inoculated intradermally into the nose and/or feet in the case of detection of dermotropic parasites. When the material is suspected to be infected with parasites causing VL the inoculation should preferably be made by the intraperitoneal route. The resulting infection becomes apparent by the development of a nodule or ulcer at the site of inoculation after weeks or months, and in case of viscerotropic parasites by massive infection of internal organs after some months. The examination of Giemsa stained smears of hamster's tissue suspension/aspirate will show amastigotes. Balb/c mice are commonly used for the diagnosis of *L. major*.

Several biochemical techniques are now being used in many centres to identify the different *Leishmania* species, sub-species or strains.

**a) Isoenzyme characterisation** is the most commonly used method (1, 3, 7, 11). This technique requires a large number of parasites. Principles of enzyme electrophoresis are the following: soluble enzymes are extracted from the organisms grown in media for bulk cultivation (BHI medium, MEM:FCS:EBLB medium, Schneider's Drosophila medium). A small amount of the extracts is then placed in an inert supporting substance, the matrix, containing a buffer at a fixed pH. The matrix is usually starch gel, but it could equally well be absorbent cellulose acetate, acrylamide or agarose. The pH of the buffer in the matrix is chosen so that usually the isoenzymes are negatively charged. A direct current is passed through the matrix carried by the ions in the buffer. When electrophoresis is completed, most proteins will have moved in the matrix towards the anode, depending on the amount of negative charges. If stained at this stage with a general protein stain, very many bands will be seen. However, the high substrate and co-factor specificity of enzymes allow that only these proteins will be stained. Hence, the electrophoretic mobility of one particular enzyme can be compared between several organisms. The stained matrix with its collection of stained isoenzyme bands, is known as a zymogram. Normally one or more extracts from reference organisms, in which the enzyme banding patterns are well documented, are included in the gel to aid the interpretation of results. The majority of enzymes used for characterisation purposes are stained by methods incorporating a dehydrogenase reaction. At least 12 enzymes should be examined.
b) **Kinetoplast DNA restriction endonuclease analysis** is based on the analysis through gel electrophoresis of fragments generated by endonucleases on the abundant mitochondrial DNA which constitutes the kinetoplast (3, 4, 7, 9). The electrophoretic pattern obtained, known as a restriction "fingerprint", is a biochemical marker at the organelle genotype level that allows the identification and classification of *Leishmania* strains into schizodemes – populations having similar kDNA sequences. This technique also requires a large number of parasites.

c) **DNA hybridisation probes** are a promising tool whose principle is to allow labelled, single-stranded nuclear or kinetoplast DNA sequences from well characterised reference strains to find and hybridise with homologous DNA sequences from or within unknown *Leishmania* isolates (3, 10). Only complementary DNA sequences will form double-stranded DNA which can be detected by autoradiography if the probe is radiolabelled, or by immuno-enzymatic reaction. Only a very low number of parasites are required for identification through the *in situ* hybridisation technique.

d) **The monoclonal antibody technique** is applied to the analysis and classification of both New and Old World *Leishmania* species and sub-species (3, 7, 9). For the production of the antibodies Balb/c mice are immunised with membrane preparations from either promastigotes or amastigotes. Hybridoma cultures secreting antibodies are then selected and cloned by limiting dilutions. Specificity to *Leishmania* strains is then assessed through immunofluorescence or immunoradiometric assays. This analysis seems to be quantitative rather than qualitative, since the amount of the same surface antigen may vary among *Leishmania* species.

2. **Serological tests**

Several serological tests are now used for detecting anti-leishmanial antibodies (8).

a) **Indirect fluorescent antibody test**

The indirect fluorescent antibody (IFA) test is widely used because it is easy to perform. Both amastigotes and promastigotes can be used as antigens. Washed promastigotes (three times, with phosphate buffered saline (PBS) pH 7.2 at 1,500 rpm for 15 min) fixed in acetone or formalin, or amastigotes in frozen sections, or smears of infected organs can be used. The test is genus-specific. IFA is a more sensitive and specific test than micro-ELISA in the detection of VL. With this test a high humoral immune response can be detected in vaccinated subjects against cutaneous leishmaniasis caused by *L. major*. Amastigote antigen gives better results (97.5% sensitivity) than the promastigote antigen (92%) for the serodiagnosis of VL.

The IFA test can be used for epidemiological studies on human and canine leishmaniases, but results must be confirmed by simultaneous parasitological investigations. Suspect serum is applied directly to the slides coated with *Leishmania* antigen and then visualised by treatment with the fluorescein-conjugated anti-immunoglobulin. The highest dilution showing fluorescence
under a fluorescence microscope is taken as the antibody titre. In human VL the threshold titre is about 1/100, in CanL 1/20-1/40. The test is positive in the early stages of the disease even before the appearance of symptoms and becomes negative about one year after the patient is cured.

b) Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) can be carried out on serum or on a measured volume of blood. The blood is collected by finger-prick on to suitable absorbent paper strips and allowed to dry. The sample is eluted and tested at a single dilution previously determined as giving an acceptable sensitivity and specificity. This test can be used for sero-epidemiological surveys under field conditions.

The antigen is prepared as follows: promastigotes harvested from cultures are washed four times with PBS pH 7.2 at 5,000 rpm for 15 min. The packed promastigotes are resuspended in twice their volume of distilled water and then sonicated at medium amplitude in an ice bath. The suspension is left at 4°C overnight to allow the proteins to come into solution. After a final centrifugation at 5,000 rpm for 10 min to eliminate the cellular debris, the overlay, representing the concentrated soluble antigen, is dispensed into vials and stored at -29°C until required. For use in the test it is reconstituted with PBS to the predetermined optimal protein concentration (around 20 µg/ml) as measured by Lowry's method. The ELISA test is useful for the diagnosis of Old and New World leishmaniases. There is no cross-reaction with other diseases and, according to the Leishmania strain used, sensitivity can range from 86 to 99%.

c) Direct agglutination (DA) test

This test has recently been introduced for the diagnosis of VL and CanL but needs to be evaluated and standardised. The antigen consists of promastigotes harvested from cultures, washed in PBS pH 7.2, treated with 0.4% trypsin (for 45 min at 37°C and then washed again) and stained with 0.02% Coomassie Brilliant Blue. Twofold serial dilutions of serum in PBS are made in microtitre-plate wells. Fifty µl of antigen preparation is added to each well and the plate is then carefully shaken by hand and left 18 h at room temperature. The test is read visually against a white background. The positivity is estimated by localising a clear sharp-edged blue spot.

d) Counter immunoelectrophoresis (CIEP)

The general principle of this test is that the movement of most of the protein molecules is towards the anode, except for gamma-globulins, which move towards the cathode. This property is utilised in the CIEP test. The antibody moves towards the cathode under electroendosmotic flow and negatively charged antigen moves towards the anode and precipitates on contact with antiserum. The procedure is as follows: 3-5 ml agarose is poured onto slides and wells are punched. Serum is placed in the anodal well and leishmanial
antigen, prepared as for ELISA, in the cathodal well. The slide is placed in an electrophoresis tank filled with barbitone buffer pH 8.2 and connected to the buffer chambers with filter-paper strips. A current of about 8 mAmp is applied and after 2-3 h the slide is examined for precipitation lines. The CIEP test is a reliable diagnostic test and in CanL has given better results than ELISA.

e) **Indirect haemagglutination (IHA) test**

In this method washed sheep red blood cells are incubated with tannic acid (1/20,000 in PBS pH 7.2 at 37°C for 30 min) and then sensitised with soluble leishmanial antigen (prepared as for ELISA, 3.2 mg of protein/ml) in PBS pH 6.4 for 30 min. Sensitised red blood cells are then washed and finally suspended (2%) in PBS pH 7.2 containing 1% normal rabbit serum. The test is performed by serial double dilutions of 56°C inactivated sera in microtitre-plates. The IHA test is considered less sensitive than the tests described above. However, the fall in antibody titre below diagnostic levels may be used as a serological marker for the determination of cure in VL.

### 3. **Delayed hypersensitivity test**

Delayed hypersensitivity is an important feature of all forms of human leishmaniasis and can be measured by the leishmanin test, also known as the Montenegro reaction (5). Leishmanin is a suspension of whole (0.5-1 x 10^7/ml) or disrupted (250 μg protein/ml) promastigotes in pyrogen-free saline containing phenol. The dose is 0.1 ml given intradermally in the forearm. Promastigotes of any species of *Leishmania* are suitable as antigen, as are other kinetoplastid parasites. A delayed reaction develops and is read at 48-72 hrs. The reaction is an indurated nodule with some surrounding erythema, and can be measured and graded according to the "ballpoint pen" method. By applying moderate pressure, a line is slowly drawn with a ballpoint pen from a point about 1-2 cm from the margin of the skin reaction towards its centre. When resistance to further movement is first felt – indicating the margin of the reaction – the pen is lifted from the skin. By repeating the procedure on the opposite side of the skin reaction, this technique gives a visual record of the margins of the induration, the diameter of which can then be accurately determined by measuring the distance between the opposing lines.

The false positive reaction rate in otherwise healthy people is about 1% but this can be higher in areas where there is a background of leishmaniasis, since many of the healthy population may show quite high rates of leishmanin sensitivity. There is a complete cross-reactivity between all strains of *Leishmania* although heterologous antigens often give smaller reactions, but this may be caused by difficulty in standardisation. No cross-reactions occur with Chagas’ disease although some cross-reactions are found with cases of glandular tuberculosis and lepromatous leprosy. The leishmanin skin test has no value for the diagnosis of CanL. It is used in the clinical diagnosis of CL and MCL. In VL it will only measure past infections since during active disease a complete anergy is found. Leishmanins are no longer available commercially and need to be standardised.
B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

1. Vaccine

There is no effective vaccine available for prophylactic immunisation against leishmaniasis. Till now the only dependable vaccination against *Leishmania* has been limited to the protection of individuals from both *L. tropica* and *L. major* by prior syringe-induced infection with the parasite. The promastigotes are injected into the arm or other parts of the body. The living promastigotes used must either be freshly extracted from cultures or may be preserved in liquid nitrogen. The infection is allowed to run a natural course and after recovery, the individual is firmly immune to subsequent infection with both leishmanias. This type of immunisation has been practised on limited scale in hyperendemic areas of CL (caused by *L. major*) in Israel, Iran and the USSR. The immunising preparations consist of living, virulent and infective promastigotes.

*L. major* causes cross-protection against *L. tropica*, but the reverse is not true. However, this species cannot be considered totally safe and may convert the immunised host into a reservoir who will be a potential source of infection as long as the lesion remains unhealed. Hence this type of immunisation should be used only in people moving into high risk areas. Moreover, this is not beneficial in highly endemic areas as people contract infection long before this type of preparation confers protection. It takes about three months before immunity is acquired. Standardisation and quality control of such vaccines, presently not available, are urgently needed. Killed parasites are in general ineffective as vaccines in man and dogs. *Leishmania* patients who have recovered from infection, are normally immune to reinfection with the homologous parasites but not to other species.

2. Immunodiagnostic antigens

Neither the leishmanin used for skin tests nor the antigens employed in serodiagnosis in leishmaniasis are properly standardised as per the International Biological Standards. The leishmanin test is group-specific, not species-specific, and the leishmanin prepared from one clinical type of leishmaniasis will cause the development of delayed hypersensitivity to the same or other clinical types. Similarly, serological cross-reactions are common among leishmanial species.

a) Leishmanin

The leishmanin test is described in part A3 above. Sterility, safety and potency tests are required for leishmanin preparations.

b) Antigens for serological tests

Antigens for the IFA and IHA tests are now produced commercially, but they are still under evaluation. The main problem which causes unsatisfactory results is the poor stability of leishmanial antigens. They can be obtained in the laboratory by growing a *Leishmania* strain in a suitable culture medium. For the IFA and DA tests, crude particulate antigens, i.e. intact promastigotes are
required, whereas for ELISA, CIEP and IHA tests a soluble form of the antigen is needed.

3. **Seed management**

a) **Characteristics**

Strains of *Leishmania* species used to prepare biological products should be identified at species and sub-species level by appropriate identification tests given in Section A.1. Once the organisms have been isolated and established in the laboratory they must be assigned an International Code (7, 11, 14). This code should consist of four elements separated by oblique strokes: 1) The type of host from which the strain was isolated (M for Mammalia and I for Insecta followed by three letters indicating the generic name of the host); 2) The country where isolation was made, indicated by a two-letter code; 3) The year of isolation indicated by the last two digits, and 4) The original laboratory code given to the isolate (for example, MHOM/IN/80/DD8). The parasites must be free from contaminating organisms and should be capable of yielding a product which conforms to the norms. Standard reference strains are available on request from the WHO Collaborating Centres in London, Montpellier and Jerusalem. A list of Identification Centres has recently been published by WHO (16).

b) **Culture**

The strain of the parasite used for preparing leishmanin should be capable of producing a product conforming to national/international norms. It should be free from ingredients causing toxic or allergic reactions. There is no single specific antigen standardised for use in serodiagnostic tests, but when these antigens are prepared in the laboratory they must be standardised for their sensitivity depending upon the requirement. For the preparation of leishmanin as well as serodiagnostic antigens the organisms should be grown in a suitable culture medium (such as those recommended in Section A.1 for *Leishmania* isolation and bulk cultivation). Normally, good growth of parasites is obtained seven days after inoculation, and care must be taken that leishmanial stocks are not lost by overgrowth of the flagellates which may occur after about ten days.

c) **Cryopreservation**

Promastigote cultures and tissue infected with amastigotes may easily be conserved in the living state at low temperatures. Both forms can be cryopreserved for years at low temperatures in mechanical freezers (-70°C), in solid carbon dioxide containers (-76°C), or in liquid nitrogen containers (-196°C) (11). A sterile cryoprotectant is required – glycerol, to give a final concentration of 7.5 to 10%, or dimethyl sulphoxide (DMSO), to a final concentration of 5 to 7.5%. The cryoprotected samples are transferred to the sterile containers in which they are to be frozen. These may be 2 ml plastic freezing tubes with airtight screw caps, hard glass, heat-sealed ampoules, or glass/plastic capillaries. A slow cooling rate (about 1°C/min) is essential for the
cryopreservation of *Leishmania*. This can be obtained by cooling samples to 4°C and keeping them at this temperature for a minimum of one hour; they are then transferred to a -20°C freezer and left for 24 h, then removed to a -70°C freezer for at least 24 h. They can be permanently stored at this temperature, or else transferred into liquid nitrogen or solid carbon dioxide. If possible, a programmable freezing unit should be used. When the cryopreserved material is required, the sample is taken out and thawed rapidly in a water bath at 37°C.

d) Validation

Cultures for leishmanin or serodiagnostic antigens should be checked for sterility before use. Leishmanin is stored at +4°C and serodiagnostic antigens at -20°C or -70°C until required. The latter should be reconstituted with PBS pH 7.2 before use. Viable *Leishmania* cultures can be kept at -70°C for 3-4 years or at -196°C indefinitely. Because of non-availability of suitable vaccine, it has not been possible to validate the currently developed immunising agents. Live or attenuated promastigotes of *L. major* used in some areas are far from being satisfactory. Leishmanin should be tested for allergenicity in guinea pigs before use. Serodiagnostic antigens should be tested for their efficacy and sensitivity by properly standardising them for a particular test. If a batch of antigen has not been used for a long time, it should be rechecked before being employed in the test.

4. Manufacture

Since the immunodiagnostic antigens are either not available commercially or, if so, they are still under evaluation, they need to be prepared in the laboratory. Working in the laboratory poses a risk to the workers as infection can occur through injection. Biosafety precautions are therefore essential.

a) Leishmanin

*Leishmania* species are grown preferably in blood-free liquid media (such as Schneider's Drosophila medium and RPMI) in order to avoid blood antigen contamination. The promastigotes are harvested during the log phase, washed four times in pyrogen-free saline at 2,500 rpm for 15 min and resuspended in pyrogen-free saline containing 0.5% phenol (w/v) to obtain a final concentration of 0.5-1x10⁷/ml. Leishmanin can be made also with disrupted promastigotes obtained as above and sonicated. The filtrate is adjusted to a final protein concentration of 250 µg/ml with pyrogen-free saline containing Tween 80 (0.0005% v/v) and phenol (0.28% w/v).

b) Antigens for serological tests

Methods of antigen preparation for various tests are given in Section A.2.

5. In-process control

One or more batches of leishmanin should be tested in the guinea pig by allergic
test. Sensitivity and specificity of the leishmanin should preferably be determined by performing the test in appropriate animal models (different inbred mice according to the *Leishmania* species), or in patients who have recovered from leishmanial infections and in an unexposed control population.

6. **Batch control**

WHO has suggested guidelines for the production of leishmanin (13, 15). It is recommended that the source material be controlled by using isoenzyme analysis to type the *Leishmania* strains used in preparing leishmanin.

a) **Sterility**

Each filling lot should be tested for bacterial and mycotic sterility according to WHO (12). Absence of live leishmaniae is checked by inoculating one sample of each lot in an appropriate blood-agar medium which is then incubated at 23°C for at least 15 days. One sample is injected intradermally (for dermotropic leishmaniae) or intraperitoneally (for viscerotropic leishmaniae) in mice/hamsters. These animals are observed during a period of 30-90 days.

b) **Safety**

Samples from each filling lot should be tested for abnormal toxicity by appropriate tests in guinea pigs and mice. For each lot five mice weighing 17-22 g and two guinea pigs weighing 250-350 g are injected subcutaneously and intraperitoneally with one human dose of the product. The animals are then observed for at least seven days for death or signs of disease.

c) **Potency**

The leishmanin is tested on animal models (according to the *Leishmania* species involved) which have been previously infected by the same strain used for leishmanin production. Lots of at least five infected animals and control animals are injected intradermally into one of the posterior footpads with 50 μl of leishmanin. After 2-3 days all the infected animals should show a significant enlargement of the footpad compared to control animals.

An International Reference Preparation has yet to be established. The Leishmaniasis Section of WHO’s Tropical Disease Research Programme is funding programmes aimed at reaching a satisfactory standardisation of leishmanin production.

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MALIGNANT CATARRHAL FEVER
(B80)

SUMMARY

Malignant catarrhal fever (MCF) of cattle, domestic buffaloes, deer and many other bovid species, occurs broadly as two epidemiological types, namely: (i) a wildebeest-derived (WD) form caused by a T-cell tropic, lymphoproliferative gamma herpesvirus called Alcelaphine herpesvirus 1 (AHV-1). It occurs primarily in Africa, causing an inapparent infection of wildebeest of the subfamily Alcelaphinae; (ii) a sheep/goat-associated (SGA) form, probably caused by a virus closely related antigenically to the wildebeest viruses. It has not yet been cultivated in vitro or characterised physicochemically either within or outside infected cells. It is usually acquired by close contact with sheep, particularly at lambing time, and is probably maintained predominantly in sheep and goats as a latent infection.

Both forms can be transmitted parenterally, the SGA form with difficulty, and their incidence is sporadic or enzootic. The morbidity is usually low but the case mortality rate is always high. Both forms produce similar clinico-pathological signs. Diagnosis is mainly based on clinical signs, epidemiology and histopathology.

Identification of the agent: The alcelaphine viruses can be readily grown, producing cytopathic effects (CPE) in suitable monolayer cell cultures inoculated with viable lymphoreticular cell suspensions from live or recently dead sick animals, also from reservoir species. WD and some SGA agents are transmissible to rabbits parenterally, producing a characteristic, invariably lethal syndrome. Lymphoblastoid cell lines can be produced from reacting cattle, deer or rabbits; these possess natural killer (NK) cell or large granular lymphocyte (LGL) phenotypic characteristics, and may carry limited MCF viral infectivity for either the WD or SGA varieties.

No antigen tests or DNA probes are yet available for detecting viral proteins or genomes in the non-permissive lymphoreticular cells of infected animals.

Isolates of alcelaphine viruses can be reliably identified by cytopathology and immunostaining techniques, as well as by neutralisation using specific antisera to cell-free virus. Electron microscopy is also useful for preliminary characterisation.

Serological tests: Immunostaining methods detect group-specific (AHV-1, AHV-2, etc.) antibodies in the sera from reservoir species (wildebeest, sheep, etc.) and in late clinical or recovered cases in indicator species (e.g. cattle, deer). Neutralising antibodies to alcelaphine viruses have a narrower specificity and occur in lower titre, if at all, in sheep or other caprine reservoirs. Serology is used mainly for research on the epidemiology and
pathogenesis of MCF, but it is also required for confirming the identity of isolates from clinical cases.

Requirements for biological products: No vaccines are available to protect indicator species, such as cattle and deer, against MCF.

A. DIAGNOSTIC TECHNIQUES

Malignant catarrhal fever (MCF) of cattle, domestic buffaloes, deer and many other bovid species, can be broadly categorised into two epidemiological types, namely:

i) A wildebeest-derived (WD), primarily African, form for which the aetiological agent is a lymphoproliferative gamma herpesvirus, now called *Alcelaphine herpesvirus* 1 (AHV-1), after the subfamily *Alcelaphinae*, to which wildebeest belong. This is T-cell tropic and causes an inapparent infection in wildebeest, which are reservoir hosts.

ii) A sheep/goat-associated (SGA) form, usually acquired by close contact with sheep, particularly at lambing time. The causal agent is probably a virus, closely related antigenically to the wildebeest viruses but not, so far, cultivable *in vitro* or characterised physicochemically either within or outside infected cells. It is assumed to be maintained predominantly in latently infected sheep.

Both forms are transmissible by parenteral inoculation, the SGA form often with considerable difficulty; they are characterised by sporadic or enzootic incidence, often with low morbidity, always with a high (ca. 95%) case mortality rate. They are probably not contagious among indicator hosts.

Both types produce a very similar and wide range of clinicopathological signs, and routine virological confirmation of a diagnosis is possible only for WD cases. Clinical signs, epidemiology and histopathology still form the basis for the majority of diagnoses and the two epidemiological types are not distinguishable on clinicopathological grounds.

Where typical cases of WD- or SGA-MCF are encountered, the epidemiology and clinicopathological signs give a reliable indication of the cause. This is particularly true with the so-called "head and eye" form, in which a combination of pyrexia, ocular and nasal discharges, mucosal necrosis and erosion, lymphadenopathy, ophthalmitis and nervous signs are virtually unique. When sudden deaths occur, or diarrhoea and dysentery are marked features, as in some SGA outbreaks particularly, differential diagnosis is more difficult and autopsies need to be carried out very carefully.

The histopathology of MCF is virtually pathognomonic, although no viral inclusion bodies are present; it includes widespread proliferation of lymphoblastoid cells, especially in perivascular and subepithelial locations, together with fibrinoid degenerative changes and cellular infiltration of vessel walls, especially small and
medium arteries in several predilection sites. As wide a range of tissues as possible should be examined.

1. **Identification of the agent**

The infectivity of WD-MCF virus can be demonstrated only in freshly harvested, living cells from sick or recently dead animals. It is lost by freezing at -20°C or at -70°C or below in the absence of cryoprotective agents. From live animals, the buffy coat cells should be separated from blood collected into anticoagulants (preferably EDTA, also heparin, etc.); at autopsy, the best sources are lymph nodes or spleen from which washed cell suspensions are prepared. The leucocytes or viable suspensions of cells from lymph nodes, etc., are inoculated in graded doses onto monolayers of permissive cells (bovine thyroid, serially-cultivated bovine testis, fetal aoudad kidney) which develop CPE, detectable in unstained preparations 3 to 20 days later, depending on the amount of virus present. Infectivity titres are commonly >10^2 TCD_{50} per millilitre in blood and >10^4 TCD_{50} per gram in lymph nodes. The CPE in stained monolayers include syncytium and intranuclear inclusion-body formation; immunostaining using MCF-specific conjugates reveals nuclear, cytoplasmic and membrane antigens. Electron microscopy will confirm the presence of herpesvirus particles.

Many infected cell cultures release very little cell-free infectivity at low passage levels but this process can be accelerated by the use of bovine testis cells and incubation at 32-34°C. When yields of free virus are adequate, virus neutralisation tests can be used to identify isolates. Bovine and rabbit antisera are available from only a few research laboratories; many of these are produced against blue wildebeest (*Connochaetes taurinus*) isolates of high (WC 11) or low (C 500) culture passage, with very low and very high cattle pathogenicity, respectively. No internationally recognised standard preparations are available but the WC 11 strain of AHV-1 was approved as an International Reference Strain by the FAO/WHO Board for Comparative Virology.

Direct demonstration of AHV-1 viral antigens in the cells of infected cattle, etc., is extremely difficult; only about 1 in 10^6 cells show specific fluorescence in lymphoid cell suspensions at explantation.

The demonstration of SGA-MCF infectivity in natural cases is seldom successful, even using massive inocula (blood and fresh lymphoid tissue suspensions). Lines of NK/LNG cells have been established from naturally-infected cattle and deer (using CSF, lymph nodes and cornea as sources), as well as from experimental rabbits and hamsters. At least one of these lines carries MCF infectivity and all are presumed to carry parts of the viral genome, although no virions or viral antigens are demonstrable.

2. **Serological tests**

a) **Virus neutralisation (VN)**

Tests have been developed for antibodies to AHV-1 in both naturally infected
reservoir and indicator hosts. The first of these is a virus neutralisation (VN) test using cell-free virus of the WC 11 strain, and another uses a hartebeest isolate (AHV-2). The test is laborious but can be performed in microtitre plates, using low passage cells or cell lines. The main applications have been in studying the range and extent of natural infection in wildlife, captive species in zoos and, to a lesser extent, sheep populations. It has also been useful in attempts to develop vaccines, all of which have had very limited success. High-titre VN antibodies can be induced but these are evidently not protective.

MCF virus stock (African strain WC11) is grown in primary or secondary cell cultures of bovine kidney, bovine thyroid, low passage bovine testis, or other permissive cell type, and stored in aliquots at -70°C. The stock is titrated to determine the dilution which will give 100 TCID$_{50}$ in 25 μl under the conditions of the test.

**Test procedure:**

i) Inactivate sera for 30 min in a waterbath at 56°C.

ii) Make doubling dilutions of test sera in cell culture medium from 1/2 to 1/16 using a 96-well flat-bottomed cell culture grade microtitre plate, 4 wells per dilution and 25 μl volumes per well. Positive and negative control sera are also included in the test. No standard sera are available, but internal positive standards should be prepared and titrated in an appropriate range.

iii) Add 25 μl per well of MCF virus stock at a dilution in culture medium calculated to provide 100 TCID$_{50}$ per well.

iv) Incubate for one hour at 37°C. The residual virus stock is also incubated.

v) Back titrate the residual virus in four 10-fold dilution steps, using 25 μl per well and at least 4 wells per dilution.

vi) Add 50 μl per well of bovine kidney cell suspension at 3 x 10$^5$ cells/ml.

vii) Incubate the plates in a CO$_2$ atmosphere for 7-10 days.

viii) Read the plates microscopically for cytopathic effects. Validate the test by checking the back titration of virus (which should give a value of 100 TCID$_{50}$ with a permissible range 30-300) and the control sera. the standard positive serum should give a titre within ±0.3 log$_{10}$ units from its predetermined mean.

ix) The test serum results are determined by the Spearman-Kärber method as the dilution of serum which neutralised the virus in 50% of the wells.

x) A negative serum should give no neutralisation at the lowest dilution tested (1/2, equivalent to a dilution of 1/4 at the neutralisation stage).
b) **Indirect fluorescent antibody (IFA) test**

The IFA is less specific; it can be used to demonstrate several varieties of "early" and "late" antigens in AHV-1 infected cell monolayers. Antibodies reacting in IFA or immunoperoxidase tests develop in cattle and rabbits during the long incubation period and later in the clinical course of the disease. Their increase is sometimes useful in supporting a diagnosis, even of SGA-MCF. However, cross-reactions with other bovid herpesviruses, especially the ubiquitous BHV-3/BHV-4 agents (bovine "cytomegaloviruses"), as well as the putative caprine herpesviruses of SGA-MCF, reduce the differential diagnostic value.

**Preparation of fixed slides:**

Inoculate nearly or newly confluent cell cultures (as listed above) with MCF virus (African strain WC11). Uninoculated control cultures should be processed in parallel. At about 5 days – when the first signs of CPE are expected to appear, but before overt CPE is visible – treat cultures as follows: discard the supernatant, remove cells with trypsin-versene solution, spin down cells at approximately 1,200 rpm for 5 min, discard supernatant, and resuspend cells in 10 ml phosphate buffered saline (PBS) for each 800 ml plastic bottle.

Make test spots of cell suspension on two wells of a polytetrafluoroethylene (PTFE)-coated multiwell slide and air dry. Fix in acetone. Stain spots with positive reference serum and anti-species conjugate. Examine picture of +/-cells under UV microscope. Adjust cell suspension by adding non-infected cells and/or PBS to give a suitable concentration which will form a single layer of cells when spotted onto the slide, with clearly defined positive cells amongst a background of negative cells.

Spot adjusted positive cell suspension and control negative suspensions onto multiwell slides in desired pattern. Air dry. Fix in acetone for 10 min. Rinse, dry and store at -70°C.

An alternative procedure, which is easier to evaluate, is to prepare monolayers of infected and noninfected cells in Leighton tubes or chamber slides. The cells are infected with 150 to 200 TCID$_{50}$ per ml of virus which is diluted in cell culture medium. The infected and noninfected slides are fixed in acetone and stored at -70°C.

**Test procedure:**

i) Rehydrate slides for 5 min with PBS, rinse in distilled water and air dry.

ii) Dilute sera 1/20 in PBS. Samples which give high background staining may be retested at higher dilutions. Apply diluted fluids to one MCF virus-positive cell spot and one negative control spot for each sample. Include positive and negative serum controls. Ideally, the test should be validated by titrating the control positive to determine its end-point.
iii) Incubate at 37°C for 30 min in a humid chamber.

iv) Drain fluids from spots. Wash slides in 2 changes of PBS, for 5 minutes each.

v) Wash in PBS for one hour with stirring and then air dry slides.

vi) Apply rabbit anti-bovine IgG FITC conjugate at predetermined working dilution.

vii) Incubate at 37°C for 20 min, drain slides, wash 2 x 10 min in PBS.

viii) Counterstain in Evans blue 1/10 for 30 seconds, wash in PBS for 2 min. Dip in distilled water, dry and mount in PBS/glycerol 50/50.

ix) Examine by fluorescence microscopy for specific binding of antibody to the infected cells.

c) Other tests

An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to the alcelaphine herpesvirus of MCF in exotic ruminants has been published (4).

Antibodies to AHV-1 are present in sheep worldwide, suggesting that they are induced by a herpesvirus related to the wildebeest virus. This hypothesis is supported by immunoprecipitation tests with AHV-1 antigens and by rising antibody (IIF) titres to AHV-1 in some cases of SGA-MCF. The development of radiolabelled DNA probes for AHV-1 holds promise for investigating the relationships of SGA- and WD-MCF, as well as for the pathogenesis and diagnosis of both.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

The use of vaccines may only be contemplated in high-risk situations. Control by preventing close contacts between reservoir and indicator hosts is normally highly effective, but may be impractical in wildlife conservation terms.

REFERENCES


BOVINE VIRUS DIARRHOEA
(B81)

SUMMARY

Bovine virus diarrhoea (BVD) virus is a pestivirus in the Togaviridae and is closely related to hog cholera virus and Border disease virus. The virus occurs in two forms, non-cytopathogenic and cytopathogenic. Each biotype has a specific role in a variety of clinical syndromes. Diagnosis of a variety of syndromes in cattle due to BVD virus depends on serology, clinical signs, detection of the virus and pathology.

Cattle of all ages are susceptible to infection with BVD virus. The clinical signs range from subclinical to the fulminating fatal condition called mucosal disease. However, most infections in the young calf are mild and go unrecognised clinically.

Infections of the bovine fetus may result in abortions, stillbirths, teratogenic effects or persistent infection in the neonatal calf. The clinical signs in these calves vary greatly and their appearance can range from the apparently normal healthy animal to the weak unthrifty calf that has difficulty in standing and suckling. They may show central nervous system defects and often die within days of birth, thus contributing to the "weak calf syndrome".

The most characteristic pathology is seen in mucosal disease. At post-mortem examination, there are 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 tonsil. Often, there are petechial haemorrhages in the serosa of the abdominal organs, in particular the gut and the kidney. Such lesions could account for the peritoneal and pleural effusions that are often present. On histological examination, there is a clear demonstration of destruction of the lymphoid tissue within the gut associated lymphoid tissue.

Persistently viraemic animals may later develop mucosal disease. They become anorexic, develop oral erosions and may have profuse diarrhoea. They invariably die, often within 1-2 weeks of developing clinical signs.

Identification of the agent: When acute infection with BVD virus is suspected in cases of diarrhoea or respiratory infection, the virus may be isolated in cell culture from blood or from nasal discharge but diagnosis by seroconversion, either the virus neutralisation test or ELISA, is more reliable.

A congenital infection which may result in a persistent viraemia can be readily diagnosed by isolation of non-cytopathogenic virus in cell cultures from blood or serum. Presence of virus should be confirmed after an interval of at least 3 weeks. These animals will also have no or low levels of antibodies.
Preliminary diagnosis of chronic infections due to BVD virus or mucosal disease will often be made on clinical signs. Confirmation can be made by isolation of the cytopathogenic biotype of BVD virus from a variety of samples but particularly intestinal tissues. Non-cytopathogenic virus should also be detected, especially in blood.

Serological tests: These are usually required to detect acute infections and the ELISA for antibody has advantages over the virus neutralisation test.

Requirements for biological products: There is no standard vaccine for BVD but a number of commercial preparations are available. The use of a modified-live virus vaccine cannot be recommended. Killed virus vaccines probably require booster vaccinations.

A. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Bovine virus diarrhoea (BVD) virus is a pestivirus (1, 2) in the Togaviridae and is closely related to hog cholera virus and Border disease virus (infecting sheep). The virus occurs in two forms (biotypes), non-cytopathogenic and cytopathogenic. Each biotype has a specific role in a variety of clinical syndromes – acute, congenital and chronic infections – and needs to be identified (3, 5); cytopathogenic by virus neutralisation test with specific antiserum and non-cytopathogenic by direct or indirect staining with fluorescent antibody or by an immunoperoxidase method (11). Cytopathogenic virus can also be identified by staining methods but the result is confused if both biotypes are present. The virus may be isolated in a number of cell cultures (e.g. kidney, lung, testis or turbinate) of bovine origin and growth of both biotypes is usually satisfactory. Non-cytopathogenic BVD virus is a common contaminant of fresh bovine tissue and cell cultures must be checked for freedom from adventitious virus by regular testing. It may be convenient to maintain a stock of cultures (e.g. as primary suspensions or secondary cells) in a liquid nitrogen refrigerator. These can then be tested over a series of passages or seeded to other susceptible cells and checked before routine usage (8). Such problems may be overcome by the use of continuous cell lines, which can be obtained BVD-free. Fetal bovine serum, selected for use in cell culture, must also be free not only of virus but, of equal importance, specific antibody. Heat treatment (56°C for 30-45 minutes) has been employed to "sterilise" serum for cell culture but this may not be adequate for low levels of BVD virus; irradiation (2.5 Mrad) is more certain. Where appropriate, it is better to substitute horse serum for bovine fetal serum.

a) Acute infections

Acute infections of cattle occur, particularly in young animals, and may be clinically inapparent or associated with diarrhoea. Affected animals may be predisposed to concurrent infections, for example respiratory disease, perhaps due to an immunosuppressive effect by the virus. During acute infections a brief viraemia may be detectable and nasal shedding of virus may occur. There
may also be a transient leucopenia or temperature response but these can vary greatly between animals and are not reliable indicators. However, a serological response is the most certain means of diagnosing a previous infection.

b) Fetal infection

If non-cytopathogenic virus infects the bovine fetus, this may result in abortions, stillbirths, teratogenic effects or a congenital infection that persists in the neonatal calf (3, 6). Confirmation that an abortion is due to BVD virus is often difficult to establish (14) but virus may be isolated from fetal tissue in some cases, or viral antigen detected by fluorescent or enzyme methods (11). An attempt should be made to detect specific antibody in samples of serum, body fluids from abdominal or thoracic cavities, or in the supernatant fluid from a suspension of tissue. Stillbirths or teratogenic effects may be associated with an active immune response to the virus.

Although abortion is a frequent sequel to congenital infection with BVD virus, it is not always recognised in the field. Infection during the first third of pregnancy can result in the abortion of a conceptus that is small and not seen 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 (15). Most typical of the CNS pathology is cerebellar hypoplasia, dysmyelination, 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.

c) Persistent viraemia

When infections of the fetus occur before about 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 non-cytopathogenic BVD virus in blood. Samples of buffy coat cells have been examined in the past but whole blood or serum are adequate. Coverslip preparations of the chosen cell culture should be seeded with the sample and examined regularly for 6-7 days before immune labelling to detect viral antigen. Inocula containing undiluted blood or serum should be replaced with fresh medium, after 1-2 hours, to reduce background levels of staining. A further passage in cell culture may be necessary to confirm a negative result (5). A microtitre culture system can be used to examine large numbers of samples and to screen herds for persistently viraemic stock. Growth of non-cytopathogenic virus is detected in the culture by an enzyme-labelled system, such as immunoperoxidase. To confirm a diagnosis of persistent viraemia, animals
should be retested after an interval of at least three weeks to ensure that seroconversion has not occurred. Furthermore, animals with a persistent viraemia will also lack specific antibody but diagnosis in the young calf, up to about two months of age, may be confused by the presence of maternal antibody to BVD virus. Maternal antibody may also interfere with virus isolation. In older animals, low levels of antibody may be present due to their ability to seroconvert to strains of BVD virus "heterologous" (antigenically different) from the persisting virus.

There are no pathognomonic lesions in the viraemic calf. The clinical signs vary greatly and their appearance can range 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 this requires their rapid identification and removal from the herd. Bulls that are persistently infected usually have poor quality, highly infective semen and, as a result, reduced fertility. In several recent cases the first clinical signs were not those associated with fertility but defects in locomotion and in the eye of the infected bull. All bulls used for natural or artificial insemination should be screened for a persistent infection with BVD virus.

d) Mucosal disease

It is well established that persistently viraemic animals may later succumb to mucosal disease (3). It has now been shown that this syndrome is a result of superinfection with the cytopathogenic biotype (4). Consequently, a requirement for diagnosis of mucosal disease is the isolation of cytopathogenic virus from affected cattle. This biotype may be isolated from blood but this can be unreliable. However, it can be recovered consistently from a variety of tissues, in particular intestinal and Peyer's patch tissue (5). Isolation may be difficult from gut samples if autolysis has occurred and suspensions from lymph nodes or tonsil should then be tested. Non-cytopathogenic virus can also be detected, particularly from blood or blood-associated organs.

Tissue for examination should be suspended in cell culture medium, the gross particles removed by centrifugation and the supernatant fluid seeded to cell cultures, some of which should contain coverslips. In some cases undiluted supernatant fluid may be toxic to cell cultures and the inoculum should be replaced with fresh medium after 1-2 hours. The cultures should be examined regularly for the appearance of cytopathic effect. This may not always be extensive and can be transient, particularly when non-cytopathogenic virus is present at high titre. Dilution of the sample will often reveal the presence of both biotypes, with the non-cytopathogenic virus at higher titre than for the
cytopathogenic form. At least one further passage may be required to confirm the absence of cytopathogenic virus. Cryostat tissue sections can be prepared from clinical cases of mucosal disease and then stained for viral antigen.

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.

At post-mortem examination, there are 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. Often there are petechial haemorrhages in the serosa of the abdominal organs, in particular the gut and the kidney. Such lesions could account for the peritoneal and pleural effusions that are often present.

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.

2. Serological tests

Antibody to BVD virus can be detected in cattle sera by a standard virus neutralisation (VN) test or by ELISA, using one of several published methods (9, 10).

a) Virus neutralisation test

Because it makes the test easier to read, most laboratories use highly cytopathogenic laboratory-adapted strains of BVD virus for VN tests, although immunoassay techniques are now available which make possible simple detection of the growth or neutralisation of non-cytopathogenic strains where this is considered desirable. No single strain is likely to be ideal for all circumstances, but in practice one should be selected which detects the highest proportion of serological reactions in the local cattle population. The most widely used are the two American cytopathogenic strains "Oregon C24V" and "NADL".

An outline protocol for a microtitre VN test is given below (7):

i) Heat inactivate the test sera for 30 minutes at 56°C.

ii) From a starting dilution of 1:5, make serial twofold dilutions of the test sera 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) To each well, add an equal volume (e.g. 50 µl) of a stock of cytopathogenic strain of BVD virus containing 100 TCID$_{50}$. A back titration of virus stock is also done in some spare wells to check the potency of the virus.

iv) Incubate for one hour at 37°C.

v) Trypsinise a flask of suitable cells (e.g. bovine turbinate, bovine testis) and adjust the cell concentration to $3 \times 10^5$ per ml. Add 50 µl cell suspension to each well of the microtitre plate.

vi) Either seal the plate, or incubate in 5% CO$_2$, for four days at 37°C.

vii) Examine the wells microscopically for cytopathic effect. 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-Karber method. A seronegative animal will show no neutralisation at the lowest dilution (1:5, equivalent to a final dilution of 1:10).

b) ELISA

Both indirect and blocking types of test can be used. 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 BVD virus. 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 X100 or 1-octyl-beta-D-glucopyranoside (OGP). Some workers have used fixed infected whole cells as antigen.

A complex-trapping-blocking ELISA has been described in ref. 13. An outline protocol for an indirect ELISA is given below (7).

i) Inoculate roller cultures of secondary calf testis cells with a high multiplicity of infection (about 1) of BVD virus strain Oregon C24V. Overlay with serum-free medium. Incubate 24 hours at 37°C.

ii) Scrape off and pellet the cells. Discard the supernatant medium. Treat the pellet with two volumes of 2% OGP in phosphate buffered saline for 15 min at 4°C. Centrifuge 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 pre-determined 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 phosphate buffered saline with 0.05% Tween 20 (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 EDTA, 1% polyvinyl pyrrolidone, pH 7.2) and added to virus- and control-coated wells for one hour at 37°C. The plates are then washed five times in PBST.

v) Rabbit anti-bovine IgG peroxidase conjugate is added at a pre-determined dilution (in serum diluent) for one 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 optical density read on an ELISA plate reader. The value obtained with control antigen is subtracted from the test reaction to give the final result for each serum.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

Infection via the oropharynx and respiratory tract is probably the most important route of transmission of the BVD virus on farms. Protection against spread in this way would have a beneficial effect in controlling disease due to the virus, particularly in the young animal. The formulation of a vaccine that will provide protection of 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 the USA. Traditionally, BVD vaccines have been based on a cytopathogenic strain of virus and fall into two classes: vaccine containing viruses treated or grown to make them less pathogenic (modified live virus) or vaccine containing inactivated viruses.

The use of vaccines containing live virus is now thought to be unacceptable because a cytopathogenic strain may precipitate mucosal disease by superinfection of persistently viraemic animals, and a non-cytopathogenic form may infect the fetus as described above. Live-virus vaccine may also be immunosuppressive and precipitate other infections. Vaccines containing killed virus are safe to use but probably require booster vaccinations to obtain satisfactory levels of immunity, which may be inconvenient.

1. **Seed management**

a) **Characteristics**

It is not yet possible to characterise, with any certainty, BVD virus isolates beyond their biotype. The separation and purification of the two biotypes from
an initial mixed culture depends on either:

- 3 cycles of a limiting dilution technique for the non-cytopathogenic virus or
- 3 cycles of plaque selection for the cytopathogenic virus.

When isolates have been cloned their identity should be confirmed by direct or indirect staining with specific antibody linked to fluorescein or enzyme.

An ideal vaccine should contain a strain (or strains) of virus which has been shown to give protection against the wide diversity of antigenicity that has been demonstrated with BVD virus.

b) Culture

Both biotypes will grow in a variety of cell cultures of bovine origin. Standard procedures may be used, with an expectation for harvesting non-cytopathogenic virus on days 6-7 and cytopathogenic 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.

c) Validation as a vaccine

It is crucial to ensure that the cell cultures and fetal bovine serum included in culture medium are free of adventitious BVD virus and antibody (described in section A) and other microorganisms.

2. Manufacture

There is no standard method for the manufacture of a BVD vaccine but conventional laboratory techniques with stationary, rolled or suspension (microcarriers) cell cultures may be utilised to prepare small batches. The techniques may also be used to prepare viral antigen for use in ELISA.

3. In-process control

Cultures should be inspected regularly to ensure they are free from contamination, and to monitor the health of the cells and the development or absence of cytopathic effect, as appropriate.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in the chapter on General Information.

b) Safety

It is essential that all the infectivity be killed during preparation of an
inactivated vaccine and samples should be subjected to several passages in cell culture to ensure the absence of live BVD virus. 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

Antigen content can be assayed by ELISA and adjusted as required to a standard level. However, the potency of the vaccine should be determined by inoculation into seronegative calves, followed by monitoring of the antibody response.

d) Duration of immunity

There is little 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. Unfortunately data is not available on the antibody levels that correlate with protection against respiratory infection or in utero infection.

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 a 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.

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SUMMARY

Viral haemorrhagic disease of rabbits (VHD) is a rapidly fatal disease of young adult and older rabbits. The causative agent of VHD has not been definitely identified, but is thought to be a calicivirus or parvovirus. The disease spreads rapidly by faecal to oral route or through infected rabbit tissue. Rabbits develop a high fever within 24-36 hours, and up to 90% die within another 24 hours with few clinical signs. Sometimes rabbits are found with a frothy bloody discharge from the nostrils resulting from terminal pulmonary congestion. The agent replicates in the small intestine, liver, and splenic lymphocytes in the marginal zone and in the red pulp. Death is usually the result of severe disseminated intravascular coagulation and massive venous thrombosis.

Identification of the agent: Post-mortem lesions of VHD are very characteristic. The liver has a fine reticular pattern of pale necrosis of the peripheral lobular tissue. Total infarction of the kidneys and spleen as well as pulmonary thrombosis and congestion of the lungs are common. The usual diagnostic test for VHD is haemagglutination of liver or spleen extract using human type O red blood cells (RBCs). Confirmatory tests include histopathology, electron microscopy, ELISA and immunopathologic staining using convalescent serum. Negative stain electron microscopy of liver or spleen extract reveals 25-35 nm particles with short surface projections.

Serological tests: Recovered rabbits can be identified serologically by the ELISA or haemagglutination inhibition (HI) tests using human type O RBCs and viral antigen prepared from infected rabbit liver.

Requirements for biological products: Antigen for the HI test is prepared from VHD infected liver, which may be inactivated with formalin or β-propiolactone inactivants. Convalescent antiserum is necessary for a positive control and for conjugation for immunostaining. Human type O RBCs are required for haemagglutination tests.

A. DIAGNOSTIC TECHNIQUES

Viral haemorrhagic disease of rabbits (VHD) is a rapidly fatal disease affecting mainly rabbits older than two months. The causative agent of VHD has not been definitely identified, but is thought to be a calicivirus or parvovirus (2, 5). The disease spreads rapidly by contact (faecal to oral route, or through infected rabbit tissue), but not by the air.

The clinical signs of VHD are few. There is a short incubation period of 24-36 hours followed by a high fever and mild lethargy. Within 12-24 hours of the onset of fever, there may be a variety of neurologic signs and distress just before death.
These may include sudden movements, collapse, opisthotonus, paddling, or a terminal squeal. A frothy, bloody discharge may flow from the nose terminally in a few cases. The terminal signs are so brief that rabbits are usually found dead.

1. Identification of the agent

Gross pathological lesions may be subtle but are characteristic. The most consistent lesion is a pale liver due to a diffuse fine reticular pattern of necrosis which can be seen on the capsular surface. The spleen is often congested and enlarged 2-3 times. The thymus is enlarged, often oedematous, and may show petechiae. The lungs are variably congested or haemorrhagic. Dark kidneys are seen in a few cases due to complete infarction of these organs. The digestive tract is usually full of ingesta. There may be a moderate catarrhal enteritis in the small intestine. Haemorrhages may occur in other tissues but are less consistent (1).

The histological lesion in the liver consists of severe, acute, diffuse portal and midzonal necrosis with nuclear swelling, margination of chromatin, and karyorrhexis. Small multiple intranuclear eosinophilic inclusion bodies can be found in swollen nuclei. Varying degrees of small intestinal necrosis can be found in most cases which are not autolysed. The necrosis is segmental and may be missed if multiple sections are not examined. There can be severe crypt and villous necrosis with nuclear changes similar to that seen in the liver, or there may be mild focal villous epithelial necrosis (1). Necrosis is also present in lymphocytes in the red pulp and marginal zone of the spleen. These nuclei are pyknotic and may contain intranuclear inclusion bodies. In addition, there is often diffuse fibrin deposition in the sinusoids of the red pulp of the spleen which obliterates most of the blood sinusoids. This latter lesion is due to a severe diffuse terminal coagulopathy which sometimes results in total infarction of the spleen. The lungs are similarly affected with major pulmonary venous thrombi. This results in acute to subacute alveolar congestion and haemorrhage, often with little or no inflammatory response. Similarly, the kidneys may be infarcted due to both renal vein thrombosis and diffuse glomerular microthrombosis. Microthrombi may be found in the brain associated with areas of acute ischaemic changes or in any other organ. The coagulopathy is remarkably severe and acute and is usually not found in rabbits killed before the development of terminal signs. Therefore, this coagulopathy is the likely cause of sudden death in this disease (2, 3).

The routine diagnostic procedure for VHD is the haemagglutination test (HA) using human type O red blood cells (RBCs) collected, stored overnight in Alsever's solution, and washed in 0.85% saline at pH 6.5 (7). Washed RBCs are suspended at 0.75% concentration in saline. Two-fold dilutions of clarified supernatant of a 10% tissue homogenate of liver or spleen are incubated with an equal volume of washed RBCs in a sealed round-bottom microtitre plate at 4°C or 25°C. After one hour of incubation, agglutination at an end point dilution of greater than 1:160 is considered positive. All positive results are confirmed with immunostaining or electron microscopy of liver tissue sections.

The double sandwich ELISA generally confirms the results of HA but may
sometimes detect antigen even in autolytic samples. Plates are coated with a trapping serum obtained from hyperimmunised diseased or vaccinated animals. Clarified tissue homogenates diluted 1:4 in phosphate buffered saline (PBS), containing 0.1% Tween 20 (PBST) are incubated in the plates for one hour at 37°C. Trapped antigen is detected with either guinea pig hyperimmune serum against highly purified VHD virus, biotinylated rabbit convalescent antibodies, or monoclonal antibodies. After washing, plates are incubated with anti-guinea pig peroxidase conjugate, streptavidin peroxidase, or anti-mouse conjugate respectively (6).

Tissues fixed in paraformaldehyde-lysine-periodate fixative and paraffin embedded, or cryosections fixed in methanol, can be immunostained using a direct method with fluorescein or biotin conjugates with the avidin-biotin alkaline phosphatase (ABC-AP) procedure of Hsu (4). Conjugates are made from the serum of recovered rabbits. Paraffin embedded tissue sections are thoroughly deparaffinised in xylene, rehydrated, trypsinised (0.1% for 30 minutes at 37°C), washed, and incubated with biotinylated antiserum (1:300) overnight. Fluorescein-conjugated antiserum may be applied directly on cryosections for one hour. Specific staining of the nuclei and cytoplasm of necrotic cells in the liver and spleen are characteristic. Some staining is also seen in the cytoplasm of macrophages in the mesenteric lymph node, appendix, colon, and thymus. Intravascular staining can be found in renal glomeruli and other capillaries. Routine formalin fixation cannot be used for this immunostaining procedure. Advanced autolysis may result in high non-specific immunostaining, especially on liver sections.

The VHD virus has not yet been cultivated in vitro despite numerous attempts in many primary cells, cell lines, embryonating eggs, and most conventional laboratory animals. Primary isolation can be carried out in laboratory rabbits. The virus can be isolated from the liver, blood, and spleen during the febrile period. The specific identification of the VHD agent as a virus has relied heavily on electron microscopy and immunopathological methods applied to infected rabbit tissue sections.

Electron microscopy of liver, spleen, and infected intestinal epithelium reveals similar cellular changes. Nuclear changes include nuclear swelling and margination of chromatin, with some nuclei in various stages of karyorrhexis or karyolysis. Most degenerate cell nuclei contain one or more dense irregular and sometimes globular inclusion bodies. Some inclusion bodies appear more organised, forming rows or patterns of small dense cores. Also found in these nuclei are numerous 25 nm hexagonal empty capsid-like structures. They are usually randomly scattered but can be found in linear and paracrystalline arrays (2).

The cell cytoplasm is swollen and vacuolated and contains abundant small (25 nm) dense spherical structures. Some of these are found in large numbers in vacuoles in the cytoplasm, often adjacent to the nucleus.

The haemagglutination inhibition (HI) test has been used, along with ABC-AP indirect immunostaining of infected spleen cryosections, with a panel of antisera. Negative staining with electron microscopy of liver or spleen extract reveals a 25-35 nm particle with short surface projections.
2. **Serological tests**

a) **Haemagglutination inhibition**

The HI test for serological diagnosis is rapid and simple (7). Antigen is prepared from infected rabbit liver taken freshly at death. The liver is homogenised in 10 volumes of Eagle's minimal essential medium, clarified by low speed centrifugation, and again at 10,000 g. The supernatant is filtered twice through a 0.22 µm filter, checked for the absence of bacterial contamination, and frozen at -70°C.

Before use in the HI test, the serum is inactivated (56°C for 30 minutes) and treated by adsorption to remove nonspecific activity. The inactivated serum (0.2 ml) is added to 1.8 ml of 25% kaolin in saline and allowed to stand for 20 minutes at 25°C and then centrifuged. The kaolin-adsorbed serum is then adsorbed with one drop of 30-60% packed human type O RBCs prepared as outlined above for HA. After 20 minutes at 25°C, the serum is clarified by centrifugation and used in the HI test. The adsorbed serum is considered to be at 1:10 dilution.

For the HI test, 0.025 ml of adsorbed serum is added to the first well of a round-bottom microtitre plate, and doubling dilutions are made in wells 2-8 using saline. An equal volume of antigen (8 HA units/0.025 ml) is added, and the plate is loosely covered and incubated at 25°C for 30-60 minutes. Human O RBCs (prepared as described previously) are added (0.025 ml) to each well and allowed to settle at 25°C for one hour. The antigen is titrated with each test to ensure that 8 HA/0.025 ml were used. The titre of the serum is the end-point dilution showing inhibition of haemagglutination. Serum HI titres greater than 1:80 are considered positive.

b) **Enzyme-linked immunosorbent assay**

A blocking ELISA based on the double sandwich ELISA described above was developed to detect antibodies against VHD virus. Plates are coated with trapping sera in carbonate/bicarbonate buffer, pH 9.6, overnight at room temperature. After washing with PBST, plates are successively incubated with an optimal dilution of antigen (clarified liver homogenate of diseased animals) for one hour at 37°C, with test sera diluted in PBST (16 h, 4°C), biotinylated antibodies to VHD virus (1 h, 37°C), and streptavidin-peroxidase (30 min, 37°C), with each step followed by washing. O-phenylenediamine is used as a substrate. After 15 min the colour reaction is stopped with 1.25 M sulphuric acid. Titres are expressed by 70% inhibition. A titre higher than 1:2 is considered positive. In some holdings antibody titres probably caused by a cross-reacting non-pathogenic agent can be detected by different serological tests (8).
B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Antigen for the HI test must be prepared from infected rabbit liver. This may be used as live antigen or may be chemically inactivated. For HI testing, a known positive convalescent control rabbit serum is required. Also, a regular source of human type O RBCs is required for HA or HI testing. Convalescent antisera may be conjugated to biotin or fluorescein for immunostaining of cryosections.

Several inactivated vaccines have been produced from extracts of VHD-infected liver. These may be inactivated using 0.5% β-propiolactone, 1.0% formaldehyde, or binary ethyleneimine. Either aluminium hydroxide or oil has been used as an adjuvant. These killed vaccines protect rabbits for up to six months and may be repeated if necessary in breeding stock. Usually meat-producing rabbits would not be vaccinated when they are marketed young (younger than 10-11 weeks). Though vaccination has been used to control major outbreaks in endemic regions, it is impossible to distinguish vaccinated rabbits from recovered rabbits clinically or serologically. Recovered rabbits may shed virus in their faeces for at least one month, which may lead to inadvertent spread of VHD into regions where the disease is not present. Eradication by 'stamping out' methods has been successful and should be considered in non-endemic areas.

These vaccines have been produced for emergency use and are not standardised. Inactivation of any vaccine must be assured by safety testing in rabbits.

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SUMMARY

Bovine spongiform encephalopathy (BSE) is a fatal neurological disease of adult cattle. The lesions in the brain, the epidemiological pattern, and the transmissibility of the disease indicate that BSE is one of the subacute spongiform encephalopathies caused by unconventional transmissible agents. BSE was first discovered in Britain in 1986. The archetype for this group of diseases is scrapie of sheep and goats (see B32).

At the time of writing (Nov. 1991), BSE has been confirmed in only one animal which has not been potentially exposed through food, although experimental transmissibility of BSE to cattle has been demonstrated following parenteral challenge with brain tissue from affected cattle. The current epidemic can be explained by oral exposure to a scrapie-like agent in the ruminant-derived protein of meat and bone meal included in proprietary concentrates or feed supplements. Since July 1988 in Great Britain and since January 1989 in Northern Ireland the feeding of ruminant-derived protein to ruminants has been prohibited, as it has now also been in the Republic of Ireland and Switzerland and to cattle in France. These last countries have reported a low incidence of BSE.

BSE is a progressive and fatal neurological disease of adult cattle with a peak incidence between four and five years of age. The clinical course is variable but can extend to several months. Clinical signs give rise to suspicion of disease and confirmation is by histopathology of the brain. Lesions have been described only in the central nervous system.

Identification of the agent: No diagnostic test for the BSE agent is available. BSE can be transmitted from brain tissue of terminally affected cattle to mice by intracerebral/intraperitoneal inoculation or by feeding, but a minimum incubation period of 292 days precludes bioassay from routine use. This is the only method currently available for detection of infectivity.

Serological tests: Immune responses have not been detected in scrapie or other transmissible spongiform encephalopathies and in the absence of antibodies there is no basis for such tests.

Other tests: Clinical signs are sufficiently distinctive to lead to suspicion of disease, particularly if differential diagnoses are eliminated.

To confirm the diagnosis of spongiform encephalopathy, neurohistological examination of the brain is necessary. The correlation between the clinical diagnosis and the neurohistological diagnosis can, with appropriate experience, be greater than 90 percent. The histopathological examination also
permits a differential diagnosis in cases with alternative pathology. The pathognomonic lesions are bilaterally symmetrical spongiform change in grey matter neuropil and neuronal vacuolation of certain brain stem nuclei.

Brain extracts treated with detergent and examined in the electron microscope show the characteristic fibrils homologous with scrapie-associated fibrils (SAF) in clinically affected animals and are regarded as diagnostic. Such fibrils are composed of a modified form of a host-encoded membrane protein (PrP) and are considered important in the pathogenesis of these diseases.

Requirements for biological products: There are no biological products available.

A. DIAGNOSTIC TECHNIQUES

BSE is invariably fatal, has an insidious onset and slowly progressive course (14, 16). Rarely, a case will present with acute signs and then deteriorate rapidly (16). Adult cattle from about two years old are affected, but most cases occur in animals four to five years of age.

There is no breed predilection but the incidence of affected herds by functional type is much greater for dairy than beef. Onset of clinical signs is not associated with season or stage of breeding cycle.

Presenting signs, though variable, usually include apprehensive and hyperaesthetic behavioural changes. For example, affected cows may be reluctant to enter the milking parlour or may kick vigorously during milking. In dry cows especially, pelvic limb incoordination and weakness can be the first clinical features to be noticed. Neurological signs predominate throughout the clinical course and may include many aspects of altered mental status and behaviour, abnormalities of posture and movement and aberrant sensation, but the most commonly reported nervous signs are apprehension, pelvic limb ataxia and hyperaesthesia to touch and sound. Affected cows will sometimes stand with low head carriage, the neck extended and the ears directed caudally (12). Abnormalities of gait include swaying of the pelvic quarters and pelvic limb hypermetria – features which are most readily appreciated when cattle are observed at pasture. Gait ataxia may also involve the pectoral limbs and, with advancing severity of locomotor signs, generalised weakness, resulting in falling and recumbency, can dominate the clinical picture. There has been no change in the clinical picture of BSE during the epidemic in the UK from 1985 to date (Nov. 1991), and it is essentially similar in other countries where BSE has occurred.

The intense and characteristic pruritic behaviour of many sheep with scrapie is not prominent in cattle with BSE, though in a proportion of cases there is rubbing and scratching activity. General clinical features of loss of bodily condition, decreasing liveweight and reduction in milk yield often accompany nervous signs as the disease progresses.
The protracted clinical course, extending usually over a period of weeks or months, eventually requires slaughter on welfare considerations. Where, however, a statutory policy for slaughter, diagnosis and destruction of cattle suspected to have BSE exists, a clinical diagnosis initiates slaughter procedures (1).

Early in the disease course the signs may be subtle, variable and non-specific and thus prevent clinical diagnosis on an initial examination. Continued observation of such equivocal cases together with appropriate clinical pathology procedures to eliminate differential diagnoses, especially metabolic disorders, will establish the essential progressive pattern of signs. Some early clinical signs of BSE may show similarities with features of nervous ketosis, hypomagnesaemia and encephalic listeriosis, and may sometimes be exacerbated following stress, such as that caused by transport.

1. **Identification of the agent**

As for scrapie, in the absence of *in vitro* methods for isolation of the causative agent, histopathological changes, confined to the central nervous system, provide the basis for confirmation of the clinical diagnosis of BSE (13, 14).

The histopathological changes are neurodegenerative and closely resemble those of sheep scrapie. The most prominent features are vacuolar and comprise a spongiform change in grey matter neuropil and single or multiple vacuoles within neuronal perikarya. The precise light microscopic appearance of the spongiform change in the transmissible spongiform encephalopathies has been defined previously (8). In contrast to natural scrapie of sheep, the spongiform change in BSE is the predominant form of vacuolar change. Both forms of vacuolation are bilaterally distributed and usually symmetrical with a constant distribution pattern throughout the brain stem (15).

The high frequency of occurrence of neuroparenchymal vacuolation in certain anatomic nuclei of the medulla oblongata at the level of the obex has, in the BSE epidemic in the UK, provided a satisfactory means of establishing a diagnosis on a single section of the medulla in greater than 99 percent of cases (13). However, observation of equivocal lesions in the medulla at this level requires examination of other brain areas to detect cases of BSE with potentially atypical or minimal lesions and to establish pathological differential diagnoses.

Neurodegenerative changes other than vacuolation are less prominent. A gliosis (astrocytosis), as seen in scrapie, is often present in sites of vacuolar change but clear demonstration of this may require specific immunostaining methods.

For the preparation of material for histopathological examination, cattle suspected of having the disease should be killed with an intravenous injection of a concentrated barbiturate solution.

The whole brain is then removed as soon as possible after death and placed in approximately 8 litres of 10 percent formol saline fixative which should be changed
twice weekly. After fixation for two weeks, the brain is cut into coronal slices. Initially a single block cut at the obex of the medulla oblongata should be selected for histological processing by conventional paraffin wax embedding methods for neural tissue. Sections, cut at 5 μm thickness and stained with haematoxylin and eosin, are examined for characteristic spongiform change and neuronal vacuolation.

The diagnosis may be confirmed if unequivocal changes are present in the medulla section, but in any other circumstance additional areas of brain, particularly the brain stem, should be examined as above.

Vacuoles within the perikarya, indistinguishable from those of BSE or scrapie, have been reported in neurones of the red and oculomotor nuclei in the midbrain as an incidental finding in cattle (4, 14), but have not been reported elsewhere in the brains of normal cattle. Thus, unlike the diagnosis of scrapie, which may be confounded by occurrence of such neuronal vacuolation scattered in the medullae of healthy sheep (see B32) (17, 18), histopathological diagnosis of BSE can be made with confidence of minimising false positive diagnoses.

As with scrapie of sheep (see B32), the possibility of BSE cases occurring in which brain lesions are minimal or undetectable by light microscopy is a potential problem which can be resolved only by diagnostic criteria independent of histopathology.

The demonstration of characteristic fibrils, the bovine counterpart of scrapie-associated fibrils (SAF) (see B32), by negative stain electron microscopy in detergent extracts of fresh brain tissue (9, 10, 14), and the detection of their constituent modified host protein by electrophoretic separation and immunoblotting techniques (5, 7) are additional diagnostic methods. They may be particularly useful when a histopathological diagnosis is difficult or impossible due to post-mortem decomposition (11).

BSE infection can be shown by intracerebral/intraperitoneal inoculation (6) or by feeding (2) of mice with brain tissue from terminally affected cattle, but bioassay is impractical for routine diagnosis because of the long incubation period required.

2. **Serological tests**

Homologies with scrapie, in which no immune response in the host has been detected, indicate that there is currently no basis for serological tests.

**B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

There are no biological products available.
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of vacuolated neurones in the medulla from apparently healthy sheep of
NEW WORLD SCREWWORM
(B84)

SUMMARY

The New World screwworm, Cochliomyia hominivorax (Coquerel), is an obligate parasite of mammals during its larval stages. The feeding of larvae several centimetres into the underlying tissues of the host causes a condition known as wound or traumatic myiasis which can be fatal. Infestations are generally acquired at sites of previous wounding due to natural causes or to animal husbandry practices, but they may also occur in the mucous membranes of bodily orifices.

Female flies are attracted to wounds, at the edges of which they lay an average of 200 eggs. The larvae emerge within 24 hours and immediately begin to feed, burrowing head-downwards into the wound. After two molts, the larvae leave the wound and drop to the ground into which they burrow to pupate. The duration of the life-cycle is temperature dependent, being shorter at higher temperatures, and may be completed in under three weeks in the tropics.

Treatment is effected by application of organophosphorus insecticides into infested wounds, both to kill larvae and to provide a residual protection against reinfection. Preventive measures include the spraying or dipping of susceptible livestock with organophosphorus compounds and strict control of the movement of animals out of infested areas.

Identification of the agent: The larvae of C. hominivorax may be easily confused with the larvae of other agents of myiasis, especially those of the Old World screwworm, Chrysomya bezziana Villeneuve, which has a very similar biology. Accurate diagnosis involves the identification of larvae extracted from the deepest part of an infested wound. The mature, third instar larvae are most reliable for this purpose and can be identified by their darkly pigmented dorsal tracheal trunks, extending from the twelfth segment forward to the tenth or ninth. This pigmentation is unique to the larvae of C. hominivorax among the species encountered in wound myiasis.

Adult flies are not directly harmful and the necessity for their identification is not so great as that for larvae. In the adult stage, species in the genus Cochliomyia may be separated from other genera by confirmation of a metallic blue-green body colour with three dark longitudinal stripes on the thorax. Separation of C. hominivorax from the very similar C. macellaria is discussed below.

Serological tests: There are no applicable serological tests, nor are they indicated in the identification of this disease.

Requirements for biological products: There are no biological products available except for the use of sterilised male flies in the sterile insect technique.
A. DIAGNOSTIC TECHNIQUES

The New World screwworm fly, Cochliomyia hominivorax (Coquerel) (Diptera: Calliphoridae), is an obligate parasite of mammals. Unlike most other species of blowfly, female screwworms do not lay their eggs on carrion. Instead, they lay them at the edges of wounds on living, injured mammals. Virtually any wound is attractive, whether natural (from fighting, predators, thorns, disease, tick and insect bites) or man-made (from shearing, branding, castrating, de-horning, docking, ear-tagging). Commonly infested natural wounds are the navels of newly-born animals and the vulval and perineal regions of their mothers, especially if traumatised. Although most larvae develop in wounds, they may also invade natural body openings such as the nostrils and associated sinuses, the eye orbits, mouth, ears and vagina, when eggs are deposited on mucous membranes.

Within 24 hours of the eggs being laid, larvae emerge and immediately begin to feed on the underlying tissues, burrowing gregariously head-downwards into the wound in a characteristic screwworm fashion. As they feed, tearing the tissue with their hook-like mouthparts, the wound is enlarged and deepened, resulting in extensive tissue destruction. Infested wounds have a characteristic odour which is attractive to gravid females which lay further batches of eggs. In a severe infestation that is left untreated, death may occur.

The larvae reach maturity about 5-7 days after hatching and leave the wound, falling to the ground into which they burrow and pupate. On completion of development, adult flies usually emerge from the puparium in the morning and mate within 1-3 days. About four days after mating, female flies are ready to oviposit. They seek a suitable host and lay an average of 200 eggs (range 10-490) in a flat, shingle-like batch, all eggs oriented in the same direction. Batches are laid at intervals of three days (14). Adult flies live on average for 2-3 weeks in the field.

The rate of development of the immature stages is influenced by environmental and wound temperatures, being slower at low temperatures although true diapause is not entered. This effect is most pronounced in the pupal stage, which can vary from one week to two months duration depending on the season (7). Thus, the complete life cycle may take 2-3 months in cold weather (8), while in temperate conditions with an average air temperature of 22°C it is completed in ca 24 days (7) and in tropical conditions of about 29°C it is completed in ca 18 days (14).

The degree to which C. hominivorax can tolerate cold has had a major influence on its distribution. Historically, its range extended from the southern states of the USA, through Mexico, Central America, the Caribbean islands and northern countries of South America to Uruguay, northern Chile and northern Argentina (6). The distribution contracted during the winter months but expanded during the summer months, producing a seasonality at the edges of the distribution and year-round populations in the central areas of the distribution, the New World tropics. Following a major control programme in the USA and Mexico utilising the sterile insect technique (SIT), the northern limits of C. hominivorax are now the border regions between Mexico, Guatemala and Belize (5). In 1988 C. hominivorax
was introduced into Libya in North Africa and threatened to become firmly established there (2, 3), but it has since been eradicated thanks to an intensive campaign using the sterile insect technique.

The parasite most similar biologically to the New World screwworm is the Old World screwworm, *Chrysomya bezziana* Villeneuve. This is also an obligate parasite and, like *C. hominivorax*, gravid females oviposit at the edges of wounds on mammals, the emerging larvae producing wound myiasis. Its distribution is confined to the Old World as the name suggests, throughout much of Africa (from south of the Sahara to northern South Africa), the Indian subcontinent and southeast Asia (from Southern China through the Malay Peninsula and the Indonesian and Philippine islands to New Guinea) (6, 13, 15). It has also been introduced into several countries on the west coast of the Persian Gulf (11). The climatic requirements of the two screwworm species are very similar and their potential distributions if unrestrained would overlap considerably (13).

Organophosphorus insecticides such as dichlofenthion, fenchlorphos and, in particular, coumaphos are recommended for the treatment of wounds infested with screwworm (4). They have the effect of expelling the larvae, which die on the ground. To provide residual protection against reinfection they must be applied at 2- to 3-day intervals until the wound has healed. The contents of individual wound treatment sachets (e.g. 5 g of 5% coumaphos wettable powder) should be either sprinkled directly onto a wound or, more effectively, brushed into the wound as a paste after first being mixed with ordinary cooking oil (33 ml).

Organophosphorus compounds may also be applied as aerosol sprays, in which marker dyes and bacteriostats are included, or as dusts which are puffed into the wound from plastic squeeze bottles. Dichlofenthion is used as a 1% aerosol to treat cases due to *C. hominivorax* in South America and is also effective against *Ch. bezziana* (9). Any larvae that die in the wound should be removed to prevent sepsis. Close attention should always be paid to the manufacturer’s safety instructions.

Direct prevention of screwworm infestation is achieved by spraying or dipping of livestock with coumaphos (0.25% aqueous suspension of 50% wettable powder) or other organophosphorus insecticide at the maximum concentration prescribed for external parasite control. The effects of such treatment are twofold: firstly, direct killing of larvae and provision of residual protection; secondly, the killing of ticks and other external parasites means there are fewer wounds available as sites for oviposition.

Dipping or spraying would be indicated if any member of a group of animals were found infested, or if animals were transiting through or leaving an infested area, or following wounding animal husbandry practices, e.g. shearing. A single subcutaneous injection of ivermectin (200 µg/kg) was effective against *Ch. bezziana* in preventing navel strike of new-born calves (9) and scrotal strike of castrated calves (12) and also prevented restrike of treated wounds of adults. However, it may be ineffective against *C. hominivorax* (Mackley & Brown, in 5).
REMOVE LARVA FROM WOUND AND EXAMINE DORSAL ASPECT

'Hairy' larva with obvious body processes

'Smooth' larva, no obvious body processes

Chrysomya albiceps
Chrysomya rufifacies

EXAMINE DORSAL TRACHEAL TRUNKS IN POSTERIOR SEGMENTS

Trunks darkly pigmented forwards from 12th to 10th or 9th segment

Cochliomyia hominivorax

Trunks not darkly pigmented

Other species

Fig. 1. Key for the diagnosis of third instar larvae of Cochliomyia hominivorax from cases of wound myiasis.
Indirect prevention of screwworm infestation includes the avoidance of wounding procedures at the time of year when screwworm are numerous, the careful handling of livestock to minimise wounding, the removal of sharp objects (e.g. wire strands) from livestock pens, and the use of measures to reduce other parasites which cause wounding, in particular ticks, e.g. by dipping and by insecticide-impregnated ear-tags.

To prevent the spread of the disease beyond present limits, strict observation of the requirements for international trade, as set out in the *OIE International Animal Health Code*, is necessary.

1. Identification of the agent

The identification of the eggs and first instar larvae of the agents of myiasis is difficult and as these stages are seldom encountered during the collection of specimens from infested wounds they will not be considered further.

Larvae being collected for diagnosis should be removed from the deepest part of the wound to reduce the possibility of collecting non-screwworm species which may infest the shallower parts of the wound. Living specimens should be examined for pigmentation of the tracheal trunks (Fig. 1 and see below) and then be placed directly into a tube containing 70% alcohol and returned to the laboratory for examination under a dissecting microscope at up to x 50 magnification (for further techniques see 10, 15).

Second instar larvae: Second instar larvae of *C. hominivorax* may be collected in routine sampling and can be diagnosed by the presence of dark pigmentation of the dorsal tracheal trunks for over half their length in the terminal segment (Fig. 2). Other species have less marked pigmentation of the dorsal tracheal trunks, e.g. for no more than a third of their length in the twelfth segment of *Ch. bezziana*. More positive identification may be gained by rearing the larvae to third instar on a medium of 1 l water, 1 kg ground meat, 70 g dried blood and 1.5 ml formalin, mixed and maintained at 35-38°C and 70% RH.

Fig. 1. Dorsal aspect of terminal segment of second instar of Cochliomyia hominivorax showing pigmentation of dorsal tracheal trunks.

Third instar larvae: Third instar larvae of *C. hominivorax* have a robust, typical maggot shape, with a cylindrical body from 6-17 mm long and 1.6-3.5 mm wide (7).
Fig. 3. Diagnostic characteristics of third instar larva of Cochliomyia hominivorax: A) whole larva, lateral aspect; B) posterior face of terminal segment; C) posterior spiracle (a, anterior spiracle; b, button adjacent to opening in peritreme; p, peritreme; sl, slit; sp, spines). (After Laake et al. (7).)
Fully mature larvae average 15-16 mm in length and develop a reddish tinge over the creamy white colour of younger larvae. The prominent rings of spines around the body are complete on the anterior margins of segments 2-9 and almost complete on segment 10 with a slight break dorsally. The spines are large compared to non-screw worm species, the longest averaging 130 µm. The anterior spiracles have from 6-11 well separated branches each, but usually 7-9. On the posterior face of the terminal segment, the posterior spiracles have a darkly pigmented, incomplete peritreme enclosing three straight, slightly oval shaped slits, which point towards the break in the peritreme. The diagnostic features mentioned above are illustrated in Fig. 3. Of greatest diagnostic value are the dorsal tracheal trunks which extend forwards from the posterior spiracles and are darkly pigmented up to the tenth or ninth segment (Fig. 1; see also 6, 10, 15 for identification keys). This is most readily appreciated in living larvae. Those in preservative may need dissection to remove opaque tissues covering the trunks.

Adult: Identification of adult C. hominivorax is seldom required for the diagnosis of myiasis as the larval stages are those most apparent to livestock owners and veterinary personnel. However, a brief description follows. The body length averages 8-10 mm and has a deep blue to blue-green metallic colour, with three dark longitudinal stripes on the dorsal surface of the thorax. This combination of colour and pattern is not shared by any other species involved in wound myiasis.

![Image of Cochliomyia hominivorax](image)

**Fig. 4. Diagnostic characteristics of adult Cochliomyia hominivorax; note longitudinal thoracic stripes (b, basicosta; p, parafrontal, indicated from above on whole Cochliomyia hominivorax and laterally on head of typical calliphorid fly; s, lower squama, surface hairless except at base; v, stem vein with hairs on dorsal posterior surface). (After Laake et al. 7.)**
except the New World *Cochliomyia macellaria* (Fabricius). The *Cochliomyia* species may be separated by the presence of black hair on the lower half of the parafrontalia of *C. macellaria*. In addition, females of *C. hominivorax* have a dark brown-black basicosta whereas those of *C. macellaria* have a yellow basicosta (Fig. 4; see 1, 7).

2. **Serological tests**

No serological tests are available for diagnosis, nor are they indicated for this disease.

B. **REQUIREMENTS FOR BIOLOGICAL PRODUCTS**

There are no biological products such as vaccines available, but the only proven method of eradication of *C. hominivorax* relies on a biological technique, the sterile insect technique (5).

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ENTEROVIRUS ENCEPHALOMYELITIS
(previously Teschen disease)
(B85)

SUMMARY

Enterovirus encephalomyelitis (previously Teschen disease) is a severe form of encephalomyelitis of swine. A milder form of the disease in the United Kingdom (Talfan disease) and poliomyelitis suum in Denmark have also been described, and both forms of the disease are caused by a porcine enterovirus type I, Teschen Talfan virus or TTV). The viruses involved in these conditions belong to the genus Enterovirus in the Picornaviridae family.

The disease was first described in Czechoslovakia in 1929. During the '40s and '50s it caused serious losses in European countries. The clinical disease is now rare in Europe, although serological evidence in some countries indicates that apathogenic variants of the virus circulate in pig populations.

The most suitable methods for diagnosis are demonstration of characteristic microscopic lesions in the central nervous system of affected pigs, isolation and subsequent identification of TTV, and demonstration of specific antibodies. At post-mortem, microscopic lesions occur in the grey matter of the brain and in the spinal cord, which are characterised by inflammatory lesions with perivascular lymphocyte infiltration. The later stages of the disease are characterised by a degeneration of neurons and their replacement by glial connective tissue.

Identification of the agent: Tissue suspensions of brain and spinal cord from affected pigs are inoculated into tissue culture derived from swine kidney. If TTV is present, it gives rise to specific cytopathogenic effects characterised by round refractile cells. For TTV identification a suitable serological test is employed using specific antiserum against the reference strain 'Zabreh'. Virus neutralisation tests and indirect fluorescent antibody tests are preferred.

Serological tests: For screening for specific antibodies in pig populations it is usual to use the virus neutralisation test in microtitre plates or the enzyme-linked immunosorbent assay (ELISA).

Requirements for biological products: Vaccines are available. Inactivated vaccine produced from virus propagated on tissue cultures from swine kidney is recommended for active immunisation. The virus is usually inactivated by formaldehyde or other suitable inactivants having a linear rate of inactivation, and adsorbed onto aluminium hydroxide or mixed with oil adjuvant. The vaccine is injected subcutaneously if adjuvanted or intramuscularly.

For serological diagnosis it is necessary to have a reference strain of TTV propagated in cell cultures and hyperimmune serum monospecific for TTV.
A. DIAGNOSTIC TECHNIQUES

Enterovirus encephalomyelitis (previously Teschen disease) is an acute condition of swine characterised by central nervous system disorders. Teschen is the name of a town in Czechoslovakia where the disease was first recognised in 1929 (4). The causal agent is a porcine enterovirus type I (2, 5), Teschen Talfan virus (TTV), which is a member of the genus Enterovirus belonging to the family Picornaviridae (1). The porcine encephalomyelitis observed in Canada during the 1960s was caused by a coronavirus and is not discussed in this manuscript.

All strains of Teschen Talfan viruses are antigenically very similar but nevertheless they can be divided into three antigenic subtypes (9). Clinical manifestations of the disease depend on the extent of lesions in the central nervous system. Most often ataxia, leg paralysis and convulsions are observed. An important epizootiological fact is that the TTV is pathogenic for swine only, affecting weaners most frequently. Small quantities of the virus are present in faeces over a long period after clinical recovery and thus convalescent animals become a latent source of infection. The virus propagates intensively in the central nervous system of affected animals and can be detected most easily from brain and spinal cord. The pathological lesions caused by the virus are encephalitis and non-purulent myelitis associated with degeneration of motor ganglion cells.

Laboratory diagnosis of the disease is based on histological examination of brain and spinal cord, on identification of the agent in the central nervous system of affected pigs, and on the detection of specific antibodies in the blood of convalescent animals.

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. Microscopic examination reveals non-suppurative perivascular lymphocyte infiltrations (4). The infiltrations are confined to the grey matter with occasional transmission into the white matter. Changes are observed in the ventral horns of the spinal cord, in the diencephalon and cerebellum 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. Non-purulent leptomeningitis, especially in the cerebellum, is often seen.

1. Identification of the agent

Samples of brain and spinal cord are collected from pigs slaughtered during the clinical stage of the disease. The samples should be placed into a solution prepared from equal parts of phosphate buffered isotonic saline (PBS) at pH 7.4 and glycerol.

Pieces of tissues are minced to prepare a 10% (w/v) suspension in PBS solution. The suspension is centrifuged lightly and the supernatant fluid is used for inoculation of tissue cultures. Monolayer cultures of primary swine kidney cells or established cell lines derived from swine kidney, for example PK15 or IBRS-2, are most suitable for isolation of TTV.
Ten tubes with monolayers of cells are labelled and opened to discard the growth medium. Each tube is then inoculated with 0.1 ml of suspect tissue homogenate, closed and placed on a roller drum. After 1 hour, the inoculum is discarded and each tube is washed three times with PBS. Finally each tube is replenished with 1 ml of maintenance medium without calf serum. The tubes are then returned to the roller drum and incubated at 37°C. Microscopic examination should be performed daily. If the sample contains live TTV, development of characteristic cytopathic effect (CPE) will be seen after 3-4 days. The CPE is characterised by small foci of rounded refractile cells. With further incubation, the CPE generalises and all cells detach from the glass. After several passages the virus grows better and produces complete CPE after 24-48 hours. The identity of TTV can be confirmed by the use of specific antiserum. Virus neutralisation and indirect fluorescent antibody tests are the best suited for this purpose. Once an isolate has been identified serologically as type I, piglet inoculation is the only certain means of determining that the given isolate is highly virulent.

Reference antisera have been prepared by hyper-immunisation of rabbits or colostrum-deprived sucking pigs with the reference strain of virus strain 'Zabreh'.

a) Virus neutralisation test for TTV identification

The virus harvested from cell cultures is diluted in tissue culture maintenance medium over a range 10^{-4} to 10^{-6} in ten-fold steps. Two rows of dilutions are prepared, equal volumes of reference antiserum diluted 1:10 are added to the first row and of negative serum to the second row. Mixtures are incubated overnight at +2°C and thereafter inoculated into roller tube cultures or into wells of microtitre plates. Assessment is done 72 hours later, based on the appearance of CPE. The diagnosis of TTV is confirmed if the titre of the isolated virus in the presence of positive serum is at least 10^3 lower than the virus incubated with the negative serum.

b) Indirect fluorescent antibody test for the confirmation of TTV antigen in cells

The indirect fluorescent antibody 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 antiglobulin, using a microscope with UV or blue light source. The antigen is detectable in cells 12 hours after the infection with TTV, i.e. before the development of CPE. Cross-reactivity with other enterovirus serotypes may be a problem.

The method consists of the following steps:

i) Monolayers of swine kidney cells on coverslips are inoculated with the suspected material. Positive and negative specimens are examined in parallel.

ii) After 12 hours the coverslips are removed, washed twice in PBS and fixed in cold acetone for 5 minutes.
iii) The coverslips are placed into a wet box and flooded with rabbit or pig hyperimmune anti-TTV serum optimally diluted 1:10 with PBS.

iv) The wet box is closed and incubated at 37°C for 60 minutes.

v) The coverslips 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 coverslips are then washed three times with PBS, air dried and mounted in 0.1 M Tris-buffered glycerol, pH 8.6.

After processing the coverslips 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.

2. **Serological tests**

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 virus neutralisation test is the most useful. Originally the test was carried out in tubes using swine kidney cell cultures as the indicator system for infectivity. Later the microtitre modification was introduced, in the interests of economy. Recently an ELISA test has been developed which is more sensitive and rapid in use although it requires special equipment and considerable laboratory experience (3).

a) **Virus neutralisation test in microtitre plates**

Flat-bottomed plastic microtitre plates with 96 wells are used in the test (10). The serum to be tested is diluted in 2-fold steps from 1:2 up to 1:256 in volumes of 50 μl. Dilutions are prepared in duplicate. Minimum essential medium (MEM) supplemented with 5% fetal calf serum and antibiotics is used as a diluent. The virus is diluted to contain 10^2 TCID₅₀ per 50 μl and 50 μl of this working stock is added to each serum dilution. The mixtures are shaken and incubated for one hour at room temperature. A constant amount of 50 μl of cell suspension with a density of 5 x 10⁵ cells/ml is then added. After further shaking, the plates are sealed and incubated at 37°C in a CO₂ atmosphere. Controls including positive and negative reference sera and controls of cells with or without the virus are also included. Plates are examined for virus-specific CPE using an inverted microscope. The results can be assessed after 48-72 hours owing to the rapid progress of the CPE. Virus neutralisation titres are regarded as positive if the corresponding serum neutralised the virus at a dilution of 1:8 or higher. Back titration of the test virus is recommended.

b) **Enzyme-linked immunosorbent assay (ELISA)**

An alternative method for the detection and titration of specific antibodies
against TTV is the ELISA technique (3). The test is performed in microtitre plates using TTV grown on tissue cultures as antigen. The technique can be carried out using the following steps.

i) Plates are sensitised with pre-diluted antigen in 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 PBS-T (buffered saline solution containing 0.05% Tween 20). 50 μl of the diluted sera are placed into each of two wells with positive antigen and into two wells with negative antigen. The plate is incubated for one hour at 37°C.

iv) The plates are washed five times with PBS-T.

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 one 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 at 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 optical densities (OD) of the wells are measured at a wavelength of 492 nm, preferably using an automatic multi-channel spectrophotometer with print-out mechanism.

ix) The OD of a serum is the mean reading of two wells with positive antigen minus the mean reading of two wells with negative antigen. OD readings of test sera which exceed by more than two-fold the mean reading of standard negative sera are regarded as positive.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

The following biological products are necessary for the diagnosis of enterovirus encephalomyelitis and for active immunisation:

a. Reference strain of the TTV
b. Specific hyperimmune serum
c. Vaccine against enterovirus encephalomyelitis, prepared from the virus
propagated in cell cultures.

Reference strain of the TTV

**Characteristics:** Following long experience, the strain 'Zabreh', isolated in Czechoslovakia during the period of peak incidence of the disease, has been selected as the reference strain. 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.

**Stock virus:** The strain 'Zabreh' is propagated on monolayers of tissue culture either from primary swine kidney or from an established cell line, for example PK15. A 10% suspension in PBS at pH 7.4 is prepared from the brain and spinal cord of piglets infected experimentally with TTV. The suspension is centrifuged and the supernatant is used for the inoculation of cell cultures. The procedure for the cultivation of TTV 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 one hour of incubation at 37°C the fluid is decanted, the cultivation vessel rinsed with buffered saline, and the cells overlayed with serum-free Earle's medium with antibiotics. Cytopathic effect is apparent within 48 hours and the monolayer disintegrates completely during the next 48-72 hours. In the subsequent 3-5 passages in cell cultures, 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$_{50}$ 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. TTV 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 non-cytopathogenic. The stock virus should be divided into small aliquots and preserved at -60°C. Frozen virus retains its properties for several years. For use 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 rabbits or colostrum-deprived piglets with TTV. The reference strain 'Zabreh' 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 3 doses of 2 ml of virus suspension plus 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.

**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). Initially the brains and spinal cords of experimentally infected piglets were used as antigen for vaccine manufacture. After establishing the cultivation of TTV virus on monolayers of porcine kidney cells (11), the virus propagated *in vitro* is used to prepare vaccine. The following two types of vaccines have been developed:

i) Killed vaccines prepared from virus inactivated as described below (point 2) and adsorbed onto aluminium hydroxide or mixed with oil adjuvant for parenteral administration (6, 8).

ii) Live attenuated virus vaccines obtained by serial passages in swine kidney cultures. This type of vaccine has been successful when administered intranasally or perorally (6, 7), but is not commercially available.

1. **Seed management**

   a) **Characteristics**

   Local strains of TTV isolated from natural outbreaks of enterovirus encephalomyelitis are recommended as antigen for inactivated vaccine production.

   b) **Culture**

   The virus is propagated on tissue cultures and specificity is confirmed by neutralisation tests as described in part A, 2a. Viruses shown to be identical to the reference strain are tested for freedom from contamination and the virus content is estimated by titration on monolayer cultures of porcine kidney cells. The master seed is distributed in small aliquots and stored at -60°C. It is recommended that at least three such strains of TTV are maintained.

2. **Manufacture**

The viruses from master seeds are propagated on tissue culture to a sufficient volume for the production of one batch of vaccine. The best facility for virus production is a roller drum apparatus turning at approximately eight revolutions per hour. The harvested virus is checked for specificity, sterility and virus content. The virus has to be neutralised by reference hyperimmune serum and be free of any microbiological contamination. The virus should contain $10^6$ TCID$_{50}$ per ml when titrated on swine kidney cell cultures. The batch of virus is inactivated using
formaldehyde or other suitable chemicals such as binary ethylenimine. Where formaldehyde is used this can be done by employing formalin at a concentration of 1:2,500 at 37°C for 48 hours. The virus must be tested to show complete inactivation before it is adsorbed onto aluminium hydroxide or mixed with mineral oil emulsion adjuvant.

3. **Batch control**

a) **Sterility**

Tests for sterility and freedom from contamination may be found in the chapter on General Information.

b) **Safety**

The safety test is carried out by intranasal inoculation of 2-4 piglets at 2 months of age with double the field dose of the final product vaccine. The animals are observed for 21 days, during which time they must remain healthy, without any signs of disease which could be attributable to the vaccine.

c) **Potency**

The potency test is performed on five piglets, at 2 months of age, inoculated with the minimum recommended dose. After 21 days the vaccinated piglets, together with two unvaccinated controls, are challenged intranasally with 10 LD$_{50}$ of a virulent field strain of TTV. The potency of the vaccine is satisfactory if 60% of the vaccinated animals survive with no signs of disease but the two controls fall ill within 14 days. Potency may also be estimated by measuring the increase in specific antibodies as described in part A, 2a and b. A serum neutralising titre of at least 1:32 is required. For subsequent batches it is sufficient to carry out the serum neutralisation test.

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### Diseases/Maladies/Enfermedades

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*This list is updated annually and the revised list will be published in the May issue of the OIE Bulletin.*
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Tel: (12) 52.99.101, Fax: (12) 55.65.73 |
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<td>Bluetongue</td>
<td>Dr B.J. Erasmus</td>
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<td>Fièvre catarrhale du mouton</td>
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<td>Lengua azul</td>
<td>Tel: (12) 52.99.101, Fax: (12) 55.65.73</td>
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<td>Dr J.E. Pearson</td>
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<td>National Veterinary Services Laboratories, Animal and Plant Health Inspection Service, USDA, P.O. Box 844, Ames, IA 50010, UNITED STATES OF AMERICA</td>
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<td>Tel: (515) 239.85.51/82.00, Fax: (515) 239.84.58</td>
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<td>Dr J. Anderson</td>
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<td>Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey GU24 ONF, UNITED KINGDOM</td>
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<td>Tel: (483) 23.24.41, Fax: (483)-23.24.48, TLX: 859137 AVRI G</td>
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<td></td>
<td>Dr B.T. Eaton</td>
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<td>CSIRO, Australian Animal Health Laboratory, Division of Animal Health Institute of Animal Production and Processing, Ryrie Street P.O. Bag 24, Geelong, Victoria 3220, AUSTRALIA</td>
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<td></td>
<td>Tel: (52) 26.5222, Fax: (52) 23.1424, TLX: AA 38923</td>
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<td>African horse sickness</td>
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<td>Peste équine</td>
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<td>Peste equina</td>
<td>Tel: (12) 52.99.101, Fax: (12) 55.65.73</td>
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<td>Dr P.S. Mellor</td>
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<td>Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey GU24 ONF, UNITED KINGDOM</td>
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<td>Tel: (483) 23.24.41, Fax: (483)-23.24.48, TLX: 859137 AVRI G</td>
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<td></td>
<td>Dr J. House</td>
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<td>National Veterinary Services Laboratories, Animal and Plant Health Inspection Service, USDA, P.O. Box 848, Greenport, NY 11944, UNITED STATES OF AMERICA</td>
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<td>Tel: (516) 323.25.00, Fax: (516) 323.25.00625</td>
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<td></td>
<td>Dr J.M. Sanchez-Vizcaino</td>
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<tr>
<td></td>
<td>Animal Health Department, INIA, Embajadores 68, Madrid 28012, SPAIN</td>
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<td>Tel: (1) 527 39 50, Fax: (1) 527 58 41</td>
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| African swine fever             | Dr C. Mebus  
National Veterinary Services Laboratories, Animal and Plant Health Inspection Service, USDA, P.O. Box 848 Greenport, NY 11944  
UNITED STATES OF AMERICA  
Tel: (516) 323.25.00, Fax: (516) 323.25.07 |
| Peste porcine africaine         | Dr P.J. Wilkinson  
Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey GU24 ONF  
UNITED KINGDOM  
Tel: (483) 23.24.41, Fax: (483)-23.24.48, TLX: 859137 AVRI G |
| Peste porcina africana          | Dr J.M. Sanchez-Vizcaino  
Animal Health Department, INIA, Embajadores 68, Madrid 28012  
SPAIN  
Tel: (1) 527 39 50, Fax: (1) 527 58 41 |
| Classical swine fever           | Dr Z. Pejsak  
Veterinary Research Institute, 24-100 Pulawy  
POLAND  
Tel: 30.51, TLX: 0642401 |
| Peste porcine classique         | Dr M. Shimizu  
National Institute for Animal Health, Second Research Division  
3-1-1 Kannondai, Tsukuba-City, Ibaraki-pref.  
JAPAN 305  
TLG: VETLAB MITUKAIDO |
| Peste porcina clásica           | Prof. B. Liess  
Director, Hannover Veterinary School, Institute of Virology, Bischofsholer Damm 15, D-3000 Hannover 1  
GERMANY  
Tel: (511) 856.8841, 856.8842, Fax: (511) 856 8898, TLX: 922034 tiho d |
|                                 | Dr S. Edwards  
Central Veterinary Laboratory Weybridge, New Haw, Addlestone, Surrey KT15 3NB  
UNITED KINGDOM  
Tel: (932) 34.11.11, Fax: (932) 34.70.46, TLX: 262318 VetWey G |
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| Newcastle disease and highly pathogenic avian influenza (fowl plague) | Dr. D.J. Alexander  
Central Veterinary Laboratory Weybridge, New Haw, Addlestone,  
Surrey KT15 3NB  
UNITED KINGDOM  
Tel: (932) 34.11.11, Fax: (932) 34.70.46, TLX: 262318 VetWey G  
Prof. Dr. E.F. Kaleta  
Director, Institut für Geflügelkrankheiten der Justus-Liebig-Universität Giessen  
Frankfurter Strasse 87, D-6300 Giessen  
GERMANY  
Tel: (641) 702-4865 or 4867 |
| Maladie de Newcastle et influenza aviaire hautement pathogène (peste aviaire) | Dr. T. Della-Porta  
CSIRO, Australian Animal Health Laboratory, Division of Animal Health Institute of Animal Production and Processing, Ryrie Street  
P.O. Bag 24, Geelong, Victoria 3220  
AUSTRALIA  
Tel: (52) 26.5222, Fax: (52) 23.1424, |
| Enfermedades de Newcastle y influenza aviar altamente patógena (peste aviar) | Dr. B. Panigrahy  
National Vet. Services Laboratories and National Animal Disease Center  
P.O. Box 70, Ames, IA 50010  
UNITED STATES OF AMERICA  
Tel: (515) 239-8325, Fax: (515) 239-8458 |
| Aujeszky's disease | Prof. B. Toma  
Services des Maladies Contagieuses, Ecole Nationale Vétérinaire d'Alfort  
7 avenue du Général de Gaulle, 94704 Maisons-Alfort Cedex  
FRANCE  
Tel: (1) 43 68 73 34, Fax: (1) 43 96 71 31 |
| Maladie d'Aujeszky | Dr. E. Mocsari  
Director, Veterinary and Food Control Service, Central Veterinary Institute  
Tabornok u. 2., Budapest, 1149  
HUNGARY  
Tel: 36(1) 252-7278, Fax: 36(1) 252-5177, TLX: 22-6377 |
| Enfermedad de Aujeszky | Dr. T.G. Kimman  
Central Veterinary Institute, Bacteriology Department,  
P.O. Box 65, 8200 AB Lelystad  
THE NETHERLANDS  
Tel: (3200) 73 911, Fax: (3200) 73 473 |
|  | Dr. M. Frey  
National Veterinary Services Laboratories and National Animal Disease Center  
P.O. Box 70, Ames, IA 50010  
UNITED STATES OF AMERICA  
Tel: (515) 239-8325, Fax: (515) 239-8458 |
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| Leptospirosis                  | **Dr W.J. Terpstra**  
Royal Tropical Institute, N.H. Swellengrebel Lab. of Tropical Hygiene  
Meibergdreef 39, 1105 AZ Amsterdam-Z.O.  
THE NETHERLANDS  
Tel: (20) 566 5440/5441, Fax: (20) 566 4440 |
| Leptospirose                   | **Dr W.A. Ellis**  
Department of Agriculture, Veterinary Sciences Division  
Stormont, Belfast, BT4 3SD, Northern Ireland  
UNITED KINGDOM  
Tel: (232) 76.00.11, Fax: (232) 76.16.62 |
| Leptospirosis                  | **Dr M.A. Saravi**  
Director, Instituto de Bacteriología, CICV, INTA, Castelar  
Casilla de Correo 77, Moron 1708, Pcia. de Bs. Aires  
ARGENTINA  
Tel: 621 17 43, Fax: 54 111 1917 |
| Rabies                         | **Dr D. Miller and Dr Carole Bolin**  
National Vet. Services Laboratories and National Animal Disease Center  
P.O. Box 70, Ames, IA 50010  
UNITED STATES OF AMERICA  
Tel: (515) 239-9548, Fax: (515) 239-8397 |
| Rage                           | **Dr A. Wandeler**  
Pathology Section, Animal Diseases Research Institute, 3851  
Fallowfield Road  
P.O. Box 11300, Station H, Nepean, Ontario K2H 8P9  
CANADA  
Tel: (613) 998.9320, Fax: (613) 952.2285 |
| Rabia                          | **Dr J. Barrat**  
Laboratoire d’Etudes sur la Rage et la Pathologie des Animaux Sauvages, (CNEVA)  
BP 9, 54220 Malzeville  
FRANCE  
Tel: 83 29 26 08, Fax: 83 29 33 13 |
| Rabies                         | **Dr L. Schneider**  
Bundesforschungsanstalt für Viruskrankheiten der Tiere (Federal Research Centre for Animal Virus Diseases), Paul-Ehrlich-Str. 28, P.O. Box 1149, D-7400 Tubingen  
GERMANY  
Tel: (7071) 60.31, Fax: (7071) 60.32.01 |
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<th>Diseases/Maladies/Enfermedades</th>
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| Bovine tuberculosis and paratuberculosis | Dr. J. Haagsma  
Central Veterinary Institute, Bacteriology Department,  
P.O. Box 65, 8200 AB Lelystad  
THE NETHERLANDS  
Tel: (3200) 73.911, Fax: (3200) 73.473, TLX: 40227 |
| Tuberculosis bovina y Paratuberculosis | Mme Marie-Françoise Thorel  
Laboratoire Central de Recherches Vétérinaires, (CNEVA)  
22 rue Pierre Curie, BP 67, 94703 Maisons-Alfort  
FRANCE  
Tel: (1) 49 77 13 00, Fax: (1) 43 68 97 62 |
| Brucellosis | Prof. Dr. Protz  
Bundesgesundheitsamt (Federal Health Office), Institut für Veterinärmedizin,  
(Robert von Ostertag-Institut)  
Thielallee 88-92, Postfach: 330013, D-1000 Berlin 33  
GERMANY  
Tel: (30) 83 08 2225/83 08 2235, Fax: (30) 83 08 2741 |
| Brucelose | Mr. A. P. MacMillan  
Central Veterinary Laboratory Weybridge, New Haw, Addlestone,  
Surrey KT15 3NB  
UNITED KINGDOM  
Tel: (932) 34.11.11, Fax: (932) 34.70.46, TLX: 262318 VetWey G |
| Brucelosis | Dr. B. Garin-Bastuji  
Laboratoire Central de Recherches Vétérinaires, (CNEVA)  
22 rue Pierre Curie, BP 67, 94703 Maisons-Alfort  
FRANCE  
Tel: (1) 49 77 13 00, Fax: (1) 43 68 97 62 |
| Enzootic bovine leukosis | Miss M. H. Lucas  
Central Veterinary Laboratory Weybridge, New Haw, Addlestone,  
Surrey KT15 3NB  
UNITED KINGDOM  
Tel: (932) 34.11.11, Fax: (932) 34.70.46, TLX: 262318 VetWey G |
| Leucose bovine enzootique | Prof. O. C. Straub  
Bundesforschungsanstalt für Viruskrankheiten der Tiere (Federal Research  
Centre for Animal Virus Diseases), Paul-Ehrlich-Str. 28, P.O. Box 1149,  
D-7400 Tübingen  
GERMANY  
Tel: (7071) 60.31, Fax: (7071) 60.32.01 |
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<td>Infectious bovine rhinotracheitis</td>
<td>Dr J.T. van Oirschot&lt;br&gt;Central Veterinary Institute (CDI-DLO), Virology Department, Edelhertweg 15, P.O. Box 65, NL-8200 AB Lelystad&lt;br&gt;THE NETHERLANDS&lt;br&gt; Tel: (3200) 73911, Fax: (3200) 73473, TLX: 40227 cdi nl</td>
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<tr>
<td>Rhinotrachéite infectieuse bovine</td>
<td>Dr A. Moussa&lt;br&gt;Laboratoire de Pathologie Bovine, (CNEVA), 31 av. Tony-Garnier, BP 7033, 69342 Lyon Cedex 07, FRANCE&lt;br&gt;Tel. 78 72 65 43, Fax: 78 69 37 46</td>
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<tr>
<td>Rinotracheítis infecciosa bovina</td>
<td>Dr S. Edwards&lt;br&gt;Central Veterinary Laboratory Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UNITED KINGDOM&lt;br&gt;Tel: (932) 34.11.11, Fax: (932) 34.70.46, TLX: 262318 VetWey G</td>
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<tr>
<td>Dr L.A. Babiuk&lt;br&gt;Associate Director (Research), Veterinary Infectious Disease Organisation, 124 Veterinary Road, Saskatoon, Saskatchewan, CANADA S7N 0W0&lt;br&gt;Tel: (306) 966 7456, TLX: 074 2659, Fax: (306) 966 7478</td>
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<td>Caprine arthritis/ encephalitis and Maedi-Visna</td>
<td>Dr Michelle Rémond&lt;br&gt;Laboratoire Central de Recherches Vétérinaires, (CNEVA)&lt;br&gt;22 rue Pierre Curie, BP 67, 94703 Maisons-Alfort, FRANCE&lt;br&gt;Tel: (1) 49 77 13 00, Fax: (1) 43 68 97 62</td>
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<td>Arthrite/encéphalite caprine et Maedi-Visna</td>
<td>Mr M. Dawson&lt;br&gt;Central Veterinary Laboratory Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UNITED KINGDOM&lt;br&gt;Tel: (932) 34.11.11, Fax: (932) 34.70.46, TLX: 262318 VetWey G</td>
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<tr>
<td>Artritis/encefalitis caprina y Maedi-Visna</td>
<td>Dr D.P. Knowles, Jr&lt;br&gt;Veterinary Medical Officer, USDA-ARS, Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164-7030&lt;br&gt;UNITED STATES OF AMERICA&lt;br&gt;Tel: (509) 335.6022, Fax: (509) 335.8328</td>
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<tr>
<td>Contagious caprine pleuropneumonia Contagiosa caprina</td>
<td>Dr G. Bolske&lt;br&gt;National Veterinary Institute, P.O. Box 7073, S-750 07 Uppsala&lt;br&gt;SWEDEN&lt;br&gt;Tel: 18.16.90.00, Fax: 18.30.91.62, TLX: 76238</td>
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<td>Pleuropneumonie contagieuse caprine</td>
<td>Dr P.C. Lefèvre&lt;br&gt;Institut d’Elevage et de Médecine Vétérinaire des Pays Tropicaux (IEVMV)&lt;br&gt;10 rue Pierre Curie, 94704 Maisons-Alfort Cedex, FRANCE&lt;br&gt;Tel: (1) 43 68 88 73, Fax: (1) 43 75 23 00</td>
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<td>Pleuroneumonía contagiosa caprina</td>
<td>Dr F. Rurangirwa&lt;br&gt;Kabete Veterinary Laboratories&lt;br&gt;P.O. Box Kabete Nairobi, KENYA&lt;br&gt;Tel: 63 24 13/15, Fax: 72 28 44</td>
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<td>Scrapie/BSE Tremblante et Encéphalopathie spongiforme bovine Temblor epidémico y Encefalopatía espongiforme bovina</td>
<td>Mr R. Bradley Central Veterinary Laboratory Weybridge, New Haw, Addlestone, Surrey KT15 3NB UNITED KINGDOM Tel: (932) 34.11.11, Fax: (932) 34.70.46, TLX: 262318 VetWey G</td>
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<td>Salmonellosis Salmonellose Salmonelosis</td>
<td>Dr C. Wray Central Veterinary Laboratory Weybridge, New Haw, Addlestone, Surrey KT15 3NB UNITED KINGDOM Tel: (932) 34.11.11, Fax: (932) 34.70.46, TLX: 262318 VetWey G</td>
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<td>Contagious equine metritis Métrite contagieuse équine Metritis contagiosa equina</td>
<td>Mrs J.E. Shreeve Central Veterinary Laboratory Weybridge, New Haw, Addlestone, Surrey KT15 3NB UNITED KINGDOM Tel: (932) 34.11.11, Fax: (932) 34.70.46, TLX: 262318 VetWey G</td>
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<td>Eastern/Western/ Venezuelan equine encephalomyelitis Encéphalomyélite équine vénézuélienne/Est/ Ouest Encefalomielitis equina venezolana/ Este/Oeste</td>
<td>Dr J.E. Pearson National Veterinary Services Laboratories, Animal and Plant Health Inspection Service, USDA, P.O. Box 844, Ames, IA 50010 UNITED STATES OF AMERICA Tel: (515) 239.85.51/82.00, Fax: (515) 239.84.58</td>
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<td>Equine infectious anaemia</td>
<td>Dr J.E. Pearson</td>
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<td>Anémie infectieuse des équidés</td>
<td>National Veterinary Services Laboratories, Animal and Plant Health Inspection Service, USDA, P.O. Box 844, Ames, IA 50010 UNITED STATES OF AMERICA Tel: (515) 239.85.51/82.00, Fax: (515) 239.84.58</td>
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<tr>
<td>Anemia infecciosa equina</td>
<td>Dr H. Sentsui</td>
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<td>National Institute of Animal Health, Hokkaido Branch, 4 Hitsujigaoka, Toyoohira-ku, Sapporo JAPAN 004 Tel: 81 11 851 5226, Fax: 81 11 853 0767</td>
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<td>Prof B. Toma</td>
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<td></td>
<td>Services des Maladies Contagieuses, Ecole Nationale Vétérinaire d’Alfort 7 avenue du Général de Gaulle, 94704 Maisons-Alfort Cedex FRANCE Tel: (1) 43 68 73 34, Fax: (1) 43 96 71 31</td>
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<tr>
<td>Equine influenza</td>
<td>Dr W. Eichhorn</td>
</tr>
<tr>
<td>Grippe équine</td>
<td>Institute for Medical Microbiology, Infectious and Epidemic Diseases, Veterinary Faculty, University of Munich Veterinärstrasse 13, 8000 München 22 GERMANY Tel: (89) 2180-2528; 2180-2531; 2180-2520; 2180-2598, Fax: (89) 2180-2597</td>
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<td>Gripe equina</td>
<td>Dr J.A. Mumford</td>
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<td>Animal Health Trust, P.O. Box 5, Newmarket, Suffolk CB8 7DW UNITED KINGDOM Tel: (638) 66.11.11, Fax: (638) 66.57.89, TLX: 818418 ANHLTH G</td>
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<tr>
<td>Equine rhinopneumonitis</td>
<td>Dr J.A. Mumford</td>
</tr>
<tr>
<td>Rhinopneumonie équine</td>
<td>Animal Health Trust, P.O. Box 5, Newmarket, Suffolk CB8 7DW UNITED KINGDOM Tel: (638) 661.111, Fax: (638) 665.789, TLX: 818418 ANHLTH G</td>
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<tr>
<td>Rinoneumonía equina</td>
<td>Dr G. Allen</td>
</tr>
<tr>
<td></td>
<td>University of Kentucky, College of Agriculture, Veterinary Science 08 Gluck Equine Research Center, Lexington, Kentucky 40546-0099 UNITED STATES OF AMERICA Tel: (606) 257.4757, Fax: (606) 257.8542</td>
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<tr>
<td>Infectious arteritis of horses</td>
<td>Dr P.J. Timoney</td>
</tr>
<tr>
<td>Artérite infectieuse des équidés</td>
<td>Chair &amp; Director, Gluck Equine Research Center, Veterinary Science, College of Agriculture, University of Kentucky, Lexington, Kentucky 40546-0099 UNITED STATES OF AMERICA Tel: (606) 257.4757, Fax: (606) 257.8542</td>
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<tr>
<td>Arteritis infecciosa equina</td>
<td>Miss M.H. Lucas</td>
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<td>Central Veterinary Laboratory Weybridge, New Haw, Addlestone, Surrey KT15 3NB UNITED KINGDOM Tel: (932) 34.11.11, Fax: (932) 34.70.46, TLX: 262318 VetWey G</td>
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| Transmissible gastroenteritis  | Dr P. Vannier  
Gastro-entérite transmissible  
Gastroenteritis transmissible  
Laboratoire Central de Recherches Avicole et Porcine, (CNEVA)  
UR Station de Pathologie Porcine, Les Croix BP 53, 22400 Ploufragan  
FRANCE  
Tel: 96.94.10.90, Fax: 96.78.68.61 |
| Infectious bursal disease      | Mr P.J. Wyeth  
Bursite infectieuse  
Bursitis infecciosa  
Central Veterinary Laboratory Weybridge, New Haw, Addlestone, Surrey KT15 3NB  
UNITED KINGDOM  
Tel: (932) 34.11.11, Fax: (932) 34.70.46, TLX: 262318 VetWey G |
| Marek's disease               | Dr L.N. Payne  
Maladie de Marek  
Enfermedad de Marek  
AFRC Institute for Animal Health, Houghton Laboratory, Houghton, Huntingdon, Cambs PE17 2DA  
UNITED KINGDOM  
Tel: (480) 64.101, Fax: (480) 67.870 |
| Mycoplasmosis (M. gallisepticum) | Dr S.H. Kleven  
Mycoplasmoise (M. gallisepticum)  
Micoplasmosis (M. gallisepticum)  
Research Professor and Head, the University of Georgia, College of Veterinary Medicine, Dept of Avian Medicine, Poultry Disease Research Center, 953 College Station Rd, Athens, Georgia 30605-2797  
UNITED STATES OF AMERICA  
Tel: (404) 542.1904, Fax: (404) 542.5630 |
|                               | Dr Isabelle Kempf  
Laboratoire Central de Recherches Avicole et Porcine, (CNEVA)  
UR Station de Pathologie Porcine, Les Croix BP 53, 22400 Ploufragan  
FRANCE  
Tel: 96 01 62 22, Fax: 96 01 62 23 TLX: 950743 |
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<td>Viral haemorrhagic disease of rabbits Maladie hémorragique virale du lapin Enfermedad hemorrágica viral del conejo</td>
<td>Dr D. Gregg National Veterinary Services Laboratories, Animal and Plant Health Inspection Service USDA, P.O. Box 848 Greenport, NY 11944 UNITED STATES OF AMERICA Tel: (516) 323.2500, Fax: (516) 323.2507</td>
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<td>Bee diseases Maladies des abeilles Enfermedades de las abejas</td>
<td>Dr Cécile Fléché-Seban Laboratoire de Pathologie des Petits Ruminants et des Abeilles, (CNEVA) Route des Colles, Sophia Antipolis, 06410 Biot FRANCE Tel: 92.96.00.20, Fax: 92.96.01.22</td>
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<td>Dr E. Pozio Istituto Superiore di Sanita, Laboratorio di Parassitologia Viale Regina Elena 299, 00161 Roma ITALY Tel: (6) 49 90 int. 462, Fax: (6) 44 40 077</td>
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<td>Echinococcosis/hydatidosis Echinococcose/hydatidose Equinococcosis/hidatidosis</td>
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**VETERINARY DRUGS/MÉDICAMENTS VÉTÉRINAIRES/MEDICAMENTOS VETERINARIOS**

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**IMMUNOENZYMATIC & MOLECULAR DIAGNOSTIC METHODS**

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## ALPHABETICAL LIST OF DISEASES COVERED IN THE MANUAL

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