GLOSSARY OF TERMS

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

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

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

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

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

- **Biohazard** (CWA\textsuperscript{1} 15793:2011)
  Potential source of harm caused by biological agents or toxins.

- **Biological agent** (adapted from CWA 15793:2011)
  Any microorganism including those which have been genetically modified, cell cultures, and parasites, which may be able to provoke any infection, allergy, or toxicity in humans, animals or plants. Note: for the purpose of Biorisk Analysis, prions are regarded as biological agents.

- **Biosafety**
  Laboratory biosafety describes the principles and practices for the prevention of unintentional exposure to biological materials, or their accidental release.

- **Biosecurity**
  Laboratory biosecurity describes the controls on biological materials within laboratories, in order to prevent their loss, theft, misuse, unauthorised access, or intentional unauthorised release.

- **Biorisk** (CWA 15793:2011)
  Combination of the probability of occurrence of harm and the severity of harm where the source of harm is a biological agent or toxin. Note: the source of harm may be an unintentional exposure, accidental release or loss, theft, misuse, diversion, unauthorised access or intentional unauthorised release.

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\textsuperscript{1} CWA: CEN Workshop Agreement (2011). CEN: European Committee for Standardization
• **Biorisk analysis** (adapted from the OIE *Handbook on Import Risk Analysis for Animals and Animal Products, Volume 1*)

Biorisk analysis is the process composed of biohazard identification, biorisk assessment, biorisk management and biorisk communication.

• **Biorisk assessment (CWA 15793:2011)**

Biorisk assessment is the process of evaluating the biorisk(s) arising from biohazards, taking into account the adequacy of any existing controls, and deciding whether or not the biorisk(s) is acceptable.

• **Biorisk Management Advisor (CWA 15793:2011)**

A biorisk management advisor is an individual who has expertise in the biohazards encountered in the organization and is competent to advise top management and staff on biorisk management issues.

• **Biorisk Management (adapted from OIE *Handbook on Import Risk Analysis for Animals and Animal Products, Volume 1*)**

Biorisk management is the process of identifying, selecting and implementing measures that can be applied to reduce the level of biorisk.

• **Biorisk Management System (CWA 15793:2011)**

A biorisk management system is part of an organization’s management system used to develop and implement its biorisk policy and manage its biorisks.

• **Cell line**

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

• **Centrifugation**

Throughout the text, the rate of centrifugation has been expressed as the Relative Centrifugal Force, denoted by ‘g’. The formula is:

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

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

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

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

• **Cross-reaction**

See ‘False-positive reaction’.

• **Cut-off/threshold**

A test result value selected for distinguishing between negative and positive results; may include indeterminate or suspicious zone.

• **Dilutions**

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

  • v/v – This is volume to volume (two liquids).

  • w/v – This is weight to volume (solid added to a liquid).
• **Dilutions used in virus neutralisation tests**

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

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

• **Efficacy**

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

• **Equivalency testing**

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

• **False-negative reaction**

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

• **False-positive reaction**

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

• **Final product (lot)**

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

• **Harmonisation**

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

• **Incidence**

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

• **In-house checks**

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

• **In-process control**

Test procedures carried out during manufacture of a biological product to ensure that the product will comply with the agreed quality standards.

• **Inter-laboratory comparison (ring test)**

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

• **Laboratory biosafety**

See *Biosafety*.
• **Laboratory biosecurity**

See Biosecurity.

• **Master cell (line, seed, stock)**

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

• **Master seed (agent, strain)**

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

• **Performance characteristic**

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

• **Phylogeography**

Phylogeography is the study of the genetic and geographic structure of populations and species.

• **Potency**

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

• **Precision**

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

• **Predictive value (negative)**

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

• **Predictive value (positive)**

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

• **Prevalence**

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

• **Primary cells**

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

• **Production seed**

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

• **Proficiency testing**

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

Quality of a biological product prepared to a final form and:

a) Relatively free from any extraneous microorganisms and extraneous material (organic or inorganic) as determined by test methods appropriate to the product; and

b) Free from extraneous microorganisms or material which could adversely affect the safety, potency or efficacy of the product.

• **Qualitative Risk Assessment** *(Handbook on Import Risk Analysis for Animals and Animal Products, Volume 1)*

An assessment where the outputs of the likelihood of the outcome or the magnitude of the consequences are expressed in qualitative terms such as high, medium, low or negligible.

• **Quantitative Risk Assessment** *(Handbook on Import Risk Analysis for Animals and Animal Products, Volume 1)*

An assessment where the outputs of the of the risk assessment are expressed numerically.

• **Reference animal**

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

• **Reference Laboratory**

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

• **Repeatability**

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

• **Reproducibility**

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

• **Risk** *(OIE Handbook on Import Risk Analysis for Animals and Animal Products, Volume 1)*

The likelihood of the occurrence and the likelihood magnitude of the biological and economic consequences of an adverse event or effect to animal or human health.

• **Risk Communication** *(Handbook on Import Risk Analysis for Animals and Animal Products, Volume 1)*

The interactive transmission and exchange of information and opinions throughout the risk analysis process concerning risk, risk-related factors and risk perceptions among risk assessors, risk managers, risk communicators, the general public, and other interested parties.

• **Room temperature**

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

• **Safety**

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

• **Sample**

Material that is derived from a specimen and used for testing purposes.
• **Sensitivity (analytical)**

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

• **Sensitivity (diagnostic)**

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

• **Sensitivity (relative)**

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

• **Specific pathogen free (SPF)**

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

• **Specificity (analytical)**

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

• **Specificity (diagnostic)**

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

• **Specificity (relative)**

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

• **Specimen**

Material submitted for testing.

• **Standard Reagents**

• **International Standard Reagents**

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

• **National Standard Reagents**

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

• **Working Standards (reagents)**

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

• **Sterility**

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

• **Thermotolerant**

The term used to describe the ability of a vaccine and/or the parent virus/strain to retain a level of infectivity after exposure to heat, that is, the delayed heat degradation of the virus. For example, for the thermotolerant L-2 Newcastle disease vaccine, it is defined by the length of time the vaccine will retain an infectivity titre sufficient to induce a protective immune response, at a particular temperature. The term “delayed heat degradation” may also
be encountered, but the term “thermotolerant” is preferred. The terms “heat resistant” and “thermostable” are considered to create unrealistic expectations of a vaccine’s properties and should be avoided.

- **Test method**
  Specified technical procedure for detection of an analyte (synonymous with assay).

- **Tests**

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

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

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

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

- **Thermotolerant**
  The term used to describe the ability of a vaccine and/or the parent virus/strain to retain a level of infectivity after exposure to heat, that is, the delayed heat degradation of the virus. For example, for the thermotolerant L-2 Newcastle disease vaccine, it is defined by the length of time the vaccine will retain an infectivity titre sufficient to induce a protective immune response, at a particular temperature. The term “delayed heat degradation” may also be encountered, but the term “thermotolerant” is preferred. The terms “heat resistant” and “thermostable” are considered to create unrealistic expectations of a vaccine’s properties and should be avoided.

- **Working seed**
  Organism at a passage level between master seed and production seed.
SECTION 1.1.

INTRODUCTORY CHAPTERS

CHAPTER 1.1.1.

MANAGEMENT OF VETERINARY LABORATORIES

INTRODUCTION

Reliable laboratory services can be delivered only by specialised facilities that are appropriately constructed and managed to provide the operating environment where the complex interaction of qualified staff, infrastructure and scientific methods can be coordinated to deliver specialised outputs consistently and safely. This chapter describes components of governance and management of veterinary laboratories that are necessary for the effective delivery of a diagnostic service, highlighting the critical elements that should be established as minimum requirements. Subsequent chapters set more specific standards for managing biological risks associated with laboratory facilities and for the range of aspects to be addressed to ensure confidence in laboratory test results.

The essential prerequisite for effective laboratory management is a clear understanding of the outputs required by the managing jurisdiction. National governments should support laboratory systems by developing a national laboratory policy based on the definition of the categories of laboratory test results required for effective implementation of the national animal health policy. Such clarity regarding national animal health requirements for laboratory services will guide the formation of national strategic planning for the delivery of these services. A clear statement of expectations of the laboratory service will guide governance and resourcing arrangements.

Further to these considerations, this chapter specifies components of diagnostic service management and delivery including the key support services that are considered essential. In addition to making provision for the scientific and technical aspects of the laboratory activities, the laboratory management system must address biorisk management and quality assurance. Laboratory management must also understand and meet the national and international regulatory requirements governing diagnostic laboratory operations. The outputs from a veterinary laboratory must be based on sound science, and mechanisms must be in place to prevent corrupt practices and inappropriate political influences.

A. GENERAL CONSIDERATIONS

1. Introduction

Laboratories fulfil an essential role in the delivery of veterinary services. Without the data and information supplied by veterinary laboratories animal disease detection, control and prevention would be significantly weakened (Edwards & Jeggo, 2012).

Chapter 1.1.5 Principles and methods of validation of diagnostic assays for infectious diseases lists the usual purposes for which laboratory testing is conducted, which include demonstration of freedom from infection in defined animal populations, certification of freedom from infection in individual animals or products for trade/movement purposes, contributions to the elimination of infection from defined populations, confirmation of diagnosis of suspect or clinical cases, estimation of prevalence of infection or exposure to facilitate risk analysis, and determination of the immune status of individual animals or populations.
These roles can be provided by governments (public sector laboratories), by industry (private sector laboratories), by universities (university laboratories) or by external organisations. Combinations of such providers in a complex matrix of services create challenges in the management and expectations of service delivery.

The governance of public sector veterinary laboratories will vary from country to country according to their public sector processes. This chapter sets out the general principles of veterinary laboratory governance and management that should ensure that Veterinary Services have access to reliable, trustworthy laboratory services, data and advice. The governance framework should ensure strong and effective delivery of services in a manner that is politically accountable, transparent, ethical, forward-looking and fair to staff and customers.

2. Accountability and oversight

A veterinary laboratory is held accountable for a range of issues apart from the delivery of basic diagnostic services. These may include health and safety, biosecurity, animal welfare and ethics, environmental contamination, genetic manipulations and quality assurance. It is essential that processes are established for the management and reporting of these issues and that individual staff are held accountable for their formally delegated responsibilities. As part of the process, it is critical to recognise and manage the resource implications, as failure to deliver to these accountabilities can bring the laboratory service into disrepute, detracting from the credibility of national animal health services.

There must be a clearly communicated and effective process by which the laboratory management is assessed and held accountable for delivery of all aspects of service delivery and accountability. This may be through a formally constituted governing body or through line management by the veterinary services or other qualified arm of government. Where a governing board is appointed, an independent chairman should be selected who understands both the political and the scientific environments in which the laboratory operates. The governing board should advise the laboratory director on how to meet the expectations of the customers and owners of the laboratory, but should also represent the laboratory’s interests by ensuring that these customers and owners have realistic expectations of the laboratory’s capability.

A laboratory should develop a medium-term strategic plan and a more detailed business plan for the year ahead, including budgets and resources to be deployed on various activities. The director of the laboratory should be responsible for presenting these plans to the governing board or departmental line management for formal approval. The laboratory should also prepare an annual report for approval through the established oversight processes.

The governing board must not become involved with the operational management of the laboratory, which must remain firmly in the hands of the director and the management team.

It is important to review regularly the overall laboratory objectives and agreed deliverables with government to ensure transparency in meeting of expectations. Staff should be kept informed on such deliverables, understand priorities and not feel unduly threatened by the need to ensure financial security for the laboratory. There may be competing pressures with regards to the activities that need to be undertaken, and the director should continually provide leadership and guidance to staff on these issues.

3. Executive management

It is essential that operational activities in the laboratory are conducted under the authority of a single individual who is given an appropriate title, e.g. director or chief executive. The director (or equivalent) should be fully accountable for the delivery of outputs from the laboratory and for the deployment of resources within the institution. As the core role of the laboratory is to participate in the diagnosis of animal disease and disease control programmes, the director should ideally be a qualified veterinarian and also have personal experience of working in a laboratory environment. Where the director does not have a veterinary qualification, a senior deputy should be appointed in the role of veterinary director. The key attributes of the director are to have an understanding of the operating environment of laboratory work, to be fully aware of the end-user requirements so that the outputs are relevant, trustworthy and timely and to demonstrate leadership qualities that will motivate the laboratory staff to deliver their best.

The director should be supported by a senior management team whose members will lead specific aspects of the work of the laboratory. The size of this team, and the scope of their individual responsibilities, will depend on the size of the laboratory, but it will typically involve leaders of different scientific disciplines (e.g. pathology, bacteriology, virology) as well as business leaders with expertise in human resources (HR), finance, procurement, engineering, information technology (IT) and communication. At least one of the senior team should be designated deputy director; the deputy will work closely with and in support of the director and fulfil the director’s responsibilities in his or her absence.
4. Infrastructure

Laboratories are highly specialised facilities with very particular requirements in terms of buildings, services and operational environments. Although some smaller laboratories can operate within an adapted general-purpose building, it is highly recommended that veterinary laboratories are housed in purpose-built units, designed with considerable input from scientific staff, along with architects, environmental experts, safety advisers and others in the design team. The structure and functions of the laboratory must comply with all relevant national regulations, such as for biocontainment, biosafety and environmental impact. Local issues must also be taken into account, such as the likelihood of extreme conditions (high or low temperatures, earthquakes, hurricanes, floods) and the reliability of water and electricity supplies.

National authorities must recognise that laboratories, whilst very expensive to build, are equally expensive to operate and maintain. It is absolutely essential that an adequate budget be allocated for annual operating costs (see section on finance below). Factors to support include the IT data support requirements (including future-proofing), utility costs and waste management.

5. Human resources

A veterinary laboratory, like any organisation employing staff, must have a clear, transparent HR policy that is seen to treat all individuals fairly. Appropriate procedures should be in place to determine remuneration, performance management, appraisal and promotion. A robust mechanism for addressing poor performance is also essential; it should provide clear and fair procedures for dismissal, in extreme circumstances. Veterinary laboratories employ an unusually high proportion of specialised staff, and this can cause difficulties where work patterns change as new technologies are introduced. HR policies should include training and retraining programmes to ensure that all staff are developed to their full potential and contribute to a flexible work force.

6. Compliance

6.1. Health and safety

Veterinary laboratories are hazardous environments. There are risks from handling dangerous pathogens, hazardous chemicals, physical hazards (ionising radiation, fire, high-pressure steam, low-temperature vessels) and animals (bites, kicks and other trauma to staff). Health and safety (H&S) must comply with the applicable national H&S legislation where such exists and be managed in a transparent and documented manner. The laboratory must have policies and procedures in place to assess all risks to staff (and visitors) and to mitigate those risks to acceptable levels.

The requirements for H&S policies and procedures will be achieved only with adequate support. Appointment of an H&S professional should be a serious consideration for larger laboratories, and this should be linked to an appropriate H&S budget. The role of the H&S professional must be clearly defined; and other staff members should understand that the presence of an H&S professional does not mean that they are any less responsible for carrying out their work in a safe and responsible manner, in compliance with agreed protocols. The H&S professional must have the full support of laboratory senior management.

A H&S committee should be established consisting of representatives from both staff and management of the laboratory. A requirement for such committee structures and operations is usually included in national legislation, and the laboratory managers must be fully conversant with these defined processes, including the appointment of H&S representatives, actions and reporting procedures for all H&S incidents, H&S training requirements and the minimum laboratory infrastructures and processes to meet these requirements.

6.2. Biosecurity

In addition to general health and safety issues veterinary laboratories have a responsibility to contain pathogens and to prevent their accidental release that might threaten neighbouring human or animal populations. Standards on biorisk management are given in Chapter 1.1.3a Standard for managing Biorisk in the veterinary laboratory and animal facilities and in the WHO1 Laboratory Biosafety Manual (WHO, 2006). All veterinary laboratories must comply with the relevant standards in these documents and also adhere to national standards and regulations. In many countries there is a national compliance monitoring authority for biosecurity and/or biocontainment. This authority will inspect the

1 WHO: World Health Organization
laboratory on a regular basis. The laboratory managers must understand the regulations and ensure that sufficient resources are available to ensure compliance.

Whilst minimum legal requirements exist, individual laboratories should examine their processes and procedures to determine where elements of biosecurity risk may arise and how best these should be managed on a local basis. A microbiological manual that contains standard operating procedures (SOPs) for all activities should be maintained. Such SOPs should highlight biosecurity controls, and it is recommended that local procedures are put in place to manage non-compliance. This is a matter of good laboratory practice, regardless of the legislative background.

Laboratory biorisk management should specifically recognise the potential for bioterrorist threats including the concept of the insider threat (e.g. the bioterrorist threat posed by a staff member). A process should be developed by which this threat can be managed. An annual staff threat appraisal would be a minimum requirement in such circumstances. In addition, measures must be in place to control access by visiting scientists to this class of pathogens.

6.3. Animal welfare

Veterinary laboratories must ensure their activities comply with animal welfare standards (Section 7 of the Terrestrial Animal Health Code, particularly Chapter 7.8 Use of animals in research and education, and Section 7 of the Aquatic Animal Health Code). It is also essential to understand fully the national legislation governing the ethical use of animals and put in place processes to ensure compliance.

6.4. Gene regulation

Many laboratories now use modified genes or gene products in their activities. Compliance with national regulations governing their use must be ensured including establishment of systems in the laboratory to monitor and ensure such compliance.

6.5. Environment

Laboratory waste may create concerns of environmental pollution. The risk of environmental damage from carcass disposal and disposal of other biological material is an issue that requires specific attention. Understanding and managing, as far as possible, any potential negative impacts of the laboratory on the surrounding environment is important and may be subject to national and local regulations. Certification of compliance with standard ISO\(^2\) 14001:2004 Environmental Management Systems (ISO, 2004) should be a target for laboratory managers.

B. SCIENTIFIC SERVICES

1. Diagnostic service delivery

The national Veterinary Services must be very clear in specifying the purposes for which laboratory capability is required, and hence the test methods and technologies to be supported. The defined purposes will include the list of diseases or infectious agent groups in scope, the nature of the government programmes to be supported in terms of the purposes of testing outlined in chapter 1.1.5, the likely scheduling and volume of submissions, and the required turnaround time for test reports. The cost implications must be identified and agreed. These discussions should be recorded in a service level agreement or similar documentation.

A key component of the delivery of scientific services is the routine monitoring, calibration and maintenance of scientific equipment. This is a real challenge in terms of both the resources to maintain the process and the availability of trained engineers and calibration equipment. Managing these processes should be a priority for resource allocation as test results generated on unmaintained and uncalibrated equipment cannot be trusted to be accurate.

Provision should also be made for the laboratory services that will be required in a disease emergency. The laboratory maximum (surge) capacity for processing samples should be defined as well as a plan for scale-up of operations. This may include a diversion of resources from lower priority tasks. Test turnaround times are also an important element in this specification.

\(^2\) ISO: International Organization for Standardization
All countries should support OIE designated Reference Centres through submission of specimens, isolates of infectious agents and other information of potential regional or international significance. It is only through receipt of such submissions that the Reference Centres can fulfil their OIE mandated role on behalf of the international community. Involvement with the designated Reference Centres is necessary for international public good.

In turn, national veterinary laboratories with special expertise in particular areas may seek recognition from international bodies such as the OIE, FAO or WHO as reference laboratories or collaborating centres. This is encouraged, as it facilitates the harmonisation of laboratory procedures worldwide, and strongly supports the work of the OIE and other international organisations. Funding for reference laboratory status needs to be allocated from national sources, and this should be part of the national planning with the Veterinary Services.

Many veterinary laboratories carry out work for a range of different customers. As well as meeting the needs of Veterinary Services, the laboratory may conduct contract work for national or international parties, provide diagnostic and surveillance procedures for private veterinarians, veterinary organisations or livestock industries, test food or environmental samples for food safety or other public health reasons, perform regulatory testing of veterinary medicinal products, and carry out contract testing for the private sector, e.g. for pharmaceutical companies. It is the responsibility of the laboratory director and management team to ensure that a balanced approach is taken in the allocation of resources in order to deliver this complex array of services. There should be clear recognition of priorities to facilitate dealing with unexpected events such as disease emergencies.

2. Quality assurance

Veterinary laboratories must be managed under a quality assurance system as specified in Chapter 1.1.4 Quality management in veterinary testing laboratories and should preferably be accredited to an international standard such as ISO/IEC 17025 General requirements for the competence of testing and calibration laboratories (ISO, 2005). The laboratory should ensure that all of its procedures, not just those concerning the laboratory bench but also those for supporting documentation and computer records, are robust, reliable and repeatable.

The quality standards require that diagnostic tests used in the laboratory should be validated as fit for purpose. The international standard for validation of diagnostic tests is established by the OIE, and is set out in chapter 1.1.5. Validation is not a once-for-all-time procedure but requires continual monitoring and refinement as the test is used. Laboratories should strive at all times to use tests that have reached at least stage 3 on the OIE validation pathway (chapter 1.1.5) and to continue refining the validation data as explained in the text.

3. Research

Laboratories are likely to engage in research, such as development or adoption of new tests or test methods, or pathogenesis or epidemiological studies of infections important in the particular country. It is essential to manage effectively the balance between research and diagnostic service delivery and the potential for competition for resources, including staff time.

C. SUPPORT SERVICES

1. Internal governance: policies and procedures

To ensure adequate standards of laboratory management across the spectrum of roles and responsibilities as identified in this chapter the responsible authority for the laboratory must ensure that laboratory management has adequate arrangements in place to deliver the required outcomes. These arrangements will include clearly defined policies and procedures supported by a management structure that is adequately resourced for implementation, audit and review.

Laboratory management should agree and document its policies for all aspects of operational activities. The processes by which such policies are implemented should also be documented in the form of clear procedures that are communicated to all staff who are involved in the particular activity. This approach has been introduced in some laboratories through the development of a quality assurance system, but is applicable to all aspects of laboratory activity. The responsibilities of designated staff for oversight and implementation of policies and procedures should be included in the documentation, and communicated clearly to all staff on the laboratory site.

3  FAO: Food and Agriculture Organization of the United Nations
2. Information management

Modern laboratories are increasingly dependent on computerised systems to manage their data. This can include an all-encompassing laboratory information management system (LIMS), bespoke systems for controlling individual laboratory equipment, and sophisticated analytical systems for use by specialised information scientists in disciplines such as molecular biology, informatics, epidemiology, risk analysis and statistics. There will also be office support systems for word processing, finance, HR and bibliographic databases. Systems for internal and external communications such as websites and email services will be needed. As with other elements of the laboratory’s activities, it is essential that the computer systems are managed by competent professionals and that the scientific staff are consulted in specifying the services they require. Measures must be in place to protect the integrity of the data, for archiving and retrieval, and for privacy protection of personal or sensitive items. It is important that the laboratory clearly determines its needs and procures the necessary resources, either through a service contract with an IT support company or through the direct employment of IT professionals, so as to provide adequate support in this essential area.

3. Finance

The budget is an integral part of the annual laboratory business plan and will set a basis for negotiation with customers and funders. The director should be personally accountable for delivering the work programme of the laboratory within budget, while individual managers of projects or activities should be set delegated delivery and financial targets. For any but the smallest of laboratories, the director should be supported in this area by one or more finance professionals, and for larger laboratories the senior finance officer should be a member of the executive management team.

Laboratory management should identify all costs and their allocation to the appropriate area of activity, so that the total cost of delivering any particular service can be identified. The operating costs should include directly attributable items (such as reagents and equipment), staff time per procedure, administration (booking in samples, generating reports), capital equipment (the cost of which may need to be spread across multiple activities or projects) and an appropriate proportion of overhead costs (covering such items as management, buildings, utilities, IT services, safety and quality procedures, and storage and archiving of samples and records). Making use of all this information, the management team should determine the total costs of operating the laboratory, broken down into specific areas, to enable an indicative budget to be prepared for approval by the governing body.

Cost control is an essential part of laboratory management. Continual efforts should be made to improve efficiency without compromising on quality. It is to be expected that customers will seek to minimise the costs to them of the services received; however, it is also important that the Veterinary Services or other laboratory customers recognise the complexity of the expenses in running a laboratory.

For many laboratories revenue generation through the sale of services and products is an important component of their finances. There may be political or regulatory constraints that determine whether such activities can make a profit, break even or be subsidised from the government allocated funds, but in all cases the laboratory should have a transparent pricing policy.

An important aspect of financial management is procurement of equipment, laboratory supplies and services. It is likely that there will be government regulations with which the actual procurement processes must comply. However it is important that the scientific staff of the laboratory should prepare detailed specifications of their requirements, whether for reagent supplies, equipment, or external provision of services. If the specification is well prepared, then the procurement process should be able to secure appropriate supplies of the product at the required quality. Clear rules must be in place to prevent undue pressure or bribery being applied to procurement officers by suppliers. This risk must be monitored closely by the senior management of the laboratory and, if necessary, by the governing body.

4. Engineering and maintenance

A modern veterinary laboratory requires substantial and adequate engineering maintenance and support. It is possible to outsource many of these maintenance requirements, but in many cases an in-house capability may better serve the need. Most laboratories have site-specific needs and requirements that are best met with a reasonable complement of engineering and trade skills on site, with staff who are familiar with local needs and issues. Laboratory management should regularly review how best to supply these support services.
5. Communications

Good communications that result in transparency of decision making and operations are vital to the success of a laboratory enterprise. This includes internal communications within the laboratory, ensuring that all staff are aware of the current priorities and how these impact on their work individually, as well as the wider activities of the laboratory and how their efforts contribute to the whole. It is essential that senior management has a system for communicating with staff throughout the laboratory and that this process genuinely works both ways. Senior managers must make efforts to be aware of the concerns and aspirations of their staff.

Externally, the director and management team must be effective advocates for the laboratory and represent it in meetings with Veterinary Services and other government officials, with scientists from other institutions, nationally and abroad, or with the wider public, including the media. It follows that the director and senior managers should be trained to interact with the media. This is a major priority particularly during a disease emergency, when effective communications with laboratory stakeholders is essential.

The key outputs from a veterinary laboratory are the scientific results and interpretation stemming from its analytical and investigational activities. These must be communicated to the customers or end-users in a clear and meaningful manner. Laboratory reports should include, where appropriate, indications of the level of uncertainty in the results, whether further results are still pending, and how to raise queries or clarifications or request further work.

A public information policy and procedures should provide a mechanism for individuals and outside bodies to ask about specific activities in the laboratory. Communications support staff should be involved in ensuring that the laboratory’s customers are kept informed about the work of the laboratory, its successes and any constraints on future work. Laboratories may provide an internet website or other IT-based strategies to assist with such communications. The management team should also ensure that procedures are in place to ensure compliance with obligatory reporting and notification requirements.

Scientific staff should be encouraged and supported to attend conferences and present papers, while the production of a steady stream of good-quality written papers in refereed journals is vital to the success of a laboratory institution. Importantly, this does not apply only to the research scientists; those working in diagnostic and surveillance work can also play an important role.

D. CONCLUSIONS

Good governance and management of a veterinary diagnostic laboratory are essential for the safe, sustainable and effective delivery of a diagnostic service. This chapter identifies the range of issues to be addressed if laboratories are to meet international standards. Many aspects of the delivery of laboratory services are now highly regulated by national authorities, and laboratory managers must be familiar with these regulations and have compliance processes in place. Key elements of staff safety, biocontainment, biosecurity, quality assurance, animal welfare and environmental management are vital components of operating such facilities. The governance and management of these aspects are as important as the delivery of the actual diagnostic service.

A well managed laboratory will further ensure that the general provisions specified in the remaining chapters of Part 1 of this Terrestrial Manual are met as well as the specific standards for the diagnostic testing for specific disease agents as outlined in Part 2. A key component in providing customer assurance is conforming with the OIE quality standard (chapter 1.1.4) supported by accreditation to quality standards such as ISO 17025. Accreditation is an important achievement of which laboratory staff can be proud, and implies that underlying compliance issues have been addressed.

Fundamental to the effective delivery of diagnostic services is the operation and maintenance of the facility and the scientific equipment. Allocation of adequate ongoing resources to this area is vital, yet is highlighted as an area of common neglect.

A successful veterinary diagnostic laboratory will have a highly trained, motivated workforce, with respect and support given to all individuals including both the frontline scientific staff and the important support teams providing vital services in areas such as finance, HR, safety, quality, procurement, engineering, IT and communications.

The achievement of all the above, and delivery of a respected and reliable service, requires a management system with checks and balances and effective review. This will include mechanisms to ensure political accountability, transparency, responsiveness, and coherent planning to ensure sustainability. A structure that includes an oversight process through the use of a laboratory governing board is strongly advocated to assist both financial management and strategic approaches to the delivery of all aspects of the laboratory’s activities.
REFERENCES


Chapter 1.1.6. PRINCIPLES OF VETERINARY VACCINE PRODUCTION

SUMMARY

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

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

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

As for all medicines, vaccine production starts within research and development (R&D) facilities, carrying out all the preclinical studies which are intended to demonstrate the quality of the products, including the safety and the efficacy. All these studies are generally carried out according to international reference standards such as good laboratory practice (GLP) for preclinical studies and good clinical practice (GCP) for clinical studies.

Before release of a vaccine for use in a country, a license or marketing authorisation must be requested from and be assessed and authorised by the competent authority to ensure compliance with local product marketing authorisation conditions. Starting materials to be used, manufacturing steps, in-process controls and controls on the finished product before release by a responsible person should be described in the marketing authorisation dossier, as should be the necessary tests to demonstrate quality, safety, and efficacy of the vaccine.

After the marketing authorisation has been granted by a competent authority, the industrial production can be launched in a manufacturing site which is authorised by the competent authority in accordance with national requirements and having the relevant equipment, facilities and personnel for production and controls. The manufacturing site should be inspected on a regular basis by experienced official inspectors.
Quality assurance is an integral part of the production of pure, safe and efficacious vaccines. This chapter outlines critical check points, with more details provided in chapters 1.1.8 Recommendations for manufacturing sites for veterinary vaccines and 1.1.9 Quality control of vaccines. It is a step-wise and iterative process. Compliance with the full standards described in these chapters can be achieved through risk analysis and step-wise process improvement.

NOMENCLATURE

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

VACCINE TYPES OR FORMS

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

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

Both live and inactivated vaccines may contain different antigenic components and may be formulated with adjuvants, stabilisers, antimicrobial preservatives and diluents. Adjuvants are designed to enhance the immunising efficacy of the vaccine. Those used frequently are typically water-in-oil emulsions (either single or double), made with mineral or vegetable oil and an emulsifying agent.

Other adjuvants, such as aluminium hydroxide gel or saponin, are also used. In addition to these traditional adjuvants, vaccines are being developed that include additional ingredients that induce immunomodulatory effects in the host animal and serve to enhance the efficacy of the product. These ingredients may include immunogenic components of microorganisms such as killed bacteria, which stimulate the immune response to other fractions contained in the vaccine, or cytokines, which may be used to regulate specific aspects of the immune system and are included in rDNA constructs used in products manufactured through biotechnology.

Many products obtained by biotechnology have now been licensed or approved, and more are being developed. Products of rDNA technology do not differ fundamentally from conventional products. Therefore, existing laws and regulations are fully applicable to these new products.

Each competent authority with power to regulate organisms and products derived from recombinant techniques should ensure that the public health and the environment are protected from any potentially harmful effect. Veterinary vaccines derived through rDNA technology may be divided into three broad categories. The division is based on the products’ biological properties and on the safety concerns they present.
Chapter 1.1.6. – Principles of veterinary vaccine production

Category I consists of non-viable or killed products that pose negligible risk to the environment and present no new or unusual safety concerns. Such products include inactivated microorganisms, either whole or as subunits, created by using rDNA techniques.

Category II products contain live microorganisms modified by adding or deleting one or more gene(s). Added genes may code for marker antigens, enzymes, or other biochemical by-products. Deleted genes may code for virulence, oncogenicity, marker antigens, enzymes, or other biochemical by-products. The marketing authorisation application must include a characterisation of the DNA segments added or deleted, as well as a phenotypic characterisation of the altered organism. The genetic modifications must not result in any increase in virulence, pathogenicity, or survivability of the altered organism in comparison with the wild-type form. It is important that the genetic modification does not cause deterioration in the safety characteristics of the organism.

Category III products make use of live vectors to carry recombinant-derived foreign genes that code for immunising antigens. Live vectors may carry one or more foreign gene(s) that have been shown to be effective for immunising target host animals. The use of DNA vaccines containing recombinant-derived foreign genes that code for immunising antigens (plasmid DNA vaccines) constitutes a new approach to vaccine development. The proper categorisation of this type of rDNA-derived product will be established as biological properties and safety characteristics are determined. These new vaccines may find application in a wide variety of situations much as conventional products have.

VACCINE PRODUCTION

1. Quality assurance

Quality assurance is a wide-ranging concept that covers all matters that individually or collectively influence the quality of a product. It is the total sum of the organised arrangements made with the object of ensuring that medicinal products are of the quality required for their intended use, ranging from process control, improvement and inspection, testing of the quality, efficacy and safety of the vaccines to assurance achieved through competent authority procedures. It is a step-wise and iterative process, and compliance with the standards described in these chapters can be achieved through risk analysis and step-wise process improvement. The basic concepts of quality assurance, good manufacturing practice (GMP), quality risk management and quality control are inter-related. See chapter 1.1.9 for full details.

2. Production facilities

Facilities used for the production of vaccines should be designed to protect the purity of the product throughout the production process and to safeguard the health of the personnel.

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

The requirements for vaccine production facilities are described in more detail in chapter 1.1.8.

3. Documentation of the manufacturing process and record keeping

A detailed Outline of Production, a series of SOPs, or other documents should also be prepared to describe the protocol for the manufacture and testing of each product produced in an establishment.

Criteria and standards for source materials should be clearly and accurately documented.

Guidelines for the preparation of such documents for veterinary vaccines are published by competent control authorities. This documentation is intended to define the product and to establish its specifications and standards. It should serve, along with the blueprints and blueprint legends (or production plan and SOPs), as a uniform and consistent method of producing the product that should be followed in the preparation of each batch/serial (one master batch record for each product).
The producer should establish a detailed record-keeping system capable of tracking the performance of successive steps in the preparation of each biological product. Records kept should indicate the date that each essential step was taken, the name of the person who carried out the task, the identity and quantity of ingredients added or removed at each step, and any loss or gain in quantity in the course of the preparation. Records should be maintained of all tests conducted on each batch/serial. All records relevant to a batch/serial of product should be retained for at least 2 years after the expiry date on the label, or in line with the requirements of the competent control authority.

Details of documentation required at a manufacturing site are described in chapter 1.1.8.

4. Production

Because of the wide variety of products, the frequently large number of stages involved in the manufacture of vaccines and the nature of the biological processes, each stage must be constantly monitored. Adherence to validated operating procedures and in-process controls is critical.

The specifications and source of all product ingredients should be defined in the Outline of Production, SOPs, or other appropriate documents. The Outline of Production must be approved by the competent authority. All ingredients of animal origin that are not subject to a validated sterilisation procedure should also be tested to ensure freedom from extraneous bacteria, fungi, mycoplasma, and viruses as specified in Chapter 1.1.7. Tests for sterility and freedom from contamination of biological materials. Their country of origin should be known.

Measures should be implemented by the manufacturer to avoid the risk of transmissible spongiform encephalopathy (TSE) agent contamination by ingredients of animal origin.

Some control authorities discourage the use of preservatives, especially antibiotics as a means of controlling adventitious contamination during production and prefer the use of strict aseptic techniques to ensure purity. However, they sometimes allow the use of preservatives in multidose containers to protect the product during use. These control authorities usually limit any addition of antibiotics in the manufacture of the product to cell culture fluids and other media, egg inocula, and material harvested from skin or possibly other tissues. Some control authorities prohibit the use of penicillin or streptomycin in vaccines administered by aerosol or parenterally. If the antibiotics used are not recommended for use in the target species, they should be shown to have no harmful effects in the vaccinated animals and not result in the contamination of food derived from vaccinated animals.

Details of vaccines production required at a manufacturing site, including requirements for starting materials, cell bank systems and seed-lot systems are described in chapter 1.1.8.

5. Process validation

Prior to obtaining a marketing authorisation for any new product, each establishment should produce in its facilities three consecutive production batches/serials of completed product to evaluate the consistency of production.

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

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

6. Stability tests

It is important to monitor the stability of each product through a programme of on-going stability. Additional information is given in the chapter 1.1.9.

All vaccines are sensitive to heat to some extent, but some are more sensitive than others. There is increasing interest in the development of vaccines that can tolerate adverse storage conditions. In this Terrestrial Manual, thermo-tolerant is defined as the ability of live vaccines to retain a level of infectivity after exposure to heat, that is,
the delayed heat degradation temperatures above 8°C. It is defined by the length of time the vaccine will retain a
potency sufficient to induce a protective immune response. By the latter criterion the term can also be applied to
killed vaccines.

7. Tests to demonstrate safety and efficacy of a vaccine

All laboratory procedures and tests should be conducted in compliance with an international standard such as
Good Laboratory Practice (GLP), see chapter 1.1.9. Similarly tests in animals should comply with Good Clinical
Practice (GCP). Submission of the results of the tests described below would normally be required in a dossier
supporting a request for the granting of a marketing authorisation or license.

7.1. Safety tests

7.1.1. Target animal safety tests

Harmonised international guidelines for safety tests are published by the International
Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medical
Products (VICH) in VICH GL 44 Target animal safety for veterinary live and inactivated vaccines
test is required for live vaccines shown to retain residual pathogenicity by induction of disease-specific signs or lesions. In general other vaccines do not require overdose testing.

For vaccines that require a single life-time dose or primary vaccination series only, the primary
vaccination regimen should be used. For vaccines that require a single dose or primary
vaccination series followed by booster vaccination, the primary vaccination regimen plus an
additional dose should be used.

The intrinsic safety of vaccines should be demonstrated early in product development and
documented as part of the licensing dossier. Safety studies during development and licensing
for all products should include the safety of a single dose, of an overdose and of repeated single
doses. Additional data are derived for live vaccines from the increase in virulence tests and by
assessing risk to the environment and in-contact animals, as discussed below. Safety should be
demonstrated in each species for which the product is indicated.

For inactivated virus or bacterial products, where host animals are used for potency testing,
safety may be determined by measuring local and systemic responses following vaccination
and before challenge in the potency tests. Further evidence concerning the safety of products is
derived from field safety trials (discussed below). Vaccines derived through biotechnology
should be evaluated as discussed in the classification of biotechnology-derived vaccines and
release of live rDNA vaccines below.

7.1.2. Increase in virulence tests

With live vaccines, there is concern that the organism might be shed from the host and
transmitted to contact animals, causing disease if it retains residual virulence or reverts to
virulence with repeated host passages. Guidelines for testing are published by VICH: GL 41:
Examination of live veterinary vaccines in target animals for absence of reversion to virulence

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

7.1.3. Assessing risk to the environment

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

7.2. Efficacy tests

7.2.1. Laboratory efficacy

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

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

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

7.2.2. Interference tests

Consideration must be given to possible interference between two different vaccines from the same manufacturer recommended to be given to the same animal within a 2-week period. The safety and the efficacy of this association should be investigated.

7.2.3. Field tests (safety and efficacy)

7.2.3.1. All vaccines

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

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

A risk assessment is carried out that should contain the following information:

- i) the purpose and need for the proposed action;
- ii) the alternatives considered;
- iii) a list of the government agencies, organisations, and persons consulted;
- iv) the affected environment and the potential environmental consequences.

The topics discussed should include:

- i) the characteristics of the vaccine organism,
- ii) human health risks,
- iii) animal health risks for both target and nontarget animals,
- iv) persistence in the environment, and increase in virulence.

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

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

If, in the course of preparing a risk assessment, competent authorities conclude that the proposed action may have a significant effect on the human environment, an environmental impact statement (EIS) should be prepared. The EIS provides a full and fair discussion of the significant environmental impacts, and informs decision-makers and the public of any reasonable alternatives that would avoid or minimise the adverse impacts. Environmental documents are considered in the United States Code of Federal Regulations [CFR] Title 40 part 1508. The EU has issued guidelines under Directive 2001/18/EC: Guideline on Live Recombinant Vector Vaccines for Veterinary Use, see http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC50004590.pdf

8. Updating the Outline of Production

Before production procedures are changed, the corresponding Outline of Production or other documentation of the manufacturing process should be changed. Establishments should have internal review procedures to evaluate all changes in production before they are initiated. Changes should also be reviewed and approved by competent authorities prior to their implementation.

In cases where a significant production step is altered, revisions may require additional data to support the purity, safety, potency, and/or efficacy of the product. In countries with regulatory systems that include confirmatory testing the final product at national laboratories, revisions should entail testing of the revised product by competent authorities.
QUALITY CONTROLS IN VACCINE PRODUCTION

1. Principle

Quality control is concerned with sampling, specifications and testing as well as the organisation, documentation and release procedures. Quality control ensures the necessary and relevant tests are carried out, and that materials are not released for use, nor products released for sale or supply, until their quality has been judged satisfactory. Quality control is not confined to laboratory operations, but must be involved in all decisions that may concern the quality of the product. The independence of quality control from production is considered fundamental to the satisfactory operation of quality control. Details of quality control are described in the chapter 1.1.9.

2. Batch/serial release for distribution

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

2.1. Batch/serial purity test

Purity is determined by testing for a variety of contaminants. Tests to detect contaminants are performed on each batch/serial of final product prior to release. Purity test procedures have been published, for example in CFR Title 9 part 113, in the annex to EU Directive 2001/82/EC (as amended), in the European Pharmacopoeia, or in this Terrestrial Manual (chapter 1.1.7), for the detection of extraneous viruses, bacteria, mycoplasma and fungi. Examples include tests for: Salmonella, Brucella, chlamydial agents, haemagglutinating viruses, avian lymphoid leucosis (virus), pathogens detected by a chicken inoculation test, or a chicken embryo inoculation test, lymphocytic choriomeningitis virus, cytopathic and haemadsorbing agents, and pathogens detected by enzyme-linked immunosorbent assay, polymerase chain reaction, or the fluorescent antibody technique.

2.2. Batch/serial safety test

Batch/serial safety tests are required by some regulatory authorities for the release of each batch/serial and typical tests are described in CFR Title 9 part 113, in this Terrestrial Manual and elsewhere. Standard procedures are given for safety tests in mice, guinea-pigs, cats, dogs, horses, pigs, and sheep and are generally conducted using fewer animals than are used in the safety tests required for licensing. Batches/serials are considered satisfactory if local and systemic reactions to vaccination with the batch/serial to be released are in line with those described in the marketing authorisation dossier and product literature. Some authorities do not permit batch/serial safety testing in laboratory animals, requiring a test in one of the target species for the product. The European Pharmacopoeia no longer requires a batch safety test in target animal species for the release of vaccine batches/serials.

2.3. Batch/serial potency test

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

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

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

3. Other tests

3.1. Tests on the finished product

Depending on the form of vaccine being produced, certain tests may be indicated and should be provided as appropriate in the Outline of Production or other documentation of the manufacturing process. These tests may concern: the level of moisture contained in desiccated/lyophilised products, the level of residual inactivant in killed products, the complete inactivation of killed products, pH, the level of preservatives and permitted antibiotics, physical stability of adjuvants, retention of vacuum in desiccated/lyophilised products, and a general physical examination of the final vaccine. A loss of potency may result when residual inactivating agent in a killed liquid product used as a diluent for a desiccated/lyophilised live fraction reduces the viability of the live organisms because of virucidal or bactericidal activity. Each batch/serial of liquid killed vaccine that is to be used as a diluent for live vaccines must, therefore, be tested for virucidal or bactericidal activity prior to release.

Tests for these purposes may also be found in CFR Title 9 part 113, in EU Directive 2001/82/EC (as amended), in the European Pharmacopoeia, or in this Terrestrial Manual.

3.2. Tests on other products

3.2.1. Purity

Purity is determined by testing for a variety of contaminants. Tests to detect contaminants are performed on master seeds, primary cells, master cell stocks (MCS), ingredients of animal origin if not subjected to sterilisation (e.g. fetal bovine serum, bovine albumin, or trypsin).

Procedures used to ensure that fetal or calf serum and other ingredients of bovine origin are free of pestiviruses should be of high concern and well documented. Tests to be used to ensure purity vary with the nature of the product, and should be prescribed in the Outline of Production or other documentation of the manufacturing process.

3.2.2. Tests for the detection of TSE agents

As tests for the detection of TSE agents in ingredients of animal origin have not been developed, vaccine manufacturers should document in their Outlines of Production or SOPs the measures they have implemented to minimise the risk of such contamination in ingredients of animal origin. This relies on three principles: first, verification that sources of all ingredients of animal origin in production facilities are from countries recognised as having the lowest possible risk of bovine spongiform encephalopathy; second, that the tissues or other substances used are themselves recognised as being of low or nil risk of containing TSE agents; third, where relevant, that the processes applied to the material have been validated for inactivation of TSE agents. Methods of production should also document the measures taken to prevent cross contamination of low risk materials by higher risk materials during processing.

MARKET MONITORING

1. Performance monitoring

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

After release of a product, its performance under field conditions should continue to be monitored by competent authorities and by the marketing authorisation holder/manufacturer itself. Consumer complaints may serve as one source of information; however, such information needs to be investigated to determine whether the reported observations are related to the use of the product. Users of veterinary vaccines should be informed of the proper procedures for making their complaints. The manufacturer of the product should be informed of all complaints received by competent authorities. Competent authorities should also ascertain whether they have received other similar complaints for this product and, if so, whether the manufacturer has taken appropriate action. Control laboratories may test samples of the batch/serial of product involved, if necessary.

Exporting countries and importing countries should ensure that marketing authorisation holders or manufacturers establish a reliable system to monitor adverse reaction notification (vaccinovigilance, post-licensing monitoring) is established to identify, at the earliest stage, any serious problems encountered from the use of veterinary vaccines. Vaccinovigilance should be on-going and an integral part of all regulatory programmes for veterinary vaccines, especially live vaccines. The marketing authorisation holder or manufacturer plays a big part in the conduct of this continuous overall vaccinovigilance evaluation. When it is determined that a product has a quality defect, immediate action should be taken to notify animal health authorities and to remove the product from the market.

2. Enforcement

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

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

INSPECTION OF PRODUCTION FACILITIES

Establishments that are approved to produce veterinary biologicals should be subject to in-depth inspections of the entire premises by national competent authorities to ensure compliance with the Outline of Production and blueprints and legends, SOPs, or other documentation of the manufacturing process. These inspections should be carried out on a regular basis and should allow the assessment of the manufacturing sites with regards to GMP standards.

These inspections may include such items as: personnel qualifications; record keeping; general sanitation and laboratory standards; production procedures; operation of sterilisers, pasteurisers, incubators, and refrigerators; filling, desiccating, and finishing procedures; care and control of animals; testing procedures; distribution and marketing; and product destruction.

Details regarding the inspection of production facilities and requirements for inspectorates are described in chapter 1.1.9.

FURTHER READING

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

Chapter 1.1.6. – Principles of veterinary vaccine production

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


PIC/S GUIDE AVAILABLE AT THE FOLLOWING ADDRESS: WWW.PICSCHEME.ORG


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


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APPENDIX 1.1.6.1.

RISK ANALYSIS FOR BIOLOGICALS
FOR VETERINARY USE

GENERAL CONSIDERATIONS

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

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

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APPENDIX 1.1.6.2.

RISK ANALYSIS FOR VETERINARY VACCINES

INTRODUCTION

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

PRINCIPLES

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

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

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

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

MANUFACTURING PRACTICES

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

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

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

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

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

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

CATEGORISATION OF VETERINARY VACCINES

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

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

VACCINOVIGILANCE

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

RISK COMMUNICATION

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

Vaccination may be applied in a number of different circumstances and ways and with different objectives, including the following:

1. Disposition of the animals:
   a) vaccination to live strategy: vaccinated animals are allowed to live their productive life unless they become infected.
   b) suppressive or dampening down vaccination, the animals are vaccinated to reduce or suppress infectious agent transmission and then they are culled, whether or not they become infected, when the resources for carcass disposal become available.

2. Types of vaccination:
   a) Emergency vaccination: is one of several measures that may be deployed to control outbreaks of disease as it provides a valuable adjunct to the application of the essential zoosanitary measures. These measures include rapid diagnosis, tracing movements of susceptible animals and potential vectors of the virus, movement controls and cleansing and disinfection of contaminated premises and transport. The control measures may also include the stamping out of infected and in-contact animals and the preventive slaughter of animals at risk of being exposed to infected and in-contact animals.

      The terms ‘emergency vaccine’ and ‘emergency vaccination’ can have different connotations, but are usually applied to differentiate between routine, prophylactic (preventive) vaccination against a known spectrum of disease agents and emergency vaccination, the latter being applied as an immediate response to an outbreak of disease caused by an agent previously not found in the territory.

   b) Ring vaccination: outside of and around an outbreak of the disease to inhibit outward spread.

   c) Barrier vaccination: vaccination in an area along the border of an infected zone or country to prevent the spread from the infected zone or country to the free zone or country.

   d) Blanket vaccination: Vaccination of the susceptible species in an entire country or zone.

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

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

4. Criteria that determine the successful application of vaccination, particularly in emergency situations include:
   a) Rapid access to vaccines that:
i) contain agent strain(s) of sufficient antigenic relatedness to the outbreak agent strain(s);

ii) are of the required type of vaccine formulation, for example in relation to host species in the target population;

iii) have acceptable safety and potency, the standards being the requirements of the OIE Terrestrial Manual.

b) Appropriate availability, including quantity and immediacy of supply;

c) Meet considerations of cost;

d) Sufficient capacity and logistics to dispatch vaccines to the place of application, by strict maintenance of the cold chain (when relevant), appropriate handling of unused quantities of vaccines and documenting the vaccination campaign.

e) The evident need to hold strategic reserves, or banks, of such valuable commodities is best exemplified by foot and mouth disease (FMD) vaccines. They are specified in contingency plans for use in an FMD outbreak and have led to an increase in the establishment of national and international FMD antigen and vaccine reserves for use all over the world (Forman & Garland, 2002), providing assurance that appropriate vaccine would be readily available and at the disposal of the country requiring it.

Emergency FMD vaccines may be formulated to a higher potency than their standard potency counterparts and there are banks that stipulate a requirement of at least 6 PD$_{50}$ (50% protective dose) per dose for cattle in contrast to the minimal statutory requirement of 3 PD$_{50}$. Higher potency can be achieved by simply increasing the antigen payload per dose and its benefits can include rapidity, magnitude and duration of the protective response or the partial compensation of a suboptimal match between the virus strain contained in the vaccine and the strain circulating in the field. High potency vaccines have been shown to induce protection within serotype against heterologous challenge in FMD. However, standard potency vaccines may also be used in an emergency, particularly when vaccine of appropriate strain composition is immediately available or where revaccination might be desired in an already pre-immune population.

f) The concept of vaccine banks, exemplified by FMD, and the increased reliance on such banks is indicative of it being a very practical adjunct to other control measures using vaccination that could usefully be adopted for a number of other diseases such as avian influenza, African horse sickness, bluetongue, classical swine fever, peste des petits ruminants, rabies (for dog vaccination) and Rift Valley fever.

### A. DEFINITION OF A VACCINE BANK

Strategic antigen or vaccine reserves, or vaccine banks as they are more commonly referred to, can be of different types. They may hold (i) the antigen component, (ii) the final end product, a ready-to-use formulated vaccine and/or (iii) be based on service contracts. Some vaccine banks are not based on antigen banks (e.g. FMD and rabies).

Antigens have to be finished into vaccines before deployment, can be stored for a very long time at ultra-low temperatures in the vapour phase of liquid nitrogen, and the vaccine formulation can be adjusted according to the need. The antigen bank type has been more commonly adopted for foot and mouth disease (FMD) because of the economic benefits, the possibility to combine core strains and optional strains for the formulation of different vaccines (mono- or polyvalent vaccines, or changes of strains over time), and this strategy avoids constantly replacing vaccines that exceed their shelf-life.

Ready-to-use vaccines can be deployed rapidly (for urgent deliveries), are stable for 1, 2 or 3 years depending on the vaccines, stored in appropriate cold facilities, and have a fixed formulation.

Some vaccine banks also use more sophisticated mechanisms based on service contracts with selected providers that include replenishment mechanisms and production on demand for non-urgent or planned deliveries.
Stockpiles of antigens, ready-to-use vaccines, or service contracts will be referred to as 'banks' in this chapter.

B. TYPES OF BANKS

A country may hold its own national bank and/or it may be part of a larger group of countries that share a bank and either have predefined drawing rights, as exemplified by the North American FMD bank, or a mechanism to determine these drawing 'rights ad-hoc as in the European Union (FMD antigen and vaccine bank). Such regional consortia may share a common geographical region, or have similar disease status and approach to control the disease. The bank may be held on the territory of one or several of its members or be retained by the manufacturer, and, if held as antigen, would be formulated for use either by the manufacturer, or in a dedicated facility maintained by the bank members. However licensing authorities demand that independent manufacturing facilities meet the same standard as the commercial vaccines for vaccines used in food producing animals in an emergency vaccination to live scenario.

With the financial support of donors, in the context of international aid, or with the use of more sophisticated financial mechanisms (trust funds or co-funding) an international organisation (regional or global, including the OIE) may manage regional or global antigen and vaccine banks retained by the manufacturers selected through specific international calls for tenders. Eligibility criteria are defined as well as guidelines for use of the regional and global antigen/vaccine banks (drawing rights) by eligible countries. Depending on the diseases this may include (i) antigens that have to be finished into vaccines before deployment, (ii) ready-to-use vaccines that can be deployed rapidly and/or (iii) vaccines produced and delivered on demand (planned deliveries). Such mechanisms can be used (i) to save on cost (economy of scale), (ii) to facilitate the delivery of determined quantities, including small quantities, of high quality vaccines complying with international standards, the standards being the requirements of the OIE Terrestrial Manual, (iii) to reduce the risks associated to the storage of vaccines, or (iv) to facilitate the implementation of regional or global control strategies with vaccination. Specific financial mechanisms can also allow countries to reimburse or to purchase directly from such banks.

In the case of an antigen bank, when using a service contract, a contract between the authorities and the selected vaccine manufacturer (formulation and filling) has to define clearly the details of formulation of the vaccine, e.g. time between reception of order and delivery, availability of buffers and vials, import permits, transportation, appropriate cold chain, vaccine labels and inserts, etc.

The location of stored antigens is of vital importance since the need to formulate vaccine may require antigen to be returned from the storage site to the original manufacturer, incurring a delay in supply. Even if the antigens are held by the commercial sector, delay following a request for the supply of emergency vaccine might still occur if the manufacturer is currently in the middle of production of a product. For example the time to produce the vaccine should be about 48–72 hours for FMD vaccine. Delays in the production and despatch of emergency vaccine to control an outbreak may lead to wider spread of the disease and further difficulty in its control. Therefore the decision to resort to emergency vaccination should not be delayed. Tried-and- proven contingency plans should envisage the organisation of a vaccination campaign at an early stage of the disease evolution. In addition the storage of antigens has the advantage that a large part of the necessary testing on those antigens can be carried out during the storage period.

Formulated and ready-to-use vaccines allow for immediate access and rapid delivery. On the other hand this also constitutes a stockpile of vaccines with a determined shelf life (expiry date). Replenishment mechanisms allow for the availability of ready-to-use vaccines with long shelf life and may also give an opportunity to adapt the strains (use of different antigens). However, storage can be organised as a surplus quantity of vaccines arising from routine vaccination campaigns taking advantage of the flexibility within the shelf life established by the manufacturer and the licensing authorities.

The economic benefits of regional (or global) vaccine banks are obvious. As they often use international tenders for procurement, they also provide the potential to deliver greater numbers of doses at a lower cost (economy of scale) and a wider number of vaccine strains. Furthermore, they reduce the number of procurement mechanisms, the fixed costs, the problem of deciding on the introduction of narrow spectrum vaccine strains. Collaboration between vaccine banks and international organisations would also be an economic way of increasing the amount of emergency vaccines available. Care would be required to ensure that collaborating vaccine banks and international organisations operate to the same standards, that drawing rights were clearly defined, and that regular contact is maintained between vaccine banks and international organisations to confirm the safety, efficacy and availability of the vaccines. In the case of shared banks, issues related to regulatory compliance would also need to be addressed at an early stage to ensure that vaccine produced from the bank would be authorised for use in any of the participating countries.

The vaccine banks often hold physical stocks of antigens or vaccines. It is also possible to establish virtual vaccine banks based on contractual relationships (service contracts) between the bank holder and manufacturers.
operating any of the above solutions, with clear obligations, price limits, maximum delays for delivery and severe contractual penalties in case of failure.

C. SELECTION OF VACCINES FOR A BANK

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

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

D. QUANTITIES OF VACCINE REQUIRED IN A BANK

The decision as to how many doses of vaccine are required is complex and problematic, embracing questions of serotypes, strains, use of mono or polyvalent vaccines, and type of formulation. Factors bearing on the decision include the type of disease, the different circumstances and ways of applying vaccination, including emergency vaccination, as described in the introduction, storage facilities available, number, species and location of livestock that are to be protected, geographical considerations, knowledge of the current and predicted global epidemiological situation, and the analyses of risks of introduction and spread of disease, together with cost–benefit studies. In determining the supply of emergency vaccines, decisions on the quantity of the product inevitably involve a compromise between the fixed cost of the maintenance of the antigen/vaccine bank, cost of purchase, storage and replacement, cold chain capacities of the beneficiaries and the likely number of doses required.

The minimum vaccine requirement might therefore be based on the number of doses that could be distributed and applied in the first week of vaccination, the expectation being that additional supplies could by then have been procured, either from other banks or from commercial sources. For example, 500,000 bovine doses of different FMD vaccine strains were routinely maintained by an international FMD vaccine bank, and withdrawing rights by member countries, which were widely dispersed over the globe, varied from 100,000 to 500,000 bovine doses. Nevertheless, this would soon be exhaustible if used in an area of high livestock density. For example, the EU antigen bank serving its Member States with densely populated livestock areas requires a minimum of 2 million vaccine equivalent cattle doses of each of the antigens stored.

When relevant, this can also be balanced with a repartition between antigens (core strains and optional strains), and ready-to-use vaccines (for rapid deliveries). For example, the OIE antigen and vaccine bank for FMD in South-East Asia (initially funded by the European Union) started operations with 800,000 vaccine equivalent cattle doses for each of the main antigens stored (five core strains in total) and 500,000 vaccine equivalent cattle doses for each of the optional strains (six in total). In this case, most vaccines are produced on demand. The use of specific mechanisms for regional FMD antigen/vaccine banks allows the strains to be adapted (antigens, ready-to-use vaccines and vaccines formulated on demand) to the strains circulating in the eligible countries of the region concerned or to which these countries may be exposed.
Chapter 1.1.10. – International standards for vaccine banks

In some other cases the physical stock of ready-to-use vaccines to set up the vaccine bank can be reduced to limit the initial fixed cost, to limit storage risks and to ask the selected manufacturer to produce vaccines mainly on demand (while protecting a long period of validity of the vaccines delivered).

E. ACQUISITION OF ANTIGENS OR VACCINES FOR A BANK

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

Regulatory concerns on existing, or potential, immunological veterinary medicinal products (IVMPs) and the advisability to use approved, authorised medicines, will predispose a bank to acquire, or maintain, its vaccines and antigens selectively. It is recommended that appropriately licensed manufacturers that have the necessary Marketing Authorisation (MA) and internationally accepted standards of Good Manufacturing Practice (GMP), Quality Assurance (QA) and Qualified Person (QP) product release should be used as authorised sources. It is recommended that the vaccine supplier be in possession of a valid official certificate of Good Manufacturing Practice provided by relevant official national authorities, and be able to demonstrate compliance with relevant standards laid down in the OIE Terrestrial Manual.

This has certainly been exemplified in recent years by FMD, PPR and rabies vaccine banks in which there has been a strong legal necessity for purchasing and holding antigens/vaccines within the commercial manufacturing sector.

Disease control authorities should consider the option of using international calls for tenders for antigens/vaccine with more than one supplier, particularly where regulatory considerations are of paramount importance. They may wish to seek advice from appropriate official licensing authorities on the necessary standards required. Requests for tenders can then ensure not only a competitive price but a veterinary medicinal product manufactured to an acceptable level of quality, the standards being those set out in this OIE Terrestrial Manual, an ultimate precondition for recognition of official national control programmes or for determination of the official status as regards FMD or PPR. It is recommended that the selection process of suppliers is not solely focused on the lowest bidder principle but also takes into consideration technical and quality criteria as well as delivery capacities. It should consider suppliers that can produce the desired vaccines/antigens and dose amounts within a specified time period that meet necessary, or indeed mandatory, tests of compliance such as safety and efficacy.

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

F. REGULATORY STANDARDS – SAFETY, EFFICACY AND QUALITY

Regulatory requirements for a veterinary medicinal product must be considered by any country wishing to have the necessary authorisation to use vaccine in an outbreak situation. For example, all veterinary medicinal products that are placed on the market in the European Union (EU) must hold a marketing authorisation and the EU lays down the requirements for such authorisations. The EU also has emergency provisions that permit, under certain conditions, the release of a vaccine without an authorisation in the country requiring it. The North American countries also have this emergency provision in their legislation, which allows the animal health authorities to exempt biological products from some requirements if they are used under official supervision in the prevention, control or eradication of animal diseases. Relevant national authorities are encouraged to have a specific procedure in place so they can authorise the importation and the use of mechanisms such as an early release certificate that allows the release of the vaccines, subject to certain conditions, before the end of quality controls on finished products in emergency situations.

In 2004, the European Medicines Agency summarised the particularities as regards regulatory requirements for the licensing of FMD vaccines, that may in a similar way apply to other vaccines used in controlling diseases caused by pathogens which are represented by several not mutually cross protecting serotypes, which undergo rapid antigenic changes and may occur simultaneously in a wide range of host species.

Therefore, it is important that licensed products be used; unlicensed products are very much a last resort.
Quality, safety and efficacy are all important and these will vary depending on the disease. In addition to the standards set out in the OIE Terrestrial Manual, certain immunologicals are covered by individual monographs in official Pharmacopoeias (e.g. FMD vaccine [inactivated] in the European Pharmacopoeia – Monograph 01/2008:0063) where the standards for safety, efficacy, sterility and quality are laid down. For the other case where the immunological comes under the Pharmacopoeia general section on Vaccines for Veterinary Use then those minimum standards should apply, while disease control authorities may wish to add additional specific individual requirements. These standards might include antigen strain identity, freedom from adventitious agents, safe sourcing of the ingredients in regard to transmissible diseases, inocuity, absence of toxicity, quantity of antigen payload per dose, safety, potency and sterility, and manufacture in officially approved quality assured good manufacturing practice (GMP) premises.

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

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

Antigen will be tested for inactivation ("inocuity"), sterility, safety and potency to ensure rapid release of further batches of product under the provisions of the European Pharmacopoeia, which stipulates that: "in situations of extreme urgency – [in this context the phrase "extreme urgency" refers to outbreaks (not to a specific degree of urgency time wise) – and subject to agreement by the competent authority, a batch of vaccine may be released before completion of the tests and the determination of potency if a test for sterility has been carried out on the bulk inactivated antigen and all other components of the vaccine and if the test for safety and the determination of potency have been carried out on a representative batch of vaccine prepared from the same bulk inactivated antigen. In this context, a batch is not considered to be representative unless it has been prepared with not more than the amount of antigen or antigens and with the same formulation as the batch to be released"

G. STORAGE OF VACCINES/ANTIGENS IN A BANK

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

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

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

The need for routine testing of stocks for stability is evident, and therefore depositories of antigens/vaccines should include a large number of small samples that are representative of the larger stock for such purposes stored side by side with it.
Chapter 1.1.10. – International standards for vaccine banks

It is recommended that security aspects are also considered (restricted access to premises, logbooks, continuity of access to electric power).

H. DEPLOYMENT PLANNING

For an antigen/vaccine bank to be optimal it has to be associated with a diagnostic laboratory with the ability to rapidly characterise the agent causing the disease and match the field strain with the available stockpiled antigens or the ready-to-use vaccines.

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

It would be advisable for member countries to monitor the literature published on important advances that are being made in subjects relating to vaccine bank technology. On-going research does lead to improvements of product, equipment, manufacture and distribution and therein more efficient and practical use of banks.

REFERENCES


CHAPTER 2.1.12.

Q FEVER

SUMMARY

Definition of the disease: Query (Q) fever (or Coxiellosis) is a zoonosis that occurs in most countries. Humans generally acquire infection through air-borne transmission from animal reservoirs, especially from domestic ruminants, but other domestic and wildlife animals (pets, rabbits, birds, etc.) can be involved. The causal agent is the obligate intracellular bacterium, Coxiella burnetii, which displays different morphological forms in its developmental cycle. Some forms can survive extracellularly and even accumulate in the environment. Because this microorganism is classified as a Group 3 pathogen, handling viable $C. burnetii$ must be done in biosafety level 3 facilities. All manipulations with potentially infected or contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis.

Description of the disease: In humans, the disease exhibits a large polymorphism. Q fever occurs either as an acute form or a severe chronic form following an early infection that may go unnoticed. The acute form resolves quite quickly after appropriate antibiotic therapy, but the chronic form requires prolonged antibiotic therapy (for 2 years or more), coupled with serological monitoring. In Australia, a vaccine (named Q-Vax) is available for professionally exposed population groups.

In domestic ruminants, Q fever is mostly associated with sporadic abortions or outbreaks of abortions and dead or weak offspring, followed by recovery without complications. Moreover, data also may suggest that Q fever plays a role in infertility or problems such as metritis in cattle. Coxiella burnetii infection persists for several years, and is probably lifelong. Sheep, goats and cows are mainly subclinical carriers, but can shed bacteria in various secretions and excreta.

Identification of the agent: For laboratory diagnosis in the context of serial abortions and/or stillbirths, samples can be taken from the placenta, vaginal discharges and tissues of aborted fetuses (spleen, liver, lung or stomach content). For investigation of bacterial shedding, samples can be taken from vagina, milk, colostrum and faeces.

As an obligate intracellular bacterium, Coxiella burnetii can be isolated by inoculation of specimens into conventional cell cultures, embryonated chicken yolk sacs or laboratory animals. Inoculation of laboratory animals (guinea-pig, mouse, hamster) is helpful in cases requiring isolation from tissues, faeces, milk or environmental samples contaminated with various microorganisms.

The bacteria can be visualised in stained tissue or vaginal mucus smears using a microscope with an oil-immersion objective lens. Because it is acid resistant, the bacteria can be stained by several methods: Stamp, modified Ziehl–Neelsen, Gimenez, Giemsa and modified Koster. Because of lack of specificity, a positive finding is only presumptive evidence of Q fever and confirmatory tests should be carried out.

To date, Demonstration of the agent by immunohistochemical staining, by in-situ hybridisation or by polymerase chain reaction (PCR) has proven to be more specific and sensitive than classical staining methods. No specific antibodies for immunochemistry are commercially available, but PCR kits are proposed for ruminants and can be used easily in suitably equipped laboratories. PCR is considered to be a useful and reliable test for screening large numbers and various types of samples. Currently, PCR has become the tool of choice for Q fever diagnosis.

Two PCR-based typing methods are becoming widely used: have been described recently, MLVA (multi-locus variable number of tandem repeats analysis) and multispacer sequence typing (MST), permitting the typing of $C. burnetii$ without the need for isolation of the organism. Moreover, SNP genotyping (single nucleotide polymorphism) has been recently described.
Currently available commercial tests allow the detection of at least the anti-phase I LPS contains the phase II part. The latter has been described as a major immunogenic determinant. 98 Intermediate phases with decreasing LPS O-chain lengths and then to phase II, with truncated LPS. Thus, the long in-ovo infected animals or humans, and the attenuated phase II, obtained by repeated passages in embryonated eggs or in cell cultures. Currently available commercial tests allow the detection of phase II or of both phases II and I anti-C. burnetii antibodies.

Requirements for vaccines: Several inactivated vaccines against Q fever have been developed, but only vaccines containing or prepared from phase I C. burnetii should be considered protective. An inactivated phase I vaccine (named Coxevac) is commercially available. Repeated annual vaccination, particularly of young animals, is recommended in at-risk areas.

### A. INTRODUCTION

#### 1. Definition of the disease and transmission routes

Q fever (or Coxielliosis) is widely distributed throughout the world with the exception of New Zealand. The causal agent, *Coxiella burnetii*, although Q fever is present in virtually all animal kingdoms, including arthropods, but the disease affects mostly humans, cattle, sheep and goats (Arricau-Bouvery & Rodolakis, 2005; EFSA, 2010; Lang, 1990; Mauton & Raoult, 1999). Domestic ruminants are considered the main reservoirs of *C. burnetii*, but cats, dogs, rabbits, birds, etc., have also been reported to be implicated in human disease/infection. There is clear epidemiological and experimental evidence that the infection is principally transmitted by inhalation of desiccated aerosol particles, and through exposure in the vicinity of infected animals, their reproductive tissues or other animal products, like wool (ECDC, 2010). Ingestion has been often suggested, particularly through the consumption of dairy products derived from contaminated raw milk, but no good evidence has shown significant transmission to humans by food. Indeed, there are epidemiological indications of sero-conversion but no association with clinical Q fever in humans. Q fever also seems very rarely transmissible from person to person, although exposure during childbirth, through sexual transmission or blood transfusion is possible. In animals, vertical transmission and sexual transmission could occur but their importance is not known. Finally, arthropods, principally ticks, may be involved in Q fever transmission. The risk of transmission seems to be linked to wildlife animals. It could be associated with bites as well as with contaminated dust from dried excrement.

#### 2. Description of the causal pathogen

The aetiological agent, *Coxiella burnetii*, is a Gram-negative obligate intracellular bacterium, adapted to thrive within the phagolysosome of the phagocyte. It has been historically classified in the *Rickettsiaceae* family. However, phylogenetic investigations, based mainly on 16S rRNA sequence analysis, have shown that the *Coxiella* genus is distant from the *Rickettsia* genus of the alpha subdivision of Proteobacteria (Drancourt & Raoult, 2005). *Coxiella burnetii* has been placed in the *Coxiellaceae* family in the order *Legionellales* of the gamma subdivision of Proteobacteria. The complete genome sequencing of *C. burnetii* has been achieved and confirms its systematic position (Seshadri et al., 2003). In general, the genomes of *C. burnetii* isolates from a wide range of biologically and geographically diverse sources are highly conserved, but notable polymorphism occurs such as rearrangement of syntenic blocks (Beare et al., 2009). This genomic plasticity might contribute to different phenotypes and is of great interest for genotyping methods (Massung et al., 2012; Sidi-Boumedine & Roussel, 2011). Unlike rickettsiae, *C. burnetii* produces a small, dense, highly resistant spore-like form (Coleman et al., 2004; Heinen et al., 1999; Minnick & Raghavan, 2012). This ability has been attributed to the existence of *C. burnetii* developmental cycle variants described from in-vitro studies: large-cell variants (LCV), small-cell variants (SCV), and small dense cells (SDC) measuring 0.2 µm wide and between 0.5 and 2 µm long or 0.4 to 0.7 µm diameter (Coleman et al., 2004; Heinen et al., 1999; Minnick & Raghavan, 2012). The SDC and SCV represent the small morphological variants of the bacteria likely to survive extracellularly as infectious particles, a trait that is important for persistence in the environment and transmission (ECDC, 2010; EFSA, 2010; Kersh et al. 2010).

Another essential characteristic is that *C. burnetii* has two antigenic forms: the pathogenic phase I, isolated from infected animals or humans, and the attenuated phase II, obtained by repeated *in-ovo* or *in-vitro* passages. An LPS (lipo polysaccharide) change occurs during serial passages: phase I cells, with full-length LPS O-chains, change to intermediate phases with decreasing LPS O-chain lengths and then to phase II, with truncated LPS. Thus, the long phase I LPS contains the phase II part. The latter has been described as a major immunogenic determinant. Currently available commercial tests allow the detection of at least the anti-*C. burnetii* phase II antibodies, which appear to be present whatever the infection stage or form. In contrast, vaccination is effective with a phase I vaccine.
but not with a phase II vaccine (Arricau-Bouvery et al., 2005; EFSA, 2010; Krauss, 1989; O’Neil et al., 2013). In general, the genomes of C. burnetii isolates from a wide range of biologically and geographically diverse sources are highly conserved, but notable polymorphism occurs such as rearrangements of syntenic blocks (Beare et al., 2009). This genomic plasticity might contribute to different phenotypes and is of great interest for genotyping methods.

3. Description of the disease in humans

Q fever is a zoonosis. In humans, the infection can manifest as an acute, chronic or subclinical form (Anderson et al., 2013; ECDC, 2010; Maurin & Raoult, 1999). Diagnosis and the treatment is often delayed because of the various nonspecific clinical expressions. The acute forms commonly include a self-limiting febrile episode, pneumonia or granulomatous hepatitis. The main clinical manifestation of chronic Q fever is endocarditis in patients with valvulopathies, vascular infections, hepatitis or chronic fatigue syndrome. The acute form resolves quite quickly after appropriate antibiotic therapy, but the chronic form requires prolonged antibiotic therapy (for 2 years or more), coupled with serological monitoring. In the absence of any appropriate antibiotic treatment, complications of the chronic form may be severe to fatal. Moreover, C. burnetii infection of pregnant women can provoke placentitis and lead to premature birth, growth restriction, spontaneous abortion or fetal death. Overall, the chronic disease is more likely to develop in immuno-compromised individuals. The infection is endemic in many areas leading to sporadic cases or explosive epidemics. Its incidence is probably greater than reported. Awareness for Q fever is increased during human outbreaks, which are generally temporary and rarely comprise more than 300 acute Q fever cases. However, the largest community outbreaks of Q fever ever reported emerged in 2007 in the Netherlands. In the subsequent years, peak incidence from February to September has increased and the geographical area has expanded progressively. The country reported 982 and 2305 confirmed cases in 2008 and 2009, respectively more than 4000 human cases with a hospitalisation rate of 20%, and it is expected to result in more cases of chronic Q fever among risk groups in the coming years. However, the factors leading to outbreaks are not fully understood (ECDC, 2010; EFSA, 2010). The losses caused by this epidemic have been estimated to be approximately 307 million euros (van Asseldonk et al., 2013).

Domestic ruminants are considered the main reservoirs for C. burnetii, but cats, dogs, rabbits, birds, etc., have also been reported to be implicated in human disease/infection. There is clear epidemiological and experimental evidence that the infection is principally transmitted by inhalation of desiccated aerosol particles, and through contact with infected animals, their reproductive tissues or other animal products, like wool (Arricau-Bouvery & Rodolakis, 2005; ECDC, 2010; Maurin & Raoult, 1999). Ingestion has been often suggested, particularly through the consumption of dairy products derived from contaminated raw milk, but no good evidence has shown a significant transmission to humans by food. Indeed, there are epidemiological indications of sero-conversion but no association with clinical Q fever in humans. Q fever seems also very rarely transmissible from person to person, although exposure during childbirth, through sexual transmission or blood transfusion is possible. In animals, vertical transmission and sexual transmission could occur but their importance is not known. Finally, arthropods, principally ticks, may be involved in Q fever transmission. The risk of transmission seems to be linked to wildlife animals. It could be associated with bites as well as with contaminated dust from dried excrement.

4. Description of the disease in animals

In cows, ewes and goats, Q fever has been associated mostly with late abortion and reproductive disorders such as premature birth, dead or weak offspring (Arricau-Bouvery & Rodolakis, 2005; EFSA, 2010; Lang, 1990). Moreover, C. burnetii might be associated with metritis and infertility in cattle. Given the lack of specificity of these latter signs, it is not recommended to rely on them for clinical diagnosis of Q fever (EFSA, 2010). Domestic ruminants are mainly subclinical carriers but can shed bacteria in various secretions and excreta. In the environment, C. burnetii can survive for variable periods and can spread. The levels of bacterial contamination in the environment have been tackled using quantitative PCR (polymerase chain reaction) for detection of C. burnetii DNA, but a rapid test assessing viability is required to evaluate the infectious risk in the environment (EFSA, 2010; Kersh, 2010). For now, the lack of knowledge of shedding patterns among ruminants has made the determination of Q fever status difficult. Concomitant shedding into the milk, the faeces and the vaginal mucus may be rare (Guatteo et al., 2007; Roussel et al., 2009a). The vaginal shedding at the day of kidding may be the most frequent (Arricau-Bouvery et al., 2005). In herds or flocks experiencing abortion problems caused by C. burnetii, most animals may be shedding massive numbers of bacteria whether they have aborted or not. The global quantities are thus clearly higher than in subclinically infected herds/flocks. At the parturitions following an abortion storm, higher bacterial discharges were measured among the primiparous compared with the other females (de Cremoux et al., 2012; Guatteo et al., 2008; Roussel et al., 2009b). Moreover, the shedding may persist for several months, following either an intermittent or a continuous kinetic pattern. Animals with continuous shedding patterns might be heavy shedders. These latter animals seem mostly to exhibit a highly-seropositive serological profile (Guatteo et al., 2007). Shedding and serological responses are associated at the group level but not at the individual level.
5. Differential diagnosis in ruminants

Diagnosis of Q fever in ruminants, including differentiating it from other abortive diseases, traditionally has been made on the basis of microscopy on clinical samples, coupled with positive serological results (Lang, 1990). At present, no gold standard technique is available, but direct detection and quantification by PCR and serological ELISA (enzyme-linked immunosorbent assay) should be considered as the methods of choice for clinical diagnosis (Niemczuk et al., 2014; Sidi-Boumedine et al., 2010). Proposals have been recently elaborated for the development of harmonised monitoring and reporting schemes for Q fever, so as to enable comparisons over time and between countries (EFSA, 2010; Sidi-Boumedine et al., 2010). Q fever diagnostic tests are also required for epidemiological surveys of at risk and suspected flocks in limited areas (following recent outbreaks in humans or animals), or for exchanges between herds or flocks. Thus, however, no gold standard technique is available and efforts are encouraged both for the validation of the methods for each purpose given (see Table 1), and for development of reference reagents for quality control, proficiency and harmonisation purposes (see Chapter 1.1. Principles and methods of validation of diagnostic assays for infectious diseases). The Q fever diagnostic tests are also required for epidemiological surveys of at risk and suspected flocks in limited areas (following recent outbreaks in humans or animals), or for exchanges between herds or flocks.

6. Zoonotic risk and biosafety requirements

Concerns about the risks posed by Q fever have been raised in Europe, where the European Commission requested scientific advice and risk assessment for humans as well as animals (ECDC, 2010; EFSA, 2010). The main conclusions were that the necessary actions to stop an outbreak must be carried out by health authorities together with veterinary authorities at the national and the local levels. The overall impact of C. burnetii infection on public health is limited but there is a need for a better surveillance system. In human epidemic situations, active surveillance of acute Q fever is the best strategy for avoiding chronic cases. Measures for the control of animal Q fever should be implemented, particularly for domestic ruminants. Only a combination of measures is expected to be effective. Long term among these options, include preventive vaccination, manure management, changes to farm characteristics, wool-shearing management, a segregated kidding area, removal of risk material, visitor ban, control of other animal reservoirs and ticks control could be used. Furthermore, the culling of pregnant animals, a temporary breeding ban, stamping out, identifying and culling shedding herds or flocks and controlling animal movements are considered as suitable options may have a role in the case of a human outbreaks.

Because of its ability to cause incapacitating disease in large groups of people, its resistance in the environment as a pseudo-spore and its natural spread as an aerosol, its likely low infectious dose, resistance in the environment, and aerosol route of transmission C. burnetii is currently considered a potential agent of bioterrorism and is classified by the Centers for Disease Control and Prevention as a group B biological agent (Drancourt & Raoult, 2005; Kersh et al., 2010). Regarding biosafety and biosecurity, C. burnetii is extremely hazardous to humans. Q fever is thus a recognised occupational zoonosis. Appropriate precautions must be taken that meet the requirements for containment Group 3 pathogens as outlined in all laboratory manipulations with live cultures or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by biosisk analysis (see Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities). In particular, it is advised to wear full cover protective clothing and filtering face piece (FFP) respiratory protection and to handle infectious and potentially infectious material with two pairs of gloves under a biological safety cabinet (BSC). Centrifugation of infected materials must be carried out in closed containers placed in sealed safety cups, or in rotors that are unloaded in a biological safety cabinet. The use of needles, syringes, and other sharp objects should be strictly limited. After all manipulations where there is a known or potential exposure to aerosols, showers must be taken when leaving the laboratory. Sporicidal disinfectants are recommended. An appropriate serological survey would help when following up the evolution of the immune status of the laboratory personnel.
## B. DIAGNOSTIC TECHNIQUES

### Table 1. Test methods available for the diagnosis of Q fever and their purpose

<table>
<thead>
<tr>
<th>Method</th>
<th>Population freedom from infection</th>
<th>Individual animal freedom from infection prior to movement</th>
<th>Contribute to eradication policies</th>
<th>Confirmation of clinical cases</th>
<th>Prevalence of infection – surveillance</th>
<th>Immune status in individual animals or populations post-vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agent identification</strong>&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>+++</td>
<td>n/a</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>n/a</td>
</tr>
<tr>
<td>Culture</td>
<td>+</td>
<td>n/a</td>
<td>+</td>
<td>=</td>
<td>+</td>
<td>n/a</td>
</tr>
<tr>
<td>Staining</td>
<td>+</td>
<td>n/a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n/a</td>
</tr>
<tr>
<td>Genotyping</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>++</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Detection of immune response</strong>&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>ELISA</td>
<td>+++</td>
<td>n/a</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>IFA</td>
<td>++</td>
<td>n/a</td>
<td>++</td>
<td>++</td>
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<td>++</td>
</tr>
<tr>
<td>CFT</td>
<td>–</td>
<td>n/a</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose; n/a = not applicable.

Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; IFA = indirect immunofluorescence assay; CFT = complement fixation test; BTM = bulk tank milk.

Clearly a confirmed positive identification of *C. burnetii* from an individual animal would support a diagnosis, however as a general principle the methods for the diagnosis of Q fever allow only an interpretation at the population level and not at the individual level. Laboratory test results should be interpreted in the context of herd or flock history (abortions, vaccination, movement and introduction, etc.).

### 1. Identification of the agent

*Coxiella burnetii* can be demonstrated in various ways, depending on the type of sample and the purpose of investigations (Samuel & Hendrix, 2009; Sidi-Boumedine et al., 2010). The ability to detect and quantify *C. burnetii* DNA by real-time PCR has dramatically enhanced diagnostic and study approaches. Individual vaginal, faecal, milk or colostrum samples or milk from the tank can be taken for investigating bacterial shedding. However, detection of shedders is still unreliable as the shedding dynamics are not well known (de Cremoux et al., 2012; EFSA, 2010; Guatteo et al., 2007; Rousset et al., 2009a). Indeed, the PCR cannot be relied on to determine the infection status because of the variability of shedding by animals (different shedding routes, potentially intermittent shedding). Serological analyses should be now carried out using ELISA and indirect immunofluorescence assay (IFA) rather than the complement fixation test (CFT). Several published works showed that the relative sensitivity is lowest for the CFT. The CFT is specific but less sensitive than the ELISA or IFA (Emery et al., 2014; Horigan et al., 2011; Kittelberger et al., 2009; Niemczuk et al., 2014; Rousset et al., 2007; 2009a). The CFT is still used by laboratories in many countries. IFA has the disadvantage of being less reproducible between operators, and therefore between laboratories. The ELISA is robust and can be automated and is recommended for routine serological testing of animals for Q fever.

A serological survey is a good way to evaluate prevalence. The presence of specific anti-*C. burnetii* antibodies provides evidence of a recent infection as well as a past exposure. Serological assays are suitable for screening herds or flocks, but interpretation at the individual animal level is not possible. Indeed, a significant proportion of animals shedding *C. burnetii* bacteria, and even some Q fever aborted animals, are found to be seronegative (de

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1 A combination of agent identification methods applied on the same clinical sample is recommended. 2 One of the listed serological tests is sufficient.
Cremoux et al., 2012; Guatteo et al., 2007; Rousset et al., 2007, 2009a). Sampling should target a representative number of animals (in particular from different age categories). Sampling has to take into account a potential weak prevalence if no prevalence data are available in the studied area. Alternatively, testing bulk tank milk (BTM) or pooled individual samples (i.e. vaginal swabs or milk samples) should be assessed in terms of their relationship to the intra-herd or intra-flock shedding prevalence. For example, PCR analyses of BTM are performed every 2 months since 2009 in the Netherlands to monitor a herd or flock with proven clinical status.

The herd or flock status can be assessed by serologically by ELISA investigation of all animals (or a significant sample). However, some discordant results can be observed using different ELISA kits (Horgan et al., 2011). One option is to use at least three kits to determine the status of a serum. Available serological methods do not, unfortunately, distinguish between infected and vaccinated ruminants. Analysis by PCR in BTM or individual samples (vaginal swabs for preference at the time of parturition) is required or complementary and may need to be repeated if the purpose is to determine free status. The free status of an animal can be assessed only if the livestock is free and if no serological or clinical history of Q fever has been reported. It is difficult to ensure that the status of the animal has not changed over time because transmission is by air.

PCR is the most reliable tool for the diagnosis of infectious abortions (EFSA, 2010; Sidi-Boumedine et al., 2010).

For laboratory diagnosis in the context of serial abortions and stillbirths, samples should be collected from aborted fetuses, placenta and vaginal discharges soon after abortion or parturition. Early detection of a Q fever storm of abortions in a herd or flock and implementation of the correct measures are essential to the handling of both farm-based and environmental route of infection. The diagnosis confirmation of clinical cases should always include a differential investigation of major abortive agents and target at least two aborted animals. Early detection of a Q fever storm of abortions in a herd or flock and correct measures is essential to deal with both farm-based and environmental route of infection. The interpretation of results is possible only at the herd level. A positive case is a herd or flock with clinical signs (abortion and/or stillbirth) for which the presence of the agent has been confirmed. If possible, vaginal swabs at the day of parturition (or taken less than 8 days after) should be collected in order to limit the number of false-negative PCR results. Effectively, the vaginal bacterial load may decrease progressively after abortion or parturition. Bacterial quantification is helpful on vaginal or placental swabs, as high levels are more likely to be associated with clinical cases. The fetal organs constitute less useful samples, as a negative result remains questionable. Bacteria are likely to spread to different organs (spleen, lung, liver, stomach contents, etc.) depending on the progression of the infection, so that the absence in one organ cannot exclude its presence somewhere else.

When difficulties in interpretation of diagnostic results are encountered, an association with a positive serological result at the herd or flock level is useful. Serological cut-off values used to diagnose Q fever are given by the kit suppliers. Interpretation of the results requires samples from at least six ewes or goats and ten cows (with priority to those that have aborted). Milk from the tank, individual milk or colostrum, vaginal and faecal samples can be taken for investigating bacterial shedding. However, detection of shedders is still fastidious as the shedding dynamics are not well known (EFSA, 2010; Guatteo et al., 2007; Kim et al., 2005; Rousset et al., 2009a) Testing bulk tank milk or pooled individual samples (i.e. vaginal swabs and/or milk samples) should be assessed in terms of the relationships with the intra-herd or flock prevalence of shedding.

Determination of the immune status of populations post-vaccination should be based on the more sensitive tests (ELISA or IFA): if possible, it should be linked to PCR testing of vaginal swabs collected at kidding. If the infection pressure is high, vaccination may only limit the magnitude of infection and shedding without inducing solid protection. The combination of seroconversion with the absence of vaginal shedding, at the following kidding, is indicative of immune protected status.

1. Identification of the agent

1.1. Isolation of the agent

For specific laboratory investigations, it may be necessary to isolate the agent. Where microscopic examination has revealed large numbers of C. burnetii combined with a low contamination rate with other bacteria, direct isolation by inoculation of embryonated chicken eggs or cell culture is possible (Maurin & Raoult, 1999; Samuel & Hendrix, 2009). To achieve isolation, a concentration above $10^5$ bacteria per ml is recommended.

**Embryonated chicken eggs:** A portion of placenta is homogenised in phosphate-buffered saline (PBS) containing antibiotics (streptomycin 100–200 µg/ml and penicillin or gentamicin 50–100 µg/ml). After low-speed centrifugation, dilutions of the supernatant fluid are inoculated into 6- to 7-day-old embryonated chicken eggs via the yolk sac. Eggs are preferably from specific pathogen free (SPF) hens. Embryos that die during the first 5 days after inoculation are discarded. The yolk sacs are harvested after 10–15 days of incubation. Stained smears of the yolk sac wall are examined to ensure the absence of bacterial contamination and to determine the presence of C. burnetii. PCR analysis can
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Chapter 2.1.12. – Q fever

also be used to detect the presence of \textit{C. burnetii} and to monitor the process of isolation. Further passages may be required to obtain an isolate in pure culture.

**Cultural cells:** A cell microculture system from a commercially available method used for virus culture, the shell vial cell culture\(^3\), has been adapted for isolating strict or facultative intracellular bacteria, including \textit{C. burnetii}. Such a method was described for \textit{C. burnetii} in 1990 (Raoult \textit{et al.}, 1990). Suspensions of samples are inoculated into human embryonic lung (HEL) fibroblasts grown on a 1 cm\(^2\) cover-slip within a shell vial. Various cell lines may be used to allow the observation of characteristic vacuoles of \textit{C. burnetii} multiplication. Centrifugation for 1 hour at 700 \(g\) enhances the attachment and penetration of bacteria into the cells. Three shell vials are used for the same sample, and by day 3, 10 and 21, the cytopathic effect (CPE) – \textit{C. burnetii} characteristic vacuoles in cells – are examined using an inverted microscope. After 10 days, detection of growing \textit{C. burnetii} within the cells is achieved directly on the cover-slip inside a shell vial by a direct immunofluorescence assay with polyclonal anti-\textit{C. burnetii} antibodies and an appropriate secondary antibody conjugated to fluorescein isothiocyanate (FITC). Cells of the remaining shell vial are harvested and transferred in a 25 cm\(^2\) culture flask. Incubation can be conducted for 3 months, with a culture medium change once a week (trypsinsiation is not used). The infection can be monitored by microscopy of Gimenez-stained cells cyto-centrifuged from the culture supernatant and by PCR analysis of the culture supernatant. When the CPE observations and Gimenez staining or PCR results are positive, a passage in a 75 cm\(^2\) culture flask is performed. Culture supernatant is then inoculated on confluent layers of Vero cells or L929 mouse fibroblasts in a 150 cm\(^2\) culture flask in order to establish a \textit{C. burnetii} isolate. This method was developed for humans but could be adapted for animals.

**Laboratory animals:** With heavily multi-contaminated samples, such as placentas, vaginal discharges, faeces, or milk, the inoculation of laboratory animals may be necessary as a filtration system. Biocontainment level 3 requirements are recommended for holding Experimentally infected rodents must be housed in appropriate biosafety and containment conditions, determined by bioresearch analysis (see Chapter 1.1.3). Mice and guinea-pigs are the most appropriate laboratory animals for this purpose (Scott \textit{et al.}, 1987). Following intraperitoneal inoculation with a dose of 0.5 ml per animal, body temperature and antibody status can be monitored. This method should be performed in conjunction with serological tests on other guinea-pigs or mice that have been inoculated with the same samples. Sera are collected 21 days after inoculation. A positive result confirms a diagnosis of \textit{C. burnetii} infection. If pyrexia develops, the animal is killed and the spleen is removed for isolation of the agent by inoculation into embryonated chicken eggs or in cell cultures. Microscopic examination of \textit{C. burnetii} can be done using impressions and staining of the collected spleens. Alternatively, the process can be simplified by performing PCR for detection of \textit{C. burnetii} DNA (see below) on spleens. In the mouse model, the spleens can be systematically collected around 9 days post-inoculation.

### 1.2. Staining

In the case of an abortion having a suspected infectious origin, smears of placental cotyledon are prepared on microscope slides. Spleen, lung, liver and abomasal contents of the aborted fetus or vaginal discharge may be used in the same manner. These could be stained according to several methods: Stamp, Gimenez, Macchiavello, Giemsa and modified Koster (Gimenez, 1964; Quinn \textit{et al.}, 1994; Samuel & Hendrix, 2009). The first three techniques give the best results. These methods are close to the modified Ziehl–Neelsen method involving basic fuchsin to stain bacteria. For example, the Stamp staining method is performed with 0.4% basic fuchsin solution, followed by rapid decolouration with 0.5% acetic acid solution, and counterstaining with 1% methylene blue or malachite green solution. The smears are examined microscopically with an oil-immersion objective lens (×500 or more). The Stamp method is preferred in veterinary laboratories while the Gimenez method is widespread for monitoring infected cultural cells in research laboratories. Gimenez is fastest because an acidic solution is not included for differentiation. \textit{Coxiella burnetii} are characterised by a very large number of thin, pink-stained cocacobacillary bacteria against a blue or green background. They may sometimes be difficult to detect because of their small size, but this is compensated for by their large numbers; often inclusions within the host cells appear as red masses against the blue or green background. The staining method is rapid. The limit of detection is high (×10\(^5\) bacteria/ml) and appropriate to the clinical diagnostic purpose as high levels of bacteria are present in samples found positive. Attention must be taken in the interpretation of the results as, microscopically, \textit{C. burnetii} can be confused with \textit{Chlamydia phila abortus} or \textit{Brucella} spp. However, using the same staining procedure, \textit{Chlamydophila} have sharper outlines, are round, small and may resemble globules. \textit{Brucella} spp. are larger (0.6–1.5 \(\mu\)m long × 0.5–0.7 \(\mu\)m wide), more clearly defined and stain more intensely. Control positive slides of \textit{C. burnetii}, \textit{Chlamydia phila abortus} and \textit{Brucella} must be used for comparison.
is usually adequate for routine purposes. When biological staining is inconclusive, one of the other methods may be used as a confirmatory test. PCR methods are preferred.

### 1.3. Specific detection methods

Detection of *C. burnetii* in samples can also be achieved by specific immunodetection (capture ELISA, immunohistochemistry), in-situ hybridisation or DNA amplification (Jensen *et al.*, 2007; Samuel & Hendrix, 2009; Thiele *et al.*, 1992). Immunohistology may be used with paraffin-embedded tissues or on acetone-fixed smears (Raoult *et al.*, 1994). The method is an indirect immunofluorescence or immunoperoxidase assay using specific polyclonal *C. burnetii* antibodies produced in laboratory animals (rabbit or guinea-pig). An anti-species (rabbit or guinea-pig) anti-IgG conjugate, labelled with FITC or peroxidase, is then used to visualise the bacteria. Control positive slides of *C. burnetii* antigen should be available for comparison. No specific antibodies for immunohistochemistry are commercially available.

Fluorescent in-situ hybridisation (FISH) using specific oligonucleotide probes targeting 16s rRNA may be used on paraffin embedded tissues, especially placenta samples (Jensen *et al.*, 2007).

PCR methods have been used successfully to detect *C. burnetii* DNA in cell cultures and biological samples. The PCR methods of *C. burnetii* detection are generally performed for the health investigations of ruminant herds or flocks prone to abortions (Sidi-Boumedine *et al.*, 2010). Nevertheless, as the number of *C. burnetii* is likely to be lower in milk, colostrums and faeces than in abortion material, PCR can be used for analysis of this large diversity of samples. Before performing the PCR, biological samples can be inactivated, for ensuring the safety of laboratory personnel, by heating at 90°C for 30–60 minutes, depending of the samples’ nature, their size or their weight. The inactivation process must be checked and validated before any use to ensure the safety of personnel. The PCR technique can be performed in suitably equipped laboratories using primers derived from various targets, such as multicopy insertion sequence IS1111 (accession number M80806), the most largely employed (Berri *et al.*, 2000). The use of these primers for the amplification of this sequence allows the sensitivity of the test to be increased and this because of the presence of several copies in the Coxiella genomes. The other target genes reported to be used in the PCR for specific *C. burnetii* identification are: superoxide dismutase (sodB) gene (accession number M74242); com1 encoding a 27 kDa outer membrane protein (accession number AB004712); heat shock operon encoding two heat shock proteins (htpA and htpB) (accession number M20482); isocitrate dehydrogenase (icd) (accession number AF069035); and macrophage infectivity potentiator protein (cbmip) (accession number U14170). Some primer and probe sequences can be obtained on the web site of the French national reference centre for human Q fever4.

The real-time PCR provides an additional means of detection and quantification (Kim *et al.*, 2005; Klee *et al.*, 2006; Stemmler & Meyer, 2002). As with the conventional PCR, various target genes are used: IS1111; IS30; com1; and icd. To quantify the bacteria in biological samples using the real-time PCR, it is recommended to amplify a unique and specific sequence. Indeed, recent data show that the number of the insertion sequence (IS1111) varied widely (between 7 and 110) depending on the isolate (Klee *et al.*, 2006). Whereas the use of this sequence could increase the sensitivity of the test, it may not be accurate for quantification when different strains are involved. It is nevertheless sufficiently informative and accurate for high quantities of bacteria (i.e., >10⁴ per vaginal swab) for abortive diagnosis (Sidi-Boumedine *et al.*, 2010). Regarding complex matrices, the DNA eluates should be evaluated for their ability to inhibit a PCR by adding an internal DNA control (such as a GAPDH sequence target) or an external control.

Different primers and probes used in PCR can be obtained on the web site (http://ifr48.timone.univ-mrs.fr/Fiches/Fievre_Q.html#toc22), regularly updated by the French Reference National Center for human Q fever. Ready-to-use kits are commercially available and can detect the bacteria in various sample types. Specific quantitative methods based on PCR kits have been recently validated according to a new French standard for real-time PCR validation (Rouset *et al.*, 2012). An external reference material of quantified bacteria is available from the French national reference laboratory either for method validation or for a control chart to routinely monitor quality of the assays.

**However, for the future, there is an urgent need for the development of a molecular method for the assessment of bacterial viability, especially in milk to assess samples and environmental samples as well as disinfectants and inactivation processes. Isolation is a way to show the viability of the bacteria present in the sample but is unreliable and not feasible on large series. It is still difficult to**

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4 At: http://ifr48.timone.univ-mrs.fr/Fiches/Fievre_Q.html#toc22
enumerate viable bacteria. The development of a multiplex PCR or microarray would provide a useful screening method for all infectious abortive agents in a single assay.

1.4. Genotyping methods

Q fever epidemiology is complex as represented by its wide host range, its capacity to resist in the environment and its multifactorial air-borne transmission. Although characterisation of isolates seems necessary for understanding the varying epidemiology of Q fever in different geographical areas, assessment of discriminatory typing methods for molecular epidemiology are in progress (Chmielewski et al., 2009; Klaassen et al., 2009; Massung et al., 2012; Sidi-Boumedine & Rousset, 2011; Sidi-Boumedine, 2015). These tools are very useful for epidemiological investigation, particularly to clarify links regarding source of infection, for better understanding the epidemiological emerging factors, elucidating human outbreaks, and to a lesser extent for evaluating control measures.

Several typing methods have been used for the characterisation of C. burnetii strains, such as restriction endonuclease of genomic DNA (Hendrix et al., 1991), PFGE (pulsed-field gel electrophoresis) (Hoenzen et al., 1990; Jager et al., 1998), and sequence and/or PCR-RFLP (restriction fragment length polymorphism) analysis of icd, com1 and mucZ genes. More recently, two PCR-based typing methods have been described, MLVA (multi-locus variable number of tandem repeats analysis) (Arricau-Bouvery et al., 2006; Sraka et al., 2006) and multipacer sequence typing (MST) (Glazunova et al., 2005) that permit the typing of C. burnetii without the need for isolation of the organism. Research continues on the development of new tools, such as single nucleotide polymorphism (SNP), and the comparison of their discriminatory capabilities.

To date, MLVA and MST are considered to be the most discriminating methods for C. burnetii, allowing the identification of up to 36 distinct genotypes. Moreover, databases have been established http://mlva.u-psud.fr/MLVAnet/ and http://ifif48.timone.univ-mrs.fr, respectively for MLVA and MST. The availability of such databases allows interlaboratory comparisons to be made easily and this will lead to a better understanding of the propagation of the C. burnetii isolates or to identify new emerging strains. Furthermore, their use in the characterisation of field samples or isolates is increasing (Chmielewski et al., 2009; Klaassen et al., 2009) and efforts to produce a standardised scheme for MLVA, based on common decisions for allele calling and marker panels to be used, are in progress and should be encouraged so that they can be made available in the near future (Massung et al., 2012; Sidi-Boumedine & Rousset, 2011; Sidi-Boumedine et al., 2009).

2. Serological tests

Among the various techniques that can be employed, the three most commonly used serological tests are the IFA, the ELISA and the CFT. Older serological tests that are no longer used in routine diagnosis include the microagglutination technique, the capillary agglutination test and the indirect haemolysis test. Overall, ELISAs are preferred for practical reasons. Currently, no IFA is commercially available for ruminants. The production of the commercial antigen for CFT will probably cease in the future. Numerous reports showed a weak sensitivity of CFT compared with other methods (EFSA, 2010; Kittelberger et al., 2009), however, this is mainly due to the difficulty to collect blood samples from infected animals. Serological tools allowing specific antibody detection in sera from different animal species (not only ruminants) should be developed (Jaspers et al., 1994; Soliman et al., 1992).

The presence of specific IgG anti-C. burnetii antibodies provides evidence of a recent infection as well as a past exposure. Serological assays are suitable for screening herds or flocks, but the interpretation at the individual animal level is not possible. Indeed, a significant proportion of animals shedding C. burnetii bacteria and even some Q fever aborted animals are found to be seronegative (Arricau-Bouvery et al., 2005; Quattee et al., 2007; Rouset et al., 2007, 2009a). Serological cut-off values used to diagnose Q fever are given by the suppliers. It was proposed that interpretation of the results requires at least six ewes or goats and ten cows (those aborted in priority) for a positive test. Both serological responses and bacterial evidence are often necessary for establishing the presence of the infection.

2.1. Enzyme-linked immunosorbent assay (ELISA)

This technique has a high sensitivity and a good specificity according to comparative evaluations between methods (Emery et al., 2014; Horigan et al., 2011; Kittelberger et al., 2009; Niemczuk et al., 2014; Rouset et al., 2007; 2009a). It is easy to perform in laboratories that have the necessary equipment (a spectrophotometer) and reagents. The ELISA is preferred to IFA and CFT, particularly for veterinary diagnosis, because it is convenient for large-scale screening and the most robust and, as it is a reliable technique for demonstrating C. burnetii antibody in various animal species (Jaspers et al., 1994; Soliman et al., 1992). Ready-to-use kits are commercially available and can detect anti-phase II antibodies or both anti-phase I and II antibodies. The quality control for some ELISA kits was recently
improved using an external reference material, available from the French national reference laboratory, showing the standardisation between kit batches.

_Coxiella burnetii_ ELISA antigen is prepared by growth of reference strains in either embryonated hens' eggs or in cell culture, as described below under IFA. Wells of the microplate are coated with _C. burnetii_ whole-cell inactivated antigen. Diluted serum samples are added to the wells and react to antigens bound to the solid support. Unbound material is removed by washing after a suitable incubation period. Conjugate (horseradish-peroxidase-labelled anti-ruminant Ig) reacts with specific antibodies bound to the antigen. Unreacted conjugate is removed by washing after a suitable incubation period. Enzyme substrate is added. The rate of conversion of substrate is proportional to the amount of bound antibodies. The reaction is terminated after a suitable time and the amount of colour development is measured spectrophotometrically.

### 2.1.1. Materials and reagents

- Microtitre plates with 96 flat-bottomed wells, freshly coated or previously coated with _Q fever_ C. burnetii antigen; microplate reader (spectrophotometer; 405 and/or 450 and/or 492 nm filters); 37°C humidified incubator; 8-and 12-channel pipettes with disposable plastic tips; microplate shaker (optional).
- Positive and negative control sera; conjugate (ruminant anti-immunoglobulin or protein A/G labelled with peroxidase); tenfold concentration of diluent (PBS–Tween); distilled water; substrate or chromogen (TMB [tetramethylbenzidine], ABTS [2,2'-azino-bis-(3-ethylbenzo-thiazoline-6-sulphonic acid)] for peroxidase); hydrogen peroxide.

### 2.1.2. Test procedure

i) Dilute the serum samples, including control sera, to the appropriated dilution (usually 1/100) and distribute 0.1 ml per well in duplicate. Control sera are positive and negative sera provided by the manufacturer and an internal positive reference serum from the laboratory in order to compare the titres between different tests.

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

iii) Add the appropriate dilution of freshly prepared conjugate to the wells (0.1 ml per well).

iv) Cover each plate and incubate as in step ii. Wash again three times.

v) Add 0.1 ml of freshly prepared chromogen substrate solution to each well (for example: TMB in 0.1 M acetic acid and 30% H₂O₂ solution [0.2 µl/ml]; or 0.25 mM ABTS in citrate phosphate buffer, pH 5.0, and 30% H₂O₂ solution [0.1 µl/ml]).

vi) Shake the plate; incubate according to the manufacturer recommendations, stop the reaction by adding stopping solution to each well, e.g. 0.05 ml 2 M sulphuric acid for TMB or 10% sodium dodecyl sulphate for ABTS.

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

### 2.1.3. Interpretation of the results

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

For example: calculate the mean absorbance (Ab) of the sample serum and of the positive (Ab<sub>pos</sub>) and negative (Ab<sub>neg</sub>) control sera, and for each serum, calculate the percentage

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

Interpret the results as follows:

- Ab <30% negative serum
- Ab 30–40% doubtful serum
- Ab >40% positive serum
2.2. Indirect immunofluorescence assay (IFA)

In human medicine, the IFA adapted as a micro-immunofluorescence technique is the current method for the serodiagnosis of Q fever (Tissot-Dupont et al., 1994). The procedure can be adapted to perform an immunoperoxidase assay. Briefly, both phase I and phase II C. burnetii antigens are used; phase II antigen is obtained by growing C. burnetii Nine Mile reference strain in cell culture, while phase I antigen is obtained from the spleens of laboratory animals inoculated with phase II C. burnetii in cell cultures. A few phase I cells may still be present in the phase II population and can be selected and propagated within animals. Antigen is diluted, dropped onto the wells of a glass microscope slide, allowed to dry, and fixed with acetone. The two forms of the infection, acute and chronic, have different serological profiles: during acute Q fever, IgG antibodies are elevated against phase II only whereas during chronic Q fever, high levels of IgG antibodies to both phase I and II of the bacteria are observed (Tissot-Dupont et al., 1994). In addition, antigen-spot slide wells may be purchased from a supplier providing the phase II form, or the phase I and II forms of C. burnetii. These can be adapted by replacing the human conjugate by a conjugate adapted to the animal species. Nevertheless, the interpretation as acute or chronic forms has not been validated for ruminants.

2.2.1. Antigen preparation

An example of C. burnetii preparation for IFA serological diagnostic based on phase II and phase I antigens is given below, but other modified protocols are used around the world (Samuel et al., 2009). Significant amounts of C. burnettii (>1012 bacteria) can be obtained in 2–5 weeks in embryonated eggs or cell cultures. An infection in mice can require 7–14 days. Purification of bacteria from host material includes differential centrifugations and takes 1 or 2 days. An infection in mice can require 7–14 days.

Phase II C. burnetii Nine Mile are grown in confluent layers of Vero or L929 cells in 150 cm2 culture flasks at 35°C under 5% CO2, with minimal essential medium (MEM) supplemented with 2 mM L-glutamine and 4% fetal bovine serum. The infection is monitored by microscopic examination of intracellular vacuoles or by Gimenez-stained cells collected from the supernatants of the flasks. Recent specific real-time quantitative PCR has been extremely valuable in routine monitoring. When a heavy C. burnetii infection is seen, the supernatants of 15 flasks are individually pelleted by centrifugation (5000 g, 15 minutes) and resuspended in 1 ml of PBS with 0.1% formaldehyde and incubated for 24 hours at 4°C. After pooling, the remaining cells are broken by sonication. Cellular debris are removed by two successive centrifugation steps (100 g, 10 minutes each). The 15 ml suspension is then centrifuged through 20 ml of PBS with 25% sucrose (6000 g, 30 minutes, without a break). The resulting pellet is washed three times in PBS (6000 g, 10 minutes), resuspended in the smallest possible volume of sterile distilled water, and adjusted to 2 mg/ml by UV spectroscopy. An antibacterial preservative, such as sodium azide at a final dilution of 0.1% or thiomersal at 0.01%, is added. Antigen prepared in this manner is frozen at –20°C.

To obtain phase I antigen, mice are inoculated with C. burnetii grown in cells (mainly in phase II). The spleens are removed 9 days after infection. Each one is ground in 7.5 ml MEM, and inoculated into three 75 cm2 culture flasks containing L929 or Vero cell monolayers (2.5 ml per flask). Amplification of phase I C. burnetii is conducted for 4 weeks, with a culture medium change once a week. The infected cells are then harvested and the bacteria are purified as described above (mainly in phase I).

Antigen production can also be performed by culture of C. burnetii in SPF embryonated eggs. At 6–7 days of age, the microorganism is inoculated into the yolk sac of the embryonated eggs, which are harvested after 10–15 days of incubation. Infected yolk sacs have a characteristic straw-yellow colour. Uninfected yolk sacs are orange in colour and have a viscous consistency. Any embryos that die before 5 days of incubation are discarded. The strain used for egg inoculation is a 1/100 homogenate of yolk sac in PBS containing penicillin (500 International Units/ml) and streptomycin (0.5 mg/ml). The yolk sacs are pooled and homogenised with three parts PBS. The suspension is inactivated with 1.6% formaldehyde for 24 hours at 37°C. The lipid supernatant fluid is discarded. The suspension is then centrifuged at moderate speed (∼500 g) for 30 minutes. After removal of the supernatant fluid, more PBS is added and centrifugation is repeated. The final suspension is diluted with PBS. Sodium azide or thiomersal is added as an antibacterial preservative. The abundance of C. burnetii and the absence of bacterial contaminants in homogenates of yolk sacs suspended in PBS are verified by microscopic examination of a smear on a microscope slide, stained by Stamp’s method. In order to obtain phase I antigen, C. burnetii recovered from spleen material of infected laboratory animals can be propagated as ground spleen extracts are subsequently transferred in the yolk sacs, given that the amount of phase I cells is still high until the sixth egg passage.

Titration of antigen with at least three different known sera (with high, moderate and low titres, respectively) is sufficient to determine the appropriate dilution for further immunofluorescence tests.
Twofold dilutions of the serum under test are placed on immunofluorescence slides with wells previously coated with one or two antigens. If specific antibodies are present, they are fixed by the antigen on the slide. The complex is then detected by examination with a fluorescence microscope following the addition of the fluorescent conjugate recognising the species-specific immunoglobulins.

### 2.2.2. Materials and reagents

- Microscope equipped for fluorescence, humidified incubator, washing basin.

- Slides suitable for the antigen are necessary. The latter may be either prepared in the laboratory or purchased from a supplier (see above). The method described is adapted from the BioMérieux kit, and is given as an example. Ready-to-use slides contain 12 wells per slide, each of 7 mm diameter, coated with phase II antigen obtained from culture on Vero cells and can be stored at 4°C or –20°C.

- Concentrated fluorescent conjugate, to be diluted when required with PBS + 1% Evans blue at the dilution recommended by the manufacturer.

- PBS, buffered glycerine, Evans blue dye 1% solution.

### 2.2.3. Test procedure

i) Inactivate the sera under test for 30 minutes at 56°C, then dilute serially from 1/40 to 1/640 in PBS.

ii) Allow the previously antigen-coated slides to warm to room temperature. Do not touch the wells.

iii) Add 20 µl of each serum dilution to the wells. Add negative and positive control sera. To one well, add 20 µl of PBS to serve as antigen control.

iv) Incubate in a humid chamber for 30 minutes at 37°C. Wash the slide twice with PBS for 10 minutes each. Rinse with distilled water and air dry.

v) Add to the wells, including the controls, 20 µl of the conjugate directed against the appropriate species (e.g. FITC-labelled rabbit anti-goat or anti-sheep IgG[H+L]), freshly diluted in PBS + Evans blue. Incubate in a humid chamber for 30 minutes at 37°C. Rinse with distilled water and air-dry. Add a few drops of buffered glycerine and cover with a cover-slip. Examine under a fluorescence microscope at magnification ×400 or more.

### 2.2.4. Interpretation of the results

A positive reaction will consist of small brilliant points against a dark background. Verify that the conjugate by itself and the negative control serum give a negative result (absence of small brilliant points). Nonspecific fluorescence usually takes the form of spots of irregular shape. The positive control must give the known titre with ± one dilution.

Values for interpretations have to be validated.

### 2.3. Complement fixation test (CFT)

This cold fixation micromethod of the type developed by Kolmer is performed with 96-well U-bottomed microtitre plates. The test detects complement-fixing antibodies present in the serum. The CFT is specific but less sensitive than the ELISA or IFA (Kittelberger et al., 2009; Rousset et al., 2007, 2009a). The CFT is still used by laboratories in many countries. This method often uses antigen in phase II prepared from a mixture of two strains (Nine Mile and Henzerling) or antigen in phase I and II mixture prepared from Nine Mile strain.

The reaction is done in two stages. Antigen and complement-fixing antibodies are first mixed, and sheep erythrocytes, sensitised by the anti-sheep erythrocyte serum, are added. Fixation of the complement by the antigen/antibody complex during the first step does not permit lysis of erythrocytes; in contrast, if there are no complement-fixing antibodies, the complement induces the lysis of the sensitised erythrocytes. Then the haemolysis rate is inversely proportional to the level of specific antibodies present in the sample serum.

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5 Dade Behring, Marburg, Germany.
6 Virion, Zürich, Switzerland.
2.3.1. Reagents

Veronal/calcium/magnesium buffer (VB), pH 7.2.

The haemolytic system: a mixture of equal parts of a 2% suspension of sheep erythrocytes in VB; and haemolytic serum diluted to a specified titre in VB.

Complement: commercial freeze-dried preparation or fresh guinea-pig serum.

Antigen: use commercial antigens at the titre recommended by the manufacturer if the antigen titration is performed with this method.

Positive and negative control sera.

2.3.2. Pretitrations

i) Dilute the sheep erythrocytes to a final concentration of 2% in VB.

ii) Titrate the haemolytic serum on a microplate: 25 µl of complement at a known haemolytic concentration (e.g. 1/30); 25 µl of increasing dilutions of haemolytic serum + 2% sheep erythrocytes. Include controls without complement. Incubate for 30 minutes at 37°C. Establish the dilution equivalent to 2 haemolytic units.

iii) Dilute the antigen as recommended by the manufacturer. The antigen may also be titrated: make increasing dilutions of antigen (25 µl horizontally) and a positive serum of known titre (25 µl, vertically). Add 25 µl of the suspension of sensitised erythrocytes and incubate for 30 minutes at 37°C. The antigen titre is the highest dilution producing a positive reaction with the highest serum dilution. Verify the absence of anticomplementary activity of the antigen at different dilutions.

iv) Titrate the complement on a microplate: serially dilute the complement or guinea-pig serum in VB, for example from 1/15 to 1/200. To each well containing 25 µl of this dilution, add 25 µl of antigen and 25 µl of the haemolytic system. Incubate for 30 minutes at 37°C and establish the dilution equivalent to 2 haemolytic units of complement.

2.3.3. Test procedure

i) Make twofold dilutions of decomplemented sample sera from 1/10 to 1/320 in six wells and in four additional wells at dilutions from 1/10 to 1/80 to detect anticomplementary activity (25 µl per well).

ii) Add 25 µl of diluted antigen or 25 µl of VB to control serum wells.

iii) Add 25 µl diluted complement to all wells. Cover the plate with plastic adhesive film and incubate for 16 hours at 4°C.

iv) Remove the plates from the refrigerator, allow them to reach room temperature, and add 25 µl of freshly prepared haemolytic system. Incubate at 37°C for 30 minutes. Centrifuge the plates at 500 g for 5 minutes at 4°C. Examine the controls and read the results.

2.3.4. Interpretation of the results

Titres between 1/10 and 1/40 are characteristic of a latent infection. Titres of 1/80 or above in one or more sera from a group of from five to ten animals reveal an active phase of the infection.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

SECTION UNDER STUDY

1. Production of Coxiella burnetii antigen

Growth and purification of C. burnetii should only be performed in facilities that meet the requirements outlined in Chapter 1.1.3. Precautions assigned to Containment Group 3 pathogens must be taken either for phase I or phase II C. burnetii. As seen in the Nine Mile reference strain, the LPS phase variation could be accompanied by a permanent chromosomal deletion that makes impossible a reversion from phase II to phase I. However, a variant of the Australia QD isolate producing truncated LPS had no detectable large deletion. The molecular
changes that occurred in LPS phase variation are not clearly defined. Even with extensive repeated passage in
non-immunologically competent hosts (cultural cells, embryonated eggs), the majority of isolates are non-clonal
as growth from a single colony is difficult to establish (Samuel & Hendrix, 2009). The risk of aerosols must be
taken into account at all stages when working with viable C. burnetii. Sustained serological monitoring of Q fever
should be carried out for laboratory personnel.

2. Diagnostic biologicals

An example of C. burnetii preparation for IFA serological diagnostic based on phase II and phase I antigens is
given below, but other modified protocols are used around the world (Samuel et al., 2009). Significant amounts of
C. burnetii (>10^{10} bacteria) can be obtained in 2 to 5 weeks in embryonated eggs or cell cultures. Purification of
bacteria from host material includes differential centrifugations and takes 1 or 2 days. An infection on mice can
require 7–14 days.

Phase I C. burnetii. Nine Mile are grown in confluent layers of Vero or L929 cells in 150 cm^2 culture flasks at 35°C
under 5% CO_2 with minimal essential medium (MEM) supplemented with 2 mM L-glutamine and 4% fetal bovine
serum. The infection is monitored by microscopic examination of intracellular vacuoles or by Gimenez-stained
cells collected from the supernatants of the flasks. Recent specific real-time quantitative PCR has been extremely
valuable in routine monitoring. When a heavy C. burnetii infection is seen, the supernatants of 15 flasks are
individually pelleted by centrifugation (9000 g, 15 minutes), resuspended in 1 ml of PBS with 0.1% formaldehyde
and incubated for 24 hours at 4°C. After pooling, the remaining cells are broken by sonication. Cellular debris are
removed by two successive centrifugation steps (100 g, 10 minutes each). The 15 ml suspension is then
centrifugated through 20 ml of PBS with 25% sucrose (6000 g, 30 minutes, without a break). The resulting pellet is
washed three times in PBS (6000 g, 10 minutes), resuspended in the smallest possible volume of sterile distilled
water, and adjusted to 2 mg/ml by UV spectroscopy. An antibacterial preservative, such as sodium azide at a final
dilution of 0.1% or thiomersal at 0.01%, is added. Antigen prepared in this manner is frozen at −20°C.

In order to obtain phase I antigen, mice are inoculated with C. burnetii grown in cells (mainly in phase II). Nine
days after infection, the spleens are removed. Each one is ground in 7.5 ml MEM, and inoculated into three
75 cm^2 culture flasks containing L929 or Vero cell monolayers (2.5 ml per flask). Amplification of phase I
C. burnetii is conducted for 4 weeks, with a culture medium change once a week. The infected cells are then
harvested and the bacteria are purified as described above (mainly in phase I).

Antigen production can also be performed by culture of C. burnetii in SPF embryonated eggs. At 6-7 days of age,
the microorganism is inoculated into the yolk sac of the embryonated eggs, which are harvested after 10–15 days
of incubation. Infected yolk sacs have a characteristic straw-yellow colour. Uninfected yolk sacs are orange in
colour and have a viscous consistency. Any embryos that die before 5 days of incubation are discarded. The
strain used for egg inoculation is a 1/100 homogenate of yolk sac in PBS containing penicillin (500 International
Units/ml) and streptomycin (0.5 mg/ml). The yolk sacs are pooled and homogenised with three parts PBS. The
suspension is inactivated with 1.6% formaldehyde for 24 hours at 37°C. The lipid supernatant fluid is discarded.
The suspension is then centrifuged at moderate speed (∼500 g) for 30 minutes. After removal of the supernatant
fluid, more PBS is added and centrifugation is repeated. The final suspension is diluted with PBS. Sodium azide
or thiomersal is added as an antibacterial preservative. The abundance of C. burnetii and the absence of bacterial
contaminants in homogenates of yolk sacs suspended in PBS are verified by microscopic examination of a smear
on a microscope slide, stained by Stamp’s method. In order to obtain phase I antigen, C. burnetii recovered from
spleen material of infected laboratory animals can be propagated, as ground spleen extracts are subsequently
transferred in the yolk sac, given that the amount of phase I cells is still high until the sixth egg passage.

Titration of antigen with at least three different known sera (with high, moderate and low titres, respectively) is
sufficient to determine the appropriate dilution for further immunofluorescence tests.

3. Vaccine

The protective antigen is composed of purified particles in phase I with the nontruncated phase I LPS structure. In
some countries, vaccination is practised for occupationally exposed people, such as abattoir workers,
veterinarians and laboratory personnel. A vaccine inactivated by formaldehyde (Q-VAX, CSL Ltd, Australia),
prepared from the Hensenfeld strain of phase I C. burnetii, received the approval of the Australian authorities in
1989. Phase I vaccines are effective, but vaccination is contraindicated for individuals who had seroconverted or
had been exposed to C. burnetii prior to immunisation.

Several vaccines have been developed against animal Q fever. Results converge today towards the use of a
phase I vaccine that is helpful against Q fever in combination with other control measures. An inactivated phase I
vaccine is commercially available (Coxevac, CEVA, Hungary) for vaccination of ruminants. A review on Q fever in
Slovakia suggests that the decrease in the occurrence of human and animal Q fever could be the result of the
large-scale vaccination of cattle that was carried out there over a 10-year period, together with improved veterinary control of domestic animal transport within the country (Serbezov et al., 1999). In the Netherlands, a large vaccination programme has been implemented in goat and sheep farms, accompanied by the controlled processing of manure and checks on animal transports, but it is not clear yet whether bacterial shedding by animals is prevented or at least reduced by vaccination. Controlling the epidemic is difficult and can be compromised by the prolonged stability of the bacterium in the environment and the possible role of animal species other than small ruminants (EFSA, 2010).

This vaccine consists of highly purified whole cells prepared from Nine Mile strain in the phase I (egg passage 3 to egg passage 5) and inactivated by formaldehyde. No adjuvant is used. Recently, a French study demonstrated the efficacy of this vaccine through experimental vaccination and challenge of pregnant goats: the vaccine prevented abortion and shedding in milk, and decreased considerably the shedding in the vaginal secretions and faeces (Arricau-Bouvery et al., 2005). Ideally, vaccine efficacy must be demonstrated by tests on all the target species.

In the case of vaccination on already infected animals, some authors believe that it is preferable to select seronegative herds or animals for immunisation, and to continue vaccination over several years in young animals (Krauss, 1989). First follow-up studies on shedding herds or flocks show a contribution of the vaccination against the infection incidence and the shedding levels (Guatteo et al., 2008; Rousset et al., 2009b). Repeated annual vaccination, particularly of young animals, is recommended. However, the duration of immunity is not defined. The development of serological tools distinguishing between infected and vaccinated ruminants (DIVA) would be helpful. To date, no data are available for comparing the cost–benefit of this strategy with a nonselective strategy in the control of Q fever.

REFERENCES


Chapter 2.1.12. – Q fever


Chapter 2.1.12. – Q fever


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

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Q fever.
CHAPTER 2.1.19.

VESICULAR STOMATITIS

SUMMARY

Vesicular stomatitis (VS) is a vesicular disease of horses, cattle and pigs caused by vesiculoviruses of the family Rhabdoviridae. This disease is clinically indistinguishable from foot and mouth disease (FMD), vesicular exanthema of swine (VES), or swine vesicular disease (SVD) when horses are not involved. Sheep, goats and many other wild species can be infected. Humans are also susceptible. The disease is limited to the Americas; however, it was previously described in France and in South Africa.

Virus is transmitted directly by the transcutaneous or transmucosal route and has been isolated from sandflies and mosquitoes. Experimental transmission has been shown from black flies to both pigs and cattle. There is seasonal variation in the occurrence of VS: it disappears at the end of the rainy season in tropical areas, and at the first frosts in temperate zones. There is also some evidence that it could be a plant virus and that animals are the end of the epidemiological chain. The pathogenesis of the disease is unclear, and it has been observed that the humoral-specific antibodies do not always prevent infection with VS serogroup viruses.

Although VS may be suspected when horses are involved as well as pigs and cattle, prompt differential diagnosis is essential because the clinical signs of VS are indistinguishable from FMD when cattle and pigs are affected, and from SVD or VES when only pigs are affected.

Identification of the agent: Virus can be readily isolated by the inoculation of several tissue culture systems, unweaned mice, or embryonated chicken eggs. Viral RNA can be detected from epithelial tissue and vesicular fluid by conventional and real-time reverse transcriptase polymerase chain reaction (RT-PCR). Viral antigen can be identified by an indirect sandwich enzyme-linked immunosorbent assay (IS-ELISA) – this is the least expensive and most rapid test. The complement fixation test (CFT) is also a good alternative. The virus neutralisation (VN) test may be used, but it is elaborate and time-consuming.

Serological tests: Convalescent animals develop serotype-specific antibodies within 4–8 days of infection that are demonstrated by a liquid-phase blocking ELISA (LP-ELISA), a competitive ELISA (C-ELISA) and VN. Other described tests are CFT, agar gel immunodiffusion and counterimmunoelectrophoresis.

Requirements for vaccines: Inactivated virus vaccines with aluminium hydroxide or oil as adjuvants have been tested in the United States of America and in Colombia, respectively. Both vaccines generated high levels of specific antibodies in the sera of vaccinated cattle. However, it is not yet clear if serum antibodies would prevent the disease. An attenuated virus vaccine has been used in the field with unknown efficacy.

A. INTRODUCTION

Vesicular stomatitis (VS) was described in the United States of America (USA) by Oltsky et al. (1926) and Cotton (1927) as a vesicular disease of horses, and subsequently of cattle and pigs. Vesicles are caused by virus on the tongue, lips, buccal mucosa, teats, and in the coronary band epithelium of cattle, horses, pigs, and many other species of domestic and wild animals. Natural disease in sheep and goats is rare, although both species can be experimentally infected. Mixed infections of foot and mouth disease (FMD) and VS viruses have occurred in the same herds of cattle and can be induced experimentally. Many species of laboratory animals are also susceptible. The disease is limited to the Americas; however, it was described in France (1915 and 1917) and in South Africa (1886 and 1897) (Hanson, 1952).
Influenza-like signs, normally without vesicles, have been observed in humans who are in contact with animals with VS or who handle infective virus. All manipulations involving virus, including infective materials from animals, should be undertaken with proper biosafety procedures.

Two distinct immunological classes of vesicular stomatitis virus (VSV) are recognised: New Jersey (NJ) and Indiana (IND). Both viruses are members of the genus Vesiculovirus, family Rhabdoviridae and have been extensively studied at the molecular level. Several other closely related rhabdoviruses have been isolated from sick animals over the past decades. There are three subtypes of the IND serogroup based on serological relationships: IND-1 IND-2 and IND-3; they are also known as classical IND virus (VSV), cocal virus (COCV), and alagoa virus (VSAV), respectively (Federer et al., 1967). Strains of the serotype NJ and subtype IND-1 are endemic in livestock in areas of southern Mexico, Central America, Venezuela, Colombia, Ecuador and Peru, with VSV NJ causing the vast majority (>80%) of the clinical cases. Sporadic activity of NJ and IND-1 VSV has been reported in northern Mexico and the western United States. IND-2 has only been isolated in Argentina and Brazil and only from horses (Salto-Argentina/63, Maipu-Argentina/86, Rancheria-Brazil/66, Riberao-Brazil/79) (Alonso et al., 1991; Alonso Fernandez & Sondahl, 1985). Cattle living together with the infected horses did not develop antibodies against VSV (Alonso et al., 1991). The IND-3 subtype, (Alagoas-Brazil/64), has been identified, sporadically only in Brazil and only in horses until 1977. However, in 1977 the IND-3 serotype (Espinosa-Brazil/77 strain) was first isolated from cattle in Brazil; it has been observed that this serotype affects cattle to a lesser degree than horses (Alonso et al., 1991; Alonso Fernandez & Sondahl, 1985). This finding confirms the first descriptions, in 1926 and 1927 (Cotton, 1927; Oltisky et al., 1926), of the NJ and IND serotypes in horses, and subsequently in cattle and pigs; this same predilection has been observed in other VS outbreaks.

The mechanism of transmission of the virus is unclear. The viruses have been isolated from sandflies, mosquitoes, and other insects (Comer et al., 1992; Francy et al., 1988; Mason, 1978). Experimental transmission of VS NJ has been demonstrated to occur from black flies (Simulium vittatum) to domestic swine and cattle (Mead et al., 2004; 2009) There are also hypotheses that the VSV is a plant virus present in pasture (Mason, 1978) and that animals are the end of the epidemiological chain and, in special circumstances, the virus could undergo an adaptation process to infect animals, followed by direct transmission between susceptible animals. During the 1982 epizootic in the western USA, there were a number of cases where there was direct transmission from animal to animal (Sellers & Maarouf, 1990). While VS is not diagnosed in livestock every year in the USA, it is VSV has historically been considered to be endemic in feral pigs on Ossabaw Island, Georgia, USA (Boring & Smith, 1962); but recent evidence suggests it may have disappeared from the island (Killmaster et al., 2011).

The incidence of disease can vary widely among affected herds. Usually 10–15% of the animals show clinical signs. Clinical cases are mainly seen in adult animals. Cattle and horses under 1 year of age are rarely affected. Mortality is close to zero in both species. However, high mortality rates in pigs affected by the NJ virus have been observed. Sick animals recover in about 2 weeks. The most common complications of economic importance are mastitis and loss of production in dairy herds (Lauerman et al., 1962). Both NJ and IND-1 serotypes in the 1995, 1997 and 1998 US outbreaks primarily caused clinical disease in horses. Although some clinical signs were observed in cattle, the primary finding in cattle was seroconversion. Recent VSV outbreaks in the USA have been associated primarily with horses and the NJ serotype.

B. DIAGNOSTIC TECHNIQUES

VS cannot reliably be clinically differentiated from the other vesicular diseases, such as FMD, vesicular exanthema of swine (VES), and swine vesicular disease (SVD) when horses are not involved. An early laboratory diagnosis of any suspected VS case is therefore a matter of urgency.

The sample collection and technology used for the diagnosis of VS must be in concordance with the methodology used for the diagnosis of FMD, VES and SVD, in order to facilitate the differential diagnosis of these vesicular diseases. Note: VS serogroup viruses can be human pathogens and appropriate precautions should be taken when working with potentially infected tissues or virus (see Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

Vesicle fluid, epithelium covering unruptured vesicles, epithelial flaps of freshly ruptured vesicles, or swabs of the ruptured vesicle are the best diagnostic samples. These samples can be collected from mouth lesions, as well as from the feet and any other sites of vesicle development. It is recommended that animals should be sedated before samples are collected to avoid injury to helpers and for reasons of animal welfare. Samples from all species should be placed in containers of Tris-buffered tryptose broth with phenol red, pH 7.6. If complement fixation (CF) is to be carried out for antigen detection, samples from all species can be collected in glycerol/phosphate buffer, pH 7.2–7.6. (Note: glycerol is toxic to virus and decreases the sensitivity of virus isolation; it is therefore only recommended for collection of samples for CFT.) Samples should be kept refrigerated and if they can arrive at the laboratory within 48 hours after collection, they should be sent refrigerated. If samples are sent frozen with dry ice, precautions should be taken to protect the sample from contact with any CO₂. There are special packaging requirements for shipping samples with dry ice (see Chapter
1.1.1 Collection and shipment of diagnostic specimens, for further information on shipping of diagnostic samples).

Alternatively, samples can be shipped with commercially available freezer packs that have been frozen in an ultra-low freezer (–60°C or colder) if shipping time is of short duration.

When epithelial tissue is not available from cattle, samples of oesophageal–pharyngeal (OP) fluid can be collected by means of a probang (sputum) cup. In pigs, throat swabs can be taken for submission to a laboratory for virus isolation. This material should be sent to the laboratory refrigerated in Tris-buffered tryptose broth. If the samples will be in shipment for more than 48 hours after collection, they should be sent frozen with dry ice as described previously. Probang samples for isolation of virus should not be treated with solvents such as chloroform. Virus can be isolated from oral and nasal specimens up to 7 days post-infection.

When it is not possible to collect samples for identification of the agent, serum samples from recovered animals can be used for detecting and quantifying specific antibodies. Paired sera from the same animals, collected 1–2 weeks apart, are preferred for checking the change in antibody titre may be needed depending on the serological assay being used and prior history of VSV in the country.

Specific reagents for VS diagnosis are not commercially available and each laboratory must produce its own or obtain them from a Reference Laboratory. The two OIE Reference Laboratories for vesicular stomatitis (see Table given in Part 4 of this Terrestrial Manual), and the Pirbright Institute, UK, produce and distribute diagnostic reagents on request.

Table 1. Test methods available for the diagnosis of vesicular stomatitis and their purpose

<table>
<thead>
<tr>
<th>Method</th>
<th>Population freedom from virus circulation</th>
<th>Individual animal freedom from infection prior to movement</th>
<th>Contribute to eradication policies</th>
<th>Confirmation of clinical cases</th>
<th>Prevalence of infection = surveillance</th>
<th>Immune status in individual animals or populations post-vaccination***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus isolation*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IS-ELISA*</td>
<td>=</td>
<td>=</td>
<td>++</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>CFT*</td>
<td>=</td>
<td>=</td>
<td>++</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>RT-PCR*</td>
<td>=</td>
<td>=</td>
<td>++</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>Detection of immune response2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LP-ELISA**</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>C-ELISA**</td>
<td>+++</td>
<td>+++</td>
<td>=</td>
<td>=</td>
<td>+++</td>
<td>++</td>
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<tr>
<td>VN**</td>
<td>+++</td>
<td>+++</td>
<td>=</td>
<td>=</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>CFT**</td>
<td>=</td>
<td>=</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>=</td>
</tr>
</tbody>
</table>

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose. Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

IS-ELISA = indirect sandwich enzyme-linked immunosorbent assay; CFT = complement fixation test; RT-PCR = reverse transcriptase polymerase chain reaction; LP-ELISA = liquid-phase blocking ELISA; C-ELISA = competitive ELISA; VN = virus neutralisation.

Should only be used on animals demonstrating clinical signs compatible with VSV. A positive result is meaningful. A negative result could mean the animal is no longer shedding virus, the virus level is too low to detect, or, for virus isolation samples that

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1 Pirbright Institute, Ash Road, Pirbright, Woking, Surrey GU24 0NF, United Kingdom.
2 A combination of agent identification methods applied on the same clinical sample is recommended.
3 One of the listed serological tests is sufficient.
Chapter 2.1.19. – Vesicular stomatitis

the samples were not maintained at appropriate temperatures and received in an appropriate time period following collection for virus isolation (virus inactivated). The presence of VSV antibodies only indicates prior exposure to VSV. It does not determine whether the antibodies are due to current infection or past infection. Interpretation of results needs to be based on serological results, clinical presentation, and epidemiology. CF antibody duration in an animal is generally less than 1 year. Antibodies detected by the VN assay and competitive ELISAs can be detected for years following infection. The difference in sensitivity of the serological assays has an effect on detection during the acute phase of infection; combination testing, such as C-ELISA and CF or paired sampling showing four-fold titre change (CF, VN, LP-ELISA), is therefore necessary when an animal presents with acute clinical signs of VSV. **Indicates the presence of antibodies only; does not indicate protection from infection.

1. Identification of the agent

1.1. Direct visualisation

Due to the different morphological characteristics of the rhabdovirus (VS serogroup viruses), picornavirus (FMD virus and SVD virus), calicivirus (VES) and the large number of virus particles present in vesicular fluids and epithelial tissues, electron microscopy can be a useful diagnostic tool for differentiating the virus family involved.

1.2. In-vitro cultivation

For identification of VS serogroup viruses and the differential diagnosis of vesicular diseases, clarified suspensions of field samples suspected to contain virus should be submitted for testing. For virus isolation, the same samples are inoculated into appropriate cell cultures. The inoculation of African green monkey kidney (Vero), baby hamster kidney (BHK-21) and IB-RS-2 cell cultures with the same sample permits differentiation of the vesicular diseases: VS serogroup viruses cause a cytopathic effect (CPE) in all three cell lines; FMD virus causes a CPE in BHK-21 and in IB-RS-2, while SVD virus causes a CPE in IB-RS-2 only. Many other cell lines, as well as most primary cell cultures of animal origin, are susceptible to VS serogroup viruses.

If a CPE develops in the cultures, the suspension fluids can be used for identification of the agent by different immunological tests and the cell culture can be stained with VS-specific fluorescent antibody conjugate. Viral antigen can be detected by enzyme-linked immunosorbent assay (ELISA), complement fixation test (CFT) or polymerase chain reaction (PCR). Similar tests can be performed on homogenate suspensions of the dissected musculo-skeletal tissues of dead mice and chicken embryos and with suspensions of epithelial samples. The brain tissue from mice is an excellent source of virus.

The preferred immunological methods for the identification of the viral antigens in the laboratory are the ELISA (Alonso et al., 1991; Ferris & Donaldson, 1988), the CFT (Alonso et al., 1991; Jenny et al., 1958) and fluorescent antibody staining. The virus neutralisation (VN) test, with known positive antisera against the VS virus NJ and IND serotypes, may be used in tissue cultures or embryonated eggs, but it is more time-consuming.

1.3. In-vivo testing

Virus replicates and can be isolated in 8- to 10-day-old chicken embryos by inoculation into the allantoic sac; in 2- to 7-day-old unweaned mice by inoculation using any route, or in 3-week-old mice by intracerebral inoculation. In all three cases, virus causes death in between 2 and 5 days after inoculation.

The most susceptible route for horses and cattle is intradermal lingual administration. Pigs are inoculated in the coronary band or on the snout. Vesicular lesions may be observed in the epithelial tissues of the mouth, teats and feet, 2-4 days after inoculation. The presence of secondary vesicles after inoculation of cattle and horses depends mainly on the VS virus isolate used. The snout is normally affected in pigs.

1.4. Virus isolation

1.4.1. Test procedure

i) Inoculate cell culture in Leighton tubes and 25 cm² flasks with the clarified suspension of tissues or vesicular fluid.

ii) Incubate inoculated cell cultures at 37°C for 1 hour.

iii) Discard inoculum and wash cell cultures three times with cell culture medium and replace with cell culture medium containing 2.5% fetal bovine serum (FBS).
iv) Incubate Leighton tube cell cultures at 33–35°C and observe for CPE.

v) After 18–24 hours of incubation, the cover-slip from one Leighton tube culture per specimen inoculated is stained with New Jersey and Indiana VS virus-specific fluorescent antibody (FA) conjugate.

vi) Remaining Leighton tube cultures and 25 cm² flask cultures are incubated at 35–37°C for 6 more days and observed daily for CPE.

vii) At 7 days post-inoculation, the remaining Leighton tube cover-slips are stained with FA conjugate.

viii) If CPE is observed and the FA staining is negative, a second passage is made, as described above, using the cells from the 25 cm² flask. Note: First passage cultures with significant CPE may yield false-negative immunofluorescence results. Serial tenfold dilutions may be prepared and inoculated to provide distinct plaques of fluorescing cells.

ix) Interpretation of the results: If no fluorescence is observed and no CPE evident in the flask culture, the sample is negative for virus isolation. If specific fluorescence is observed, the sample is positive for virus isolation.

x) Alternatively cell culture in flasks can be inoculated with field samples, incubated at 35–37°C for 48 hours and observed daily for CPE. If no CPE is observed after 48 hours, the flask cultures are frozen and thawed and a sample of the supernatant is inoculated into fresh cell culture. Up to three passages are made, of 48 hours each. To detect the presence of VSV antigen, clarified supernatants of each passage are tested by ELSA or CFT.

1.5. Enzyme-linked immunosorbent assay

The indirect sandwich ELISA (IS-ELISA) (Alonso et al., 1991; Ferris & Donaldson, 1988) is currently the diagnostic method of choice for identification of viral serotypes of VS and other vesicular diseases. Specifically, the ELISA procedure with a set of polyvalent rabbit/guinea-pig antisera, prepared against virions of the representative strains of the three subtypes of the IND serotype, identifies all strains of the VS virus IND serotype (Alonso et al., 1991). For detection of VS virus NJ strains, a monovalent set of rabbit/guinea-pig antisera is suitable (Alonso et al., 1991; Ferris & Donaldson, 1988).

1.5.1. Test procedure

i) Solid phase: ELISA plates are coated either for 1 hour at 37°C or overnight at 4°C with rabbit antisera and normal rabbit serum (as described in Alonso et al., 1991 and Allende et al., 1992), and optimally diluted in carbonate/bicarbonate buffer, pH 9.6. Subsequently, the plates are washed once with phosphate-buffered saline (PBS) and blocked for 1 hour at room temperature with 1% ovalbumin Grade V in PBS. After washing the plates can be used immediately or stored at –20°C for future use.

ii) Test samples: Antigen suspensions of test samples (10–20% epithelial tissue suspension, musculo-skeletal tissue of chicken embryo or mice in PBS or minimal essential medium (MEM) or undiluted clarified cell culture supernatant fluid) are deposited in the corresponding wells and the plates are incubated for 1 hour at 37°C on an orbital shaker.

iii) Detector: Monovalent and polyvalent guinea-pig antisera to VS virus NJ and IND serotypes, respectively, that are homologous to coated rabbit serum and that have been diluted appropriately in PBS containing 0.05% Tween 20, 1% ovalbumin Grade II, 2% normal rabbit serum, and 2% normal bovine serum (PBSTB) are added to the corresponding wells and left to react for 30–60 minutes at 37°C on an orbital shaker.

iv) Conjugate: Peroxidase/rabbit or goat IgG anti-guinea-pig Ig conjugate, diluted in PBSTB, is added and left to react for 30–60 minutes at 37°C on an orbital shaker.

v) Substrate: H₂O₂-activated substrate is added and left to react at room temperature for 15 minutes, followed by the addition of sulphuric acid to stop the reaction. Absorbance values are measured using an ELISA reader.

Throughout the test, 50 µl reagent volumes are used. The plates are washed three–five times between each stage with physiological saline solution or PBS containing 0.05% Tween 20. Controls for the reagents used are included.

vi) Interpretation of the results: Absorbance values of positive and negative antigen controls wells should be within specified values for acceptance. An antiserum giving an absorbance

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≥0.3 is considered to be positive for the corresponding virus subtype. Absorbance values <0.3–0.2 are considered suspicious and values <0.2 are considered negative for the corresponding virus subtype. Suspicious and negative samples should be inoculated in cell culture and passages re-tested in ELISA.

*Interpretation of the results:* An antiserum giving an absorbance more than 20% greater than the other antisera, negative serum and controls is considered to be positive for the corresponding virus subtype.

### 1.6. Complement fixation test

The ELISA is preferable to the CFT because it is more sensitive and it is not affected by pro- or anti-complementary factors. When ELISA reagents are not available, however, the CFT may be performed. The CFT in U-bottomed microtitre plates, using the reagents titrated by CF50% test, is described.

#### 1.6.1. Test procedure

1. **Antisera:** Guinea-pig monovalent anti-NJ VS virus and polyvalent anti-IND VS virus, diluted in veronal buffer (VB) at a dilution containing 2.5 CFU50 (50% complement fixation units) against homologous virus, are deposited in plate wells. Those antisera are the detectors used in ELISA.

2. **Test samples:** The antigen suspension of test samples, prepared as described for IS-ELISA, is added to the wells with serum.

3. **Complement:** 4 CHU50 (50% complement haemolytic units) are added to the serum and antigen. (An alternative is to use 7.5, 10 and 20 CHU50 with the goal of reaching 4 CHU50 in the test.) The mixture of antisera, test samples and complement is incubated at 37°C for 30 minutes.

4. **Haemolytic system:** A suspension of sheep red blood cells (SRBC) in VB, sensitised with 10 HU50 (50% haemolytic units) of rabbit anti-SRBC serum, is added to the wells. The haemolytic system has an absorbance of 0.66 read at 545 nm, in the proportion of two volumes of haemolytic system + three volumes of distilled water. The mixture is incubated for 30 minutes at 37°C. Subsequently, the plates are centrifuged and the reaction is observed visually.

Volumes of 25 µl for antisera, test samples and complement, and 50 µl of haemolytic system, are required. Appropriate controls for the antisera, antigens, complement and haemolytic system are included.

It is possible to perform the CF50% test in tubes (Alonso et al., 1991) using reagent volumes of 200 µl (eight times greater than those indicated for the CF in microtitre plates). With the CF50% test, the reaction can be expressed as absorbance read spectrophotometrically at 545 nm.

#### 1.6.1.1. Interpretation of the results:

When controls are as expected, samples with haemolysis <20% for one antiserum in comparison with the other antiserum and controls are considered to be positive for the corresponding type.

Field samples that are negative on the ELISA or CFT should be inoculated into cell culture or unweaned mice. If there is no evidence of viral infection after three passages, the specimen is considered to be negative for virus.

### 1.7. Nucleic acid recognition methods

The RT-PCR can be used to amplify small genomic areas of the VS virus (Hofner et al., 1994; Rodriguez et al., 1993; Wilson et al., 2009). This technique will detect the presence of virus RNA in tissue and vesicular fluid samples and cell culture, but cannot determine if the virus is infectious. In general, PCR techniques have not been routinely used for screening diagnostic cases for viruses causing VS.
2. Serological tests

For the identification and quantification of specific antibodies in serum, the ELISA and the VN test are preferable. The CFT may be used for quantification of early antibodies. Antibody can usually be detected between 5 and 8 days post-infection; the length of time antibody persists has not been accurately determined for the three tests but is thought to be relatively short for the CF and for extended periods for the VN and ELISA (Katz et al., 1997).

2.1. Liquid phase blocking enzyme-linked immunosorbent assay (a prescribed test for international trade)

The liquid-phase blocking ELISA (LP-ELISA) is a method for the detection and quantification of antibodies to VS serogroup viruses. The use of viral glycoproteins as antigen is recommended because they are not infectious, allow the detection of neutralising antibodies, and give fewer false-positive results than the VN (Allende et al., 1992).

2.1.1. Test procedure

i) **Solid phase:** As described above in Section B.1.5 for the IS-ELISA.

ii) **Liquid phase:** Duplicate, two- to five-fold dilution series of each test serum, starting at 1/4, are prepared in U-bottomed microtitre plates. An equal volume of VS virus NJ or IND glycoprotein, in a predetermined dilution providing 70% reaction, is added to each well and the plates are incubated for 1 hour at 37°C. 50 µl of these mixtures is then transferred to the ELISA plates with the solid phase and left to react for 30 minutes at 37°C on an orbital shaker.

iii) **Detector, conjugate and substrate:** The same reagents steps described and methods are used as those indicated for the IS-ELISA are performed using monovalent antisera homologous to the test antigen, as detectors.

iv) **Interpretation of the results:** 50% end-point titres are expressed in log10 in reference to the 50% reduction of negative serum control OD of the antigen control, according to the Spearmann–Kärber method. Titres of >1.0 (1/10) are considered to be positive.

2.2. Competitive enzyme-linked immunosorbent assay (a prescribed test for international trade)

A competitive ELISA for detection of antibodies has also been developed. The procedure described here is based on a procedure described by Afshar et al. (1993). It uses vesicular stomatitis NJ and IND-1 recombinant antigens as described by Katz et al. (1995).

2.2.1. Test procedure

i) **Solid phase:** Antigens are diluted in carbonate/bicarbonate buffer, pH 9.6, and 75 µl is added to each well of a 96-well ELISA plate. The plates are incubated overnight at 4°C; coated plates can be frozen at −70°C for up to 30 days. The plates are thawed, antigen is decanted, and 100 µl of blocking solution (5% nonfat dry milk powder solution in PBS [for example, 5 g dry milk powder dissolved in 95 ml PBS) is added. The plates are then incubated at 25°C for 15–30 minutes and blocking solution is decanted. The plates are washed three times with PBS/0.05% Tween 20 solution.

ii) **Liquid phase:** 50 µl of serum diluted 1/8 in 1% nonfat dry milk in PBS is added to each of the duplicate wells for each sample. A positive and negative control serum for each serotype should be included on each ELISA plate. The plates are incubated at 37°C for 30 minutes. Without washing, 50 µl of polyclonal ascites fluid is added to each well and plates are incubated at 37°C for 30 minutes.

iii) **Detector:** The plates are washed three times, and 50 µl of goat anti-mouse horseradish-peroxidase conjugate diluted in 1% nonfat dry milk with 10% normal goat serum is added to each well. The plates are incubated at 37°C for 30 minutes, washed three times, and 50 µl of tetramethyl-benzidine (TMB) substrate solution is added to each well. The plates are incubated at 25°C for 5–10 minutes and then 50 µl of 0.05 M sulphuric acid is added to each well. The plates are read at 450 nm and the optical density of the diluent control wells must be > 1.0.

iv) **Interpretation of the results:** A sample is positive if the absorbance is ≤50% of the absorbance of the diluent control. Note that horses naturally infected with New Jersey virus have been known to test positive by this assay for at least 5–6 years following infection.
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2.3. Virus neutralisation (a prescribed test for international trade)

The VN test is carried out in tissue culture microtitre plates with flat-bottomed wells using inactivated serum as test sample, 1000 TCID₅₀ (50% tissue culture infective dose) of VS NJ or IND virus, and Vero M cells, or preformed monolayer (Allende et al., 1992) or a suspension IB-RS-2 cells to test for the presence of unneutralised virus.

2.3.1. Test procedure

i) Virus: VS NJ or IND virus is grown in Vero cell monolayers and stored in liquid nitrogen or frozen at –70°C.

ii) Test samples: Sera are inactivated at 56°C for 30 minutes before testing. Positive and negative control standard sera are included in the test.

iii) Virus neutralisation: Sera are diluted in a two-fold or four-fold dilution series across the plates, starting from 1/4 dilution. Two rows of wells are used per serum. The same volume of NJ or IND virus suspension containing about 1000 TCID₅₀/25 µl is added and incubated at 37°C for 60 minutes to allow neutralisation to take place. Subsequently, 50 µl of the mixtures is deposited on preformed cell monolayers in microtitre plates or 150 µl of 300,000/ml IB-RS-2 or Vero cell suspension is added to each well with the serum/virus mixtures. The plates are covered with loosely fitting lids and incubated for 48–72 hours at 37°C in an atmosphere of 5% CO₂ or sealed with pressure-sensitive tape and incubated in a normal atmosphere. (It has been determined that a virus titre of 1000 TCID₅₀/25 µl will decrease the nonspecific reactions and maintain a high test sensitivity.)

iv) Interpretation of the results: Wells without CPE are considered to be positive. End-point titres of test serum titres are determined by the Spearmann–Kärber method when the virus titres are between 750 and 1330 TCID₅₀ and when titres of positive and negative standard sera are within twofold of their mean values as estimated from previous titration. The 100% neutralisation titres of each serum are expressed at log 10. Sera with values of 1/32 or greater are considered to be positive for antibodies against VSV. Note that horses naturally infected with New Jersey virus have been known to test positive by this test method for at least 5–8 years following infection. In an alternative protocol a viral dose of 1000 TCID₅₀ per millilitre of virus/serum mixture is used and reaction incubated at 37°C for 60 minutes to allow neutralisation to take place. Subsequently, 100 µl of the mixtures is deposited on preformed cell monolayers in microtitre plates. The plates are covered with loosely fitting lids and incubated for 48 hours at 37°C in an atmosphere of 5% CO₂. Wells without CPE are considered to be positive. End-point titre of test serum is determined by the Spearmann–Kärber method when the virus titres are between 10²±0.5/100 µl and when titres of positive and negative standard sera are within twofold of their mean values as estimated from the previous titration. The 50% neutralisation titre of each serum is expressed as log 10. Sera with values of 1.3 (1/20) or greater are considered to be positive for VS antibodies (Allende et al., 1992).

2.4. Complement fixation test (a prescribed test for international trade)

A detailed description of this test is given in Section B.1.6. This is modified as follows. The CFT may be used for quantification of early antibodies, mostly IgM. For this purpose, twofold serum dilutions are mixed with 2 CFU₅₀ of known antigen and with 5% normal bovine or calf sera included in 4 CHU₅₀ of complement. The mixture is incubated for 3 hours at 37°C or overnight at 4°C. Subsequently, the haemolytic system is added followed by incubation for 30 minutes at 37°C. The serum titre is the highest dilution in which no haemolysis is observed. Titres of 1/5 or greater are considered to be positive. This CF has low sensitivity and is frequently affected by anticomplementary or nonspecific factors.
C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Vesicular stomatitis virus infections can have significant impacts on the health and production aspects of animals, resulting in considerable economic losses for producers. Reduced feed intake caused by oral lesions can result in weight loss and delays to market. Lesions on the feet can cause temporary locomotor problems affecting the ability of an animal to obtain food and water, and permanent foot problems that result in the animal being culled. Lesions of the mammary gland can impact the ability of the dam to nurse her offspring and for harvesting milk for sale. Animals may be culled if mammary or teat lesions are severe. Where vaccination is practised, vaccine is used to reduce the severity of clinical signs and the economic impacts of the disease.

Attenuated virus vaccines have been tested in the field in the USA, Panama, Guatemala, Peru and Venezuela (Lauerman et al., 1962; Mason, 1978) with unknown efficacy. Killed vaccines for the Indiana and New Jersey serotypes are manufactured in Colombia and Venezuela (2002 OIE vaccine survey). Although a commercial vaccine combining VS and FMD antigens in a single emulsion for Andean countries has been tested in vaccination–challenge experimentation and published (House et al., 2003), the vaccine is not produced/applied routinely.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Identity of the seed and the source of the serum used in growth and passage of the virus should be well documented, including the source and passage history of the organism.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The purity of the seed and cells to be used for vaccine production must be demonstrated. The master seed virus (MSV) should be free from adventitious agents, bacteria, or Mycoplasma, using tests known to be sensitive for detection of these microorganisms. The test aliquot should be representative of a titre adequate for vaccine production, but not such a high titre that hyperimmune antisera are unable to neutralise seed virus during purity testing. Seed virus is neutralised with monospecific antiserum or monoclonal antibody against the seed virus and the virus/antibody mixture is cultured on several types of cell line monolayers. A cell line highly permissive for bovine viral diarrhoea virus, types 1 and 2, is recommended as one of the cell lines chosen for evaluation of the MSV. Bovine viral diarrhoea virus is a potential contaminant introduced through the use of fetal bovine serum in cell culture systems. Cultures are subpassaged at 7-day intervals for a total of at least 14 days, then tested for adventitious viruses that may have infected the cells or seed during previous passages.

2.2. Method of manufacture

2.2.1. Procedure

Once the vaccine is shown to be efficacious, and the proposed conditions for production are acceptable to regulatory authorities, approval may be granted to manufacture vaccine. Virus seed can be grown in cell culture. Selection of a cell type for culture is dependent on the degree of virus adaptation, growth in medium, and viral yield in the specific culture system. Vaccine products should be limited to the number of passages from the MSV that can be demonstrated to be effective. Generally, large-scale monolayer or suspension cell systems are operated under strict temperature-controlled, aseptic conditions and defined production methods, to assure lot-to-lot consistency. Dose of virus used to inoculate cell culture should be kept to a minimum to reduce the potential for viral defective interfering particles. When the virus has reached its appropriate titre, as determined by CPE, fluorescent antibody assay, or other approved technique, the virus is clarified, filtered, and inactivated (for killed vaccines).
2.2.2. Requirements for substrates and media

Cell cultures should be demonstrated free of adventitious viruses. All animal origin products used in the production and maintenance of cells (i.e. trypsin, fetal bovine sera) and growth of virus should be free of adventitious agents, with special attention paid to the presence of bovine viral diarrhoea virus.

2.2.3. In-process controls

Cell cultures should be checked macroscopically for abnormalities or signs of contamination and discarded if unsatisfactory. Virus concentration can be assessed using antigenic mass or infectivity assays.

An inactivation kinetics study should be conducted using the approved inactivating agent (β-propiolactone or ethylene-imine in binary form) on each a viral lot with a titre greater than the maximum production titre and grown using the approved production method. This study should demonstrate that the inactivation method is adequate to assure complete inactivation of virus. Samples taken at regular timed intervals during inactivation, then inoculated on to a susceptible cell line, should indicate a linear and complete loss of titre by the end of the inactivation process.

During production, antigen content is measured to establish that minimum bulk titres have been achieved. Antigen content is generally measured before inactivation (if killed vaccine) and prior to further processing.

2.2.4. Final product batch tests

Vaccine candidates should be shown to be pure, safe, potent, and efficacious.

i) Sterility and purity

During production, tests for bacteria, *Mycoplasma*, and fungal contamination should be conducted on both inactivated and live vaccine harvest lots and confirmed on the completed product (see Chapter 1.1.7 Tests for sterility and freedom from contamination of biological materials).

ii) Safety

Final container samples of completed product from inactivated vaccines should be tested.

iii) Batch potency

Potency is examined on the final formulated product. Mirroring what is done for the potency test in foot and mouth disease vaccines, a vaccination-challenge test has been proposed for testing VSV vaccines (House *et al.*, 2003). The gaps in knowledge regarding the pathogenesis of VSV infection and the immune mechanism that affords protection against viral infection are limitations for development and implementation of a validated protocol for a challenge test. However, for batch release, indirect tests can also be used for practicability and animal welfare considerations, as long as correlation has been validated to protection in the target animal during efficacy tests. Frequently indirect potency tests include antibody titration after vaccination of target species. Ideally, indirect tests are carried out for each strain for one species and each formulation of vaccine to establish correlation between the indirect test results and the vaccine efficacy test results. Relative potency could can be used to determine antigen content in final product. It is necessary to confirm the sensitivity, specificity, reproducibility, and ruggedness of such assays.

2.3. Requirements for authorisation

2.3.1. Safety requirements

i) Target and non-target animal safety

Final product may be evaluated in the host animal using two animals of the minimum age recommended for use, according to the instructions given on the label; the animals are observed for 21 days. Field safety studies conducted on vaccinates, in at least three divergent geographical areas, with at least 300 animals per area, are also recommended.
For killed and modified live virus (MLV) vaccines product safety will be based on an absence of adverse reactions such as shock, abscesses at site of inoculation, etc. In the specific case of MLV vaccines, it would not be expected to see clinical signs. If clinical signs of vesicular stomatitis virus are observed, use of the vaccine should be reconsidered. Residual virus should be evaluated for prior to mixing the antigen with adjuvant. Initial safety is evaluated in a few animals for 21 days under close observation to assess for gross safety issues. If the vaccine passes this first safety test, the vaccine is used in the field in a larger number of animals to evaluate if subtle safety issues are present: adverse reactions/swelling, abscesses, shock, etc.

Reversion to virulence for live viral vaccines is often demonstrated by back passage through susceptible species. Virus is isolated from the vaccinated animal and the isolated virus is then used to inoculate additional animals. Sequential passage through animals should show that animals remain clinically healthy with no demonstration of typical vesicular stomatitis lesions.

Environmental consideration

Inactivated vesicular stomatitis vaccines probably present no special danger to the user, although accidental inoculation may result in an adverse reaction caused by the adjuvant and secondary components of the vaccine. Modified live virus vaccines may pose a hazard to the user depending on the level of inactivation of the virus. Preservatives should be avoided if possible, and where not possible, should be limited to the lowest concentration possible. Vaccine bottles, syringes, and needles may pose an environmental hazard for vaccines using adjuvants or preservatives and for modified live virus vaccines. Instructions for disposal should be included within the vaccine packaging information and based on current environmental regulations in the country of use.

2.3.2. Efficacy requirements

The gaps in knowledge regarding the pathogenesis of VSV infection and the immune mechanism that affords protection against viral infection are limitations for the development and implementation of a validated protocol for an efficacy test. Ideally vaccine efficacy should be estimated in vaccinated animals directly by evaluating their resistance to live virus challenge. Vaccine efficacy should be established for every strain to be authorised for use in the vaccine. Live reference VSV viruses corresponding to the virus strains circulating in the region are stored at ultralow temperatures. Each challenge virus is prepared as follows. Tongue tissue infected by VSV should be obtained from original field case of VS and received at the Reference Laboratory in glycerol buffer as described in Section B. Diagnostic Techniques.

The preparation of cattle challenge virus follows the process described in Chapter 2.1.5 Foot and mouth disease, Section B.1.a Virus isolation, with the view of obtaining a sterile 10% suspension in Eagles minimal essential medium with 10% sterile fetal bovine serum.

The preparation of the stock of challenge virus to be aliquoted is prepared starting from lesions collected in two cattle over 6 months of age, previously recognised to be free of VSV antibodies. These animals are tranquillised, for example using xylazine 100 mg/ml (follow instructions for use), then inoculated intradermally (i.d.) in the tongue with the suspension in about 20 sites, 0.1 ml each. The vesiculated tongue tissue is harvested at the peak of the lesions, approximately 2 days later.

A 2% suspension is prepared as above and filtered through a 0.2 μm filter, aliquoted and frozen in the gas phase of liquid nitrogen, and constitutes the stock of challenge virus. The infective titres of this stock are determined both in cell culture (TCID₅₀) and in two cattle (BID₅₀). These two cattle that have been tranquillised using xylazine 100 mg/ml (follow instructions for use), then inoculated intradermally in the tongue with the suspension in about 20 sites, 0.1 ml each. The vesiculated tongue tissue is harvested at the peak of the lesions, approximately 2 days later.

The cattle titrations are read 2 days later. Most frequently, titres are above 10⁶ TCID₅₀ for 0.1 ml and above 10⁵ BID₅₀ for 0.1 ml calculated using the Spearmann–Kärber method. The dilution for use in cattle challenge test is 10 000 DIB₅₀ in a total volume of 4× 0.1 ml by intralingual injection for both the PD₅₀ test and the PGP test (House et al., 2003).
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i) Vaccination–challenge method

For this experimental method, a group of 12 VSV sero-negative cattle of at least 6 months of age are vaccinated with a bovine dose by the route and in the volume recommended by the manufacturer at day 0 and day 40. These animals and a control group of two non-vaccinated animals are challenged 2 weeks or more after the second vaccination. The challenge strain is a suspension of bovine virus that is fully virulent and appropriate to the virus types in the vaccine under test by inoculating a total of 10,000 BID$_{50}$ intradermally into four sites (0.1 ml per site) on the upper surface of the tongue. Animals are observed at 7–8 days after challenge.

It was proposed that vaccinated animals showing no lesion on the tongue should be considered fully protected. Vaccinated animals showing lesions at one, two, or three inoculation sites should be considered partially protected, and animals showing lesions at four sites are considered not protected (House et al., 2003). Control animals must develop lesions at four sites. Vaccine should fully protect at least nine animals out of 12 vaccinated (75% protection), the remaining animals being partially or not protected. This test gives a certain measure of the protection following the injection of two commercial bovine doses of vaccine in a limited cattle population.

Although the vaccination-challenge method has been described and published (House et al., 2003) data on the validation under field conditions for the efficacy of released vaccine are not available.

ii) Efficacy in other species

Efficacy tests in other target species, such as horses, are not yet described or standardised. In general, a successful test in cattle should be considered to be sufficient evidence of the quality of a VS vaccine to endorse its use in other species.

2.3.3. Duration of immunity

The duration of immunity (D.O.I) of a VS vaccine will depend on the efficacy (formulation and antigen payload). As part of the authorisation/licensing procedure the manufacturer should be required to demonstrate the D.O.I. of a given vaccine by either challenge or the use of a validated alternative test, such as serology at the end of the claimed period of protection.

2.3.4. Stability

The stability of all vaccines including oil emulsion vaccines should be demonstrated as part of the shelf-life determination studies for authorisation/licensing. Vaccines should never be frozen or stored above the target temperature.

i) For animal production

Virus(es) used in vaccine production should be antigenically relevant to virus(es) circulating in the field. A vaccination/challenge study in the species for which the vaccine will be used will indicate the degree of protection afforded by the vaccine. Species used in vaccination/challenge studies should be free of antibodies against vesicular stomatitis. Vaccination/challenge studies should be conducted using virus produced by the intended production method, at the maximum viral passage permitted, and using an experimental animal model. It is necessary to confirm the sensitivity, specificity, reproducibility, statistical significance and confidence level of such experimental model.

Antibody levels after vaccination measured in vitro could be used to assess vaccine efficacy provided a statistically significant correlation study has been made. For vaccines containing more than one virus (for example, New Jersey and Indiana-1), the efficacy of the different components of these vaccines must each be established independently and then as a combination in case interference between different viruses exists.

The duration of immunity and recommended frequency of vaccination of a vaccine should be determined before a product is approved. Initially, such information is acquired directly using host animal vaccination/challenge studies. The period of demonstrated protection, as measured by the ability of vaccinates to withstand challenge in a valid test, can be incorporated into claims found on the vaccine label.
If the vaccine is to be used in horses, swine, cattle, or other ruminants destined for market and intended for human consumption, a withdrawal time consistent with the adjuvant used (generally 21 days) should be established by such means as histopathological examination submitted to the appropriate food safety regulatory authorities.

ii) For control
The same principles apply as for animal production usage. In addition, it should be noted that antibody responses in vaccinated animals may not be differentiated from animals exposed to field virus. Therefore, vaccinated animals will need to be clearly identified if serological methods will be used in conjunction with compatible clinical signs to assess field virus exposure.

2.3.5. Stability
Vaccines should be stored at 4–8°C, with minimal exposure to light. The shelf life should be determined by use of the approved potency test (Section C.2.2.4.iii) over the proposed period of viability.

REFERENCES


Chapter 2.1.19. – Vesicular stomatitis


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NB: There are OIE Reference Laboratories for Vesicular stomatitis (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for vesicular stomatitis.
SUMMARY

Fowl cholera (avian pasteurellosis) is a commonly occurring avian disease that can affect all types of birds and is distributed world-wide. Fowl cholera outbreaks often manifest as acute fatal septicaemia. Diagnosis depends on isolation and identification of the causative bacterium, Pasteurella multocida. Presumptive diagnosis may be based on the occurrence of typical signs and lesions and/or on the microscopic demonstration of myriad bacteria in blood smears, or impression smears of tissues such as liver or spleen. Mild or chronic forms of the disease also occur where the disease is endemic, with localised infection primarily of the respiratory and skeletal systems.

**Identification of the agent:** Pasteurella multocida is readily isolated, often in pure culture, from visceral organs such as lung, liver and spleen, bone marrow, gonads or heart blood of birds that succumb to the acute bacteraemic form of the disease, or from the caseous exudate characteristic of chronic fowl cholera lesions. It is a facultative anaerobic bacterium that grows best at 37°C. Primary isolation is usually accomplished using media such as dextrose starch agar, blood agar, and trypticase–soy agar. Isolation may be improved by the addition of 5% heat-inactivated serum. Colonies range from 1 to 3 mm in diameter after 18–24 hours of incubation and are discrete, circular, convex, translucent, and butyrous. The cells are coccobacillary or short rod-shaped, 0.2–0.4 × 0.6–2.5 µm in size, stain Gram negative, and generally occur singly or in pairs. Bipolar staining is evident with Wright or Giemsa stains.

Identification of *P. multocida* is based on the results of biochemical tests, which include carbohydrate fermentation, enzyme production, and selected metabolite production.

Serological characterisation of strains of *P. multocida* includes capsular serogrouping and somatic serotyping. DNA fingerprinting can differentiate among *P. multocida* having the same capsular serogroup and somatic serotype. These characterisations require a specialised laboratory with appropriate diagnostic reagents.

**Serological tests:** Serological tests are rarely used for diagnosis of fowl cholera. The ease of obtaining a definitive diagnosis through isolation and identification of the causative organism generally precludes the need for serodiagnosis.

**Requirements for vaccines and diagnostic biologicals:** The *P. multocida* vaccines in general use are bacterins, containing aluminium hydroxide or oil as adjuvant, prepared from multiple serotypes. Two doses of the killed vaccine are typically required. Live culture vaccines tend to impart greater protective immunity, but are used less frequently because of potential post-vaccinal sequelae such as pneumonitis and arthritis. Multivalent vaccines typically incorporate somatic serotypes 1, 3, and 4 as they are among the more commonly isolated avian serotypes. Safety and potency testing of bacterins usually use the host animal. Final containers of live cultures are tested for potency by bacterial counts.

**A. INTRODUCTION**

Fowl cholera is a contagious bacterial disease of domesticated and wild avian species caused by infection with *Pasteurella multocida*. It typically occurs as a fulminating disease with massive bacteraemia and high morbidity and mortality. Chronic infections also occur with clinical signs and lesions related to localised infections. The pulmonary system and tissues associated with the musculoskeletal system are often the seats of chronic infection. Common synonyms for fowl cholera are avian pasteurellosis and avian haemorrhagic septicaemia. Fowl cholera is not considered to have zoonotic potential as avian isolates are generally nonpathogenic in mammals.
exposed by the oral or subcutaneous routes. Other bacterial diseases, including salmonellosis, colibacillosis, and listeriosis in chickens, and pseudotuberculosis, erysipelas, and chlamydiosis in turkeys, may present with clinical signs and lesions similar to fowl cholera. Differentiation is based on isolation and identification, as *P. multocida* is readily cultured from cases of fowl cholera.

### B. DIAGNOSTIC TECHNIQUES

Fowl cholera (avian pasteurellosis) is a commonly occurring avian disease that can affect all types of birds and is often fatal (Dereux, 1978; Rimler & Glisson, 1997; Glisson *et al.*, 2008). In the peracute form, fowl cholera is one of the most virulent and infectious diseases of poultry. Diagnosis depends on identification of the causative bacterium, *P. multocida*, following isolation from birds with signs and lesions consistent with this disease. Presumptive diagnosis may be based on the observance of typical signs and lesions and/or on the microscopic demonstration of bacteria showing bipolar staining in smears of tissues, such as blood, liver, or spleen. Mild forms of the disease may occur.

All avian species are susceptible to *P. multocida*, although turkeys may be the most severely affected. Often the first sign of disease is dead birds. Other signs include: fever, anorexia, depression, mucus discharge from the mouth, diarrhoea, ruffled feathers, drop in egg production coupled with smaller eggs, increased respiratory rate, and cyanosis at the time of death. Lesions that are often observed include: congested organs with serosal haemorrhages, enlarged liver and spleen, multiple small necrotic areas in the liver and/or spleen, pneumonia, and mild ascites and pericardial oedema. Birds that survive the acute septicaemic stage or those infected with organisms of low virulence may develop chronic fowl cholera, characterised by localised infections. These infections often involve joints, foot pads, tendon sheaths, sternal bursa, conjunctivae, wattles, pharynx, lungs, air sacs, middle ears, bone marrow, and meninges. Lesions resulting from these infections are usually characterised by bacterial colonisation with necrosis, fibrino-suppurative exudate, and degrees of fibroplasia.

Diagnosis depends on isolation and identification of the causative organism.

<table>
<thead>
<tr>
<th>Method</th>
<th>Population freedom from infection</th>
<th>Individual animal freedom from infection prior to movement</th>
<th>Contribute to eradication policies</th>
<th>Confirmation of clinical cases</th>
<th>Prevalence of infection – surveillance</th>
<th>Immune status in individual animals or populations post-vaccination</th>
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</thead>
<tbody>
<tr>
<td>Culture</td>
<td>−</td>
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<td>−</td>
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<td>Serological ELISA</td>
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Table 1. Test methods available for the diagnosis of fowl cholera and their purpose

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.

Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

*Pasteurella multocida* is a facultative anaerobic bacterium that grows best at 35–37°C. Primary isolation is usually accomplished using media such as blood agar, trypticase–soy agar or dextrose starch agar, and isolation may be improved by supplementing these media with 5% heat-inactivated serum. Maintenance media usually do not require supplemental serum. Colonies range from 1 to 3 mm in diameter after 18–24 hours of incubation. They usually are discrete, circular, convex, translucent, and butyrous. Capsulated organisms usually produce larger colonies than those of noncapsulated organisms. Watery mucoid colonies, often observed with mammalian

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1 A combination of agent identification methods applied on the same clinical sample is recommended.
respiratory tract isolates, are very rare with avian isolates. The cells are coccobacillary or short rod-shaped, usually 0.2–0.4 × 0.6–2.5 µm in size, stain Gram negative, and generally occur singly or in pairs. Recently isolated organisms or those found in tissue smears show bipolar staining with Wright or Giemsa stains or methylene blue, and are usually encapsulated.

Isolation of the organism from visceral organs, such as liver, bone marrow, spleen, or heart blood of birds that succumb to the acute form of the disease, and from exudative lesions of birds with the chronic form of the disease, is generally easily accomplished. Isolation from those chronically affected birds that have no evidence of disease other than emaciation and lethargy is often difficult. In this condition or when host decomposition has occurred, bone marrow is the tissue of choice for isolation attempts. The surface of the tissue to be cultured is seared with a hot spatula and a specimen is obtained by inserting a sterile cotton swab, wire or plastic loop through the heat-sterilised surface. The specimen is inoculated directly on to agar medium or into tryptose or another broth medium, incubated for a few hours, transferred to agar medium, and incubated again.

Identification is based primarily on the results of biochemical tests. Carbohydrate fermentation reactions are essential. Those carbohydrates that are fermented include: glucose, mannose, galactose, fructose, and sucrose. Those not fermented include: rhamnose, cellobiose, raffinose, inulin, erythritol, adonitol, m-inositol, and salicin. Mannitol is usually fermented. Arabinose, maltose, lactose, and dextrin are usually not fermented. Variable reactions occur with xylose, trehalose, glycerol, and sorbitol. *Pasteurella multocida* does not cause haemolysis, is not motile and only rarely grows on MacConkey agar. It produces catalase, oxidase, and ornithine decarboxylase, but does not produce urease, lysine decarboxylase, beta-galactosidase, or arginine dihydrolase. Phosphatase production is variable. Nitrate is reduced; indole and hydrogen sulphide are produced, and methyl red and Voges–Proskauer tests are negative. Detection of hydrogen sulphide production may require lead acetate-laden paper strips suspended above a modified H$_2$S liquid medium (Rimler, 1998; Glisson, et al., 2008). Commercial biochemical test kits are available.

Differentiation of *P. multocida* from other avian *Pasteurella* spp. and *Riemerella* (*Pasteurella*) *anatipestifer* can usually be accomplished using the tests and results indicated in Table 2. Laboratory experience has shown that *P. multocida* is most easily identified by its colony morphology and appearance in Gram stains. Positive reactions to indole and ornithine decarboxylase are the most useful biochemical indications.

### Table 2. Tests used to differentiate *Pasteurella multocida* from other avian

<table>
<thead>
<tr>
<th>Test*</th>
<th><em>Pasteurella multocida</em></th>
<th><em>Pasteurella gallinarum</em></th>
<th><em>Riemerella anatipestifer</em></th>
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<tbody>
<tr>
<td>Haemolysis on blood agar</td>
<td>−</td>
<td>−</td>
<td>v</td>
</tr>
<tr>
<td>Growth on MacConkey’s agar</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Indole production</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin liquefaction</td>
<td>−</td>
<td>−</td>
<td>+u</td>
</tr>
<tr>
<td>Catalase production</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urease production</td>
<td>−</td>
<td>−</td>
<td>v</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Lactose fermentation</td>
<td>−u</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Sucrose fermentation</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Maltose fermentation</td>
<td>−u</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

*Test reaction results: − = no reaction; + = reaction; v = variable reactions; −u = usually no reaction; +u usually a reaction.*

Antigenic characterisation of *P. multocida* is accomplished by capsular serogrouping and somatic serotyping. Capsular serogroups are determined by a passive haemagglutination test (Carter, 1955; 1972). Capsular serogroups, determined by a passive haemagglutination test, are A, B, D, E, and F. All but serogroup E have been isolated from avian hosts. A nonserological disk diffusion test that uses specific mucopolysaccharidases to differentiate serogroups A, D, and F has been developed (Rimler & Glisson, 1997; Rimler, 1994).

Somatic serotypes are usually determined by an agar gel immunodiffusion (AGID) test (Heddleston, 1962; Heddleston et al., 1972). Serotypes 1 through 16 have been reported; all 16 serotypes have been isolated from avian hosts (Rimler, 1998; Glisson et al., 2008). The most effective characterisation involves determination of both serogroup and serotype. These determinations require a specialised laboratory with appropriate diagnostic
1. Somatic typing procedure using the gel diffusion precipitin test

1.1. Test procedure

i) Inoculate a dextrose starch agar (DSA) plate (20 × 150 mm containing 70 ml of medium or two 15 × 100 mm plates containing 20 ml of medium per plate) with cells from a pure culture of P. multocida by using a sterile cotton swab. Swab the entire surface of the plate(s). Incubate the plate(s) in a 37°C incubator for 18–24 hours. This procedure is used to produce antigen for positive control purposes or to prepare antigen from diagnostic cultures.

ii) Harvest the cells from the plate(s) using 2.5 ml of 0.85% saline with 0.6% formaldehyde and a sterile hockey stick. Place the cells in a tube using a sterile pipette.

iii) Autoclave the cells at 100°C for 1 hour.

iv) Centrifuge the cell suspension mixture at 13,300 g for 20 minutes.

v) Remove the supernatant and place in a sterile tube.

vi) Prepare the agar gel for use in the gel diffusion precipitin test (GDPT) by placing 17.0 g of NaCl, 1.8 g of Noble agar, and 200 ml of distilled water into a 500 ml flask. Microwave the contents of the flask with the cap loose for 2.5 minutes. Swirl the contents of the flask and microwave again for 2.5 minutes. Allow the agar to cool slightly for 10–15 minutes. Do not prepare less than 200 ml of agar in a microwave. Dehydration during the microwave process can increase the agar concentration and negatively impact or inhibit diffusion.

vii) Place 5 ml of melted agar onto the surface of a 75 × 25 mm plain glass microscope slide. It is important that the slides are level prior to dispensing the agar. Allow the agar to cool (approximately 30 minutes) completely.

viii) Cut wells in the agar bed. The wells are 3 mm in diameter and 3 mm apart from edge-to-edge. Frequently an Ouchterlony template is used to create two or three replicates of wells per slide. Each replicate has a centre well and is surrounded by four wells located at 90° angles (from centre).

ix) Always place reference antiserum in the centre well (of a replicate). Place antigen from a diagnostic or reference culture in one of the surrounding wells within a replicate. Fill each well to capacity.

x) Incubate the slides within a moist chamber in a 37°C incubator for 48 hours. Precipitin lines of a reaction can be best observed with subdued lighting from underneath the slide. When present, reactions should occur between the centre and surrounding well(s) as an arc of precipitin. Sometimes these reactions are close to the edge of a well. Examine the slides carefully. Diagnostic cultures can react to more than one reference somatic antiserum.

xi) Use positive controls. Test reference antiserum against reference antigen each time the test is performed.

DNA fingerprinting of P. multocida by restriction endonuclease analysis (REA) has proved valuable in epidemiological investigations of fowl cholera in poultry flocks. Isolates of P. multocida having both capsular serogroup and somatic serotype in common may be distinguished by REA. Ethidium-bromide-stained agarose gels are analysed following electrophoresis of DNA digested with either HhaI or HpaII endonuclease (Wilson et al., 1992).

2. Serological tests

Serological tests for the presence of specific antibodies are not used for diagnosis of fowl cholera. The ease of obtaining a definitive diagnosis by isolation and identification of the causative organism precludes the need for serodiagnosis. Serological tests, such as agglutination, AGID, and passive haemagglutination, have been used experimentally to demonstrate antibody against P. multocida in serum from avian hosts; none were highly sensitive. Determinations of antibody titres using enzyme-linked immunosorbent assays have been used with varying degrees of success in attempts to monitor seroconversion in vaccinated poultry, but not for diagnosis.
C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

C1. Inactivated vaccine

1. Background

1.1. Rationale and intended use of the product

Fowl cholera may be caused by any of 16 Heddleston serotypes of *P. multocida*, although certain serotypes appear to be more often associated with disease. The *P. multocida* vaccines in general use are inactivated, containing aluminum hydroxide or oil adjuvant, prepared from cells of serotypes selected on the basis of epidemiological information. Commercial vaccines are usually composed of serotypes 1, 3, and 4. Vaccination plays a significant role in the control of this disease. Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

**Bacterin**. Inactivated vaccine is normally administered by intramuscular injection in the leg or breast muscles, or subcutaneously at the back of the neck. Two doses are typically administered at 2- to 4-week intervals. As with most killed vaccines, full immunity cannot be expected until approximately 2 weeks after the second dose of a primary vaccination course. Live vaccines are typically administered in the drinking water. Vaccination of diseased birds or those in poor nutritional status should be avoided as a satisfactory immune response may not be generated in such circumstances.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

All strains of *P. multocida* to be incorporated into a bacterin or vaccine must be well characterised, of known serotype, pure, safe and immunogenic. The culture(s) that is evaluated and characterised is designated by lot number and called a master seed. All cultures used in the production of licensed bacterins or vaccines must be derived from an approved master seed(s) and must be within an accepted number of passages from the master seed lot. See chapter 1.1.6 for guidelines on master seeds.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

*Pasteurella multocida* seeds must be pure culture and free from extraneous bacteria and fungi (see Chapter 1.1.7 Tests for sterility and freedom from contamination of biological materials).

2.1.3. Validation as a vaccine strain

Suitability as a vaccine strain is demonstrated in efficacy and safety trials.

2.1.4. Emergency procedure for provisional acceptance of new master seed virus (MSV) in the case of an epizootic (with pathogens with many serotypes, e.g. bluetongue virus, highly pathogenic avian influenza, FMD, etc.)

Individual countries may have provisions to expedite the licensing or authorisation procedure in the event of an animal health emergency where currently available vaccines do not protect. For example, the United States of America (USA) has the authority to issue a conditional license based on a reasonable expectation of efficacy instead of requiring completion of definitive efficacy trials prior to licensure. All requirements for master seed testing and product safety must be completed prior to conditional licensure.

2.2. Method of manufacture

2.2.1. Procedure

Production cultures of each bacterial isolate to be included in the final product are prepared separately. *Pasteurella multocida* cultures may be grown in a suitable broth media or initially grown on agar media and scaled up to broth media. Cultures are subpassaged until the desired
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volume is prepared. Cultures are harvested when they reach a suitable density, frequently
measured by spectrophotometry (optical density).

Cultures are then inactivated by formaldehyde or other suitable inactivant. The inactivated
harvest may be concentrated, typically by centrifugation or filtration, or diluted to reach the
proper concentration for blending into completed product. All the standardised component
cultures are mixed, and usually blended with an adjuvant, prior to filling sterile final containers.

2.2.2. Requirements for ingredients

See chapter 1.1.6.

2.2.3. In-process controls

The purity of the cultures is determined at each stage of production prior to inactivation. This
may be achieved by microscopic examination (e.g. phase-contrast microscopy, Gram strain)
and/or by culture. Killed cultures are tested for completeness of inactivation. Analytical assays
to determine the levels of formaldehyde or other preservatives are done on bulk vaccine and
must be within specified limits. During manufacturing, production parameters must be tightly
controlled to ensure that all serials (batches) are produced in the same manner as that used to
produce the serials used for immunogenicity efficacy studies.

2.2.4. Final product batch tests

i) Sterility/purity

Sterility tests are done on filled vaccine. Each lot must pass sterility requirements, for
example those detailed in the 9 CFR Part 113.26 or 113.27 (CFR USDA, 2001-2013). (See
also Chapter 1.1.7 Tests for sterility and freedom from contamination of biological
materials.)

ii) Identity

The identity of the antigens in inactivated products is typically ensured through the master
seed concept and good manufacturing controls. Separate identity testing on completed
product batches is not required in the USA, but procedures may differ in other countries.

iii) Safety

Safety testing is conducted on each bulk or filled vaccine lot. Live vaccines are tested
according to the method described in Section C.1.2.3.2.i, except that only one
representative animal species is required. Bacterins are administered according to label
recommendations, and the may be assessed in birds are observed vaccinated for 14 days;
at least 18 of 20 birds must show no unfavourable reactions attributable to the
bacterin batch potency tests.

Certain countries or regions, such as the European Union (EU), also may require testing
each batch for endotoxin content.

iv) Batch potency

Each production lot of bacterin or live vaccine must be tested for potency by a test that is
related to, and considered predictive of, efficacy. Potency tests are performed on the
product in its final form.

Bacterins are In the USA, inactivated vaccines are typically tested for batch potency in a
vaccination–challenge trial, such as described in 9 CFR Parts 113.116-118 (USDA, 2013).
Separate groups of birds (20 vaccinates, 10 controls) must be challenged with each of
the serotypes of P. multocida for which protection is claimed. Bacterins Vaccines are
administered according to the dose and route recommended on the label. Two doses are
administered 3 weeks apart, and all birds are challenged 2 weeks after the second dose.
The birds are observed for 14 days after challenge. For a satisfactory test, according to
9 CFR, at least 14 of 20 vaccinates must survive and at least 8 of 10 controls must die.

The mean bacterial count of any vaccine lot at the time of preparation must be sufficiently
high to ensure that at any time prior to product expiration, the count is at least twice the
immunogenicity standard. (The European Pharmacopoeia requires a count that is at least
equal to the immunogenicity standard.)
In the EU, a serological test or other validated method may be used for batch potency after a batch of minimum permissible potency is initially tested in a vaccination–challenge trial (European Pharmacopoeia, 2008).

**v) Formaldehyde content**

Vaccines inactivated with formaldehyde are tested for residual formaldehyde.

### 2.3. Requirements for authorisation/registration/licensing

The following section is based on the requirements for inactivated *P. multocida* vaccines in the USA. Other countries may have slightly different requirements.

#### 2.3.1. Manufacturing process

The general method for production of manufacturers should demonstrate that the procedure used to inactivate bacteria is sufficient for complete inactivation. A test should be developed to confirm inactivation of each bacterial culture.

*P. multocida* bacterins is presented here. Production cultures of each bacterial isolate to be included in the final product are prepared. The cultures are typically started in small vessels and subpassaged into progressively larger volumes of media until the desired production volume is achieved. Each production culture is inactivated by formalin or other acceptable means. All of the component cultures are mixed, and usually blended, with an adjuvant prior to filling sterile final containers.

The following section is based on the requirements for *P. multocida* bacterins and vaccines as found in Title 9, United States Code of Federal Regulations (CFR).

#### 2.3.2. Safety requirements

**i) Target and non-target animal safety**

Inactivated vaccines should pose no hazard to non-target species. Safety in target animals may be evaluated according to harmonised requirements in VICH GL44 (VICH, 2009). The EU and USA recommend vaccinating at least 20 non-immune, unexposed birds according to label recommendations and evaluating daily for adverse reactions. The EU monitors for 21 days. In the USA, target animal safety is evaluated during the pre-challenge period of the efficacy study, which is typically 5 weeks.

Safety also should be evaluated in a field setting prior to product licensure or registration. This evaluation typically involves multiple geographical locations or husbandry conditions and much larger numbers of birds.

**ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations**

*Not applicable.*

Each of 10 birds is given an equivalent of 10 vaccine doses and observed for 10 days. At least 8 of 10 birds must show no unfavourable reactions attributable to the master seed. Additionally, the master seeds must be tested for reversion to virulence and evaluated for excretion from the host and transmission to other target species.

**iii) Precautions (hazards)**

Vaccines prepared with aluminium-based adjuvants may cause temporary nodules at the site of injection. Operator self-injection poses no immediate problems, but medical advice should be sought as there is a risk of infection via a contaminated needle.

Vaccines prepared with oil-based adjuvants may cause more severe reactions at the site of injection, which may manifest as large nodules. Care should be taken to administer these vaccines correctly. Operator self-injection requires immediate medical attention, involving prompt incision and irrigation of the site.

#### 2.3.3. Efficacy requirements

Products prepared from candidate master seeds should be shown to be effective against challenge infection. Efficacy should be demonstrated in each animal species (e.g., chickens,
turkeys) and by each route of administration for which the product will be recommended, and protection must be demonstrated against each challenge serotype for which protection is claimed. Birds used in efficacy studies should be immunologically naïve to fowl cholera and at the minimum age recommended for product use. The lot of product used to demonstrate efficacy should be produced from the highest allowable passage of master seed.

For live avian Pasteurella vaccines, efficacy of bacterins must be demonstrated similarly prior to licensure. However, no immunogenicity standards are derived from the lot that was used to demonstrate initial efficacy; each production lot is satisfactorily tested in a vaccination-challenge trial prior to release for sale and distribution.

In the USA and EU, 20 vaccinates and 10 controls are used in each efficacy trial. Birds are challenged not less than 14 (USA) or 21 (EU) days after vaccination and are observed for 14 days after challenge. In the USA, mortality is measured and a satisfactory test requires that at least eight of the controls die and at least 16 of the vaccinates survive (USDA, 2013). In the EU, birds are expected to remain free from severe signs of disease, and a satisfactory test requires at least 70% of the control birds to be affected while at least 70% of the vaccinates remain free from disease (European Pharmacopoeia, 2008).

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

Not applicable to this disease.

2.3.5. Duration of immunity

Formal duration of immunity studies are not typically required, although it is important to check the requirements of individual countries. Revaccination recommendations, beyond the primary vaccination series, are more often determined empirically.

2.3.6. Stability

Vaccine stability should be confirmed by testing the product for potency at periodic intervals through the dating period. In the USA, at least three lots of vaccine are tested and must pass established potency requirements at the end of dating. Vaccines are typically stored at 2–7°C and protected from freezing. Partly used containers should be discarded at the end of a day’s operations.

C2. Live vaccine

1. Background

1.1. Rationale and intended use of the product

Live vaccines containing modified P. multocida are not generally used except in North America. Live vaccines are typically administered in the drinking water or wing web. Vaccination of diseased birds or those in poor nutritional status should be avoided as a satisfactory immune response may not be generated in such circumstances.

2. Outline of production and minimum requirements for vaccines

Guidelines for the production of the veterinary vaccines are given in chapter 1.1.6.

2.1. Characteristics of the seed

2.1.1. Biological characteristics

All strains of P. multocida to be incorporated into a vaccine must be well characterised, of known serotype, pure, safe and immunogenic. See chapter 1.1.6 for guidelines on master seeds.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Pasteurella multocida seeds must be pure culture and free from extraneous bacteria and fungi.

2.1.3. Validation as a vaccine strain
Suitability as a vaccine strain is demonstrated in efficacy and safety trials. In addition, seeds used in live vaccines must be genetically and phenotypically stable upon repeated in-vivo passage. Ideally, they should not persist in the vaccinated animal and any shedding of the vaccine organism from vaccinated birds should be of limited magnitude and duration.

2.1.4. Emergency procedure for provisional acceptance of new master seed virus (MSV) in the case of an epizootic (with pathogens with many serotypes, e.g. bluetongue virus, highly pathogenic avian influenza, FMD, etc.)

Many countries have mechanisms for provisional acceptance in the event of an epizootic in which commercially available vaccines are not effective. As inactivated fowl cholera vaccines are typically effective and pose less safety risk, however, it is more likely that an inactivated vaccine would be considered for a fowl cholera epizootic.

2.2. Method of manufacture

2.2.1. Procedure

Production cultures of each bacterial isolate to be included in the final product are prepared separately. Pasteurella multocida cultures may be grown in a suitable broth media or initially grown on agar media and scaled up to broth media. Cultures are subpassaged until the desired volume is prepared. Cultures are harvested when they reach a suitable density, frequently measured by spectrophotometry (optical density).

Each component culture may be standardised, by concentration or dilution, to a desired concentration. All of the standardised component cultures are mixed prior to filling sterile final containers. Live vaccines are typically lyophilised, to be reconstituted with sterile diluent immediately prior to use.

2.2.2. Requirements for ingredients

See chapter 1.1.6

2.2.3. In-process controls

The purity of the cultures is determined at each stage of production. This may be achieved by microscopic examination (e.g. phase–contrast microscopy, Gram strain) or by culture. During manufacturing, production parameters must be tightly controlled to ensure that all serials (batches) are produced in the same manner as that used to produce the serials used for efficacy studies.

2.2.4. Final product batch tests

i) Sterility/purity

Sterility tests are done on filled vaccine. Each lot must pass sterility requirements, for example those detailed in the 9 CFR Part 113.27 (CFR USDA, 2013). (See also chapter 1.1.7.)

ii) Identity

Each batch of live vaccine in the USA is tested for identity. Requirements of other countries may vary. This is most commonly accomplished by characterising the bacteria in vitro.

iii) Safety

Live vaccines may be tested according to the method described in Section C1.2.3.2.i, except that frequently only one representative animal species is required.

Certain countries (e.g. EU) also may require testing each batch for endotoxin content (European Pharmacopoeia, 2008).

iv) Batch potency

The potency of live vaccine lots is determined by a bacterial count performed on reconstituted lyophilised product in its final container. In the USA, the mean bacterial count of any vaccine lot at the time of preparation must be sufficiently high to ensure that at any
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2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

See chapter 1.1.6.

2.3.2. Safety requirements

i) Target and non-target animal safety

The safety of master seeds used in the production of live vaccines must be evaluated prior to licensing. Safety must be tested in each animal species (chickens, turkeys, ducks, psittacines) for which the product is recommended. Harmonised VICH GL44 (VICH, 2006) is available for target animal safety.

Overdose studies are typically required for live vaccines. For example, each of 10 birds is given an equivalent of 10 vaccine doses and observed for 10 days. If unfavourable reactions are seen, this finding should be included in a risk assessment, and it may be appropriate to designate maximum permissible serial potency requirements.

The master seed is also tested in representative non-target species (e.g. rodents) that may be expected to come into contact with vaccine bacteria shed by vaccinated birds. Master Seed bacteria should be administered to the most sensitive species at the most sensitive age, by the route (e.g. oral) expected to occur in the field.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Master seed bacteria for live vaccines should be evaluated for their stability with repeated passage in vivo. The seed should remain avirulent and genotypically stable after multiple passages. Harmonised requirements for reversion to virulence studies are described in VICH GL40 (VICH, 2006).

Seeds for live vaccines also should be tested for their potential to shed from vaccinated animals and persist and spread in the environment. Ideally vaccine organisms should shed no more than briefly and should not persist in the environment. Exceptions from the ideal should be addressed in a risk assessment for the product.

iii) Precautions (hazards)

Inadvertent human exposure to the vaccine organism should be reported to a physician.

2.3.3. Efficacy requirements

Products prepared from candidate master seeds should be shown to be effective against challenge infection. Efficacy should be demonstrated in each animal species (e.g. chickens, turkeys) and by each route of administration for which the product will be recommended, and protection must be demonstrated against each challenge serotype for which protection is claimed. Birds used in efficacy studies should be immunologically naive to fowl cholera and at the minimum age recommended for product use. The lot of product used to demonstrate efficacy should be produced from the highest allowable passage of master seed.

For live avian Pasteurella vaccines in the USA, 20 vaccinates and 10 controls are used in each efficacy trial. Birds are challenged not less than 14 days after vaccination and are observed for 10 days after challenge. A satisfactory test requires that at least eight of the controls die and at least 16 of the vaccinates survive.

The arithmetic mean count of colony-forming units in the lot of product that is used to demonstrate efficacy is used as the minimum standard (immunogenicity standard) for all subsequent production lots of vaccine.

500 time prior to product expiration, the count is at least twice the immunogenicity standard. The EU requires a count that is at least equal to the immunogenicity standard.

501 v) Moisture content

Lyophilised vaccine is tested for moisture content. Harmonised requirements for testing moisture by a gravimetric method are found in VICH GL26 (VICH, 2003). Typically moisture is expected to be less than 5%.
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2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

Not applicable

2.3.5. Duration of immunity

Formal duration of immunity studies are not typically required, although it is important to check the requirements of individual countries. Revaccination recommendations, beyond the primary vaccination series, are more often determined empirically.

2.3.6. Stability

Vaccine stability should be confirmed by testing the product for potency at periodic intervals through the dating period. In the USA, batches of vaccine are tested until a statistically valid stability record is established. Each lot must pass established potency requirements at the end of dating. Live vaccines should be used promptly upon opening.

REFERENCES


Chapter 2.3.9. – Fowl cholera


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SECTION 2.4.

BOVINAe

CHAPTER 2.4.1.

BOVINE ANAPLASMOSIS

SUMMARY

Definition of the disease: Bovine anaplasmosis results from infection with Anaplasma marginale. A second species, A. centrale, has long been recognised; it is usually reported as of low virulence. Anaplasma marginale is responsible for almost all outbreaks of clinical disease. Anaplasma phagocytophilum and A. bovis, which infect cattle, have been recently included within the genus and have been reported rarely to infect cattle, but they are not reported to do not cause clinical disease. The organism is classified in the genus Anaplasma belonging to the family Anaplasmataceae of the order Rickettsiales.

Description of the disease: Anaemia, and jaundice and sudden death are characteristic signs of anaplasmosis. Other signs include rapid loss of milk production and weight, but the clinical disease can only be confirmed by identifying the organism. Once infected, cattle may remain carriers for life, and identification of these animals depends on the detection of specific antibodies using serological tests, or of rickettsial DNA using amplification techniques. The disease is typically transmitted by tick vectors, but mechanical transmission by biting insects or by needle can occur.

Identification of the agent: Microscopic examination of blood or organ smears stained with Giemsa stain is the most common method of identifying Anaplasma in clinically affected animals. In these smears, A. marginale appear as dense, rounded, intraerythrocytic bodies approximately 0.3–1.0 µm in diameter with most situated on or near the margin of the erythrocyte. Anaplasma centrale is similar in appearance, but most of the organisms are situated toward the centre away from the margin of the erythrocyte. It can be difficult to differentiate A. marginale from A. centrale in a stained smear, particularly with low levels of rickettsaemia. Commercial stains that give very rapid staining of Anaplasma are available in some countries. Anaplasma phagocytophilum and A. bovis can only be observed infecting granulocytes, mainly neutrophils.

It is important that smears be well prepared and free from foreign matter. Smears from live cattle should preferably be prepared from blood drawn from the jugular vein or another large vessel. For post-mortem diagnosis, smears should be prepared from internal organs (including liver, kidney, heart and lungs) and from blood retained in peripheral vessels. The latter are particularly desirable if post-mortem decomposition is advanced.

Serological tests: A competitive enzyme-linked immunosorbent assay (C-ELISA) has been demonstrated to have good sensitivity in detecting carrier animals. Card agglutination is the next most frequently used assay. The complement fixation test (CFT) is no longer considered a reliable test for disease certification of individual animals due to variable sensitivity. Cross reactivity between Anaplasma spp. can complicate interpretation of serological tests. In general, the C-ELISA has the best specificity, with cross-reactivity described between A. marginale, A. centrale, A. phagocytophilum and Ehrlichia spp. Alternatively, an indirect ELISA using the CFT with modifications is a reliable test used in many laboratories and can be prepared in-house for routine diagnosis of anaplasmosis.

Nucleic-acid-based tests have been used experimentally, and are capable of detecting the presence of low-level infection in carrier cattle and tick vectors. A nested reaction is necessary to identify low-level carriers using conventional polymerase chain reaction (PCR) and nonspecific
amplification can occur. Recently, real-time PCR assays with analytical sensitivity equivalent to nested conventional PCR have been described.

 Requirements for vaccines and diagnostic biologicals: Live vaccines are used in several countries to protect cattle against *A. marginale* infection. A vaccine consisting of live *A. centrale* is most widely used and gives partial protection against challenge with virulent *A. marginale*.

Anaplasma centrale vaccine is provided in chilled or frozen forms. Quality control is very important as other blood-borne agents that may be present in donor cattle can contaminate vaccines and be disseminated broadly. For this reason, frozen vaccine is recommended as it allows thorough post-production quality control, which limits the risk of contamination with other pathogens.

Anaplasma centrale vaccine is not entirely safe. A practical recommendation is to restrict its use, as far as possible, to calves, as nonspecific immunity will minimise the risk of some vaccine reactions that may require treatment with tetracycline or imidocarb. Partial immunity develops in 6–8 weeks and lasts for several years after a single vaccination.

A. INTRODUCTION

Outbreaks of bovine anaplasmosis are due to infection with *Anaplasma marginale*. Anaplasma centrale is capable of producing a moderate degree of anaemia, but clinical outbreaks in the field are extremely rare. New species of *Anaplasma*, a third species, *A. phagocytophilum* and *A. bovis* (Dumler et al., 2001), with a primary reservoir in rodents, have been reported rarely to infect cattle, and but does not cause clinical disease (Dreher et al., 2005; Hofmann-Lehmann et al., 2004).

The most marked clinical signs of anaplasmosis are anaemia and jaundice, the latter occurring late in the disease. Haemoglobinemia and haemoglobinuria are not present, and this may assist in the differential diagnosis of anaplasmosis from babesiosis, which is often endemic in the same regions. The disease can only be confirmed, however, by identification of the organism.

*Anaplasma marginale* occurs in most tropical and subtropical countries, and in some more temperate regions. *Anaplasma centrale* was first described from South Africa. The organism has since been imported by other countries – including Australia and some countries in South America, South-East Asia and the Middle East – for use as a vaccine against *A. marginale*.

*Anaplasma* species were originally regarded as protozoan parasites, but later further research showed they had no significant attributes to justify this description. Since the last major accepted revision of the taxonomy in 2001 (Dumler et al., 2001), the Family *Anaplasmataceae* (Order *Rickettsiales*) is now composed of four genera, *Anaplasma*, *Ehrlichia*, *Neorickettsia*, and *Wolbachia*. The genus *Aegyptianellae* is retained within the Family *Anaplasmataceae* as genus *incertae sedis*. The revised genus *Anaplasma* now contains *Anaplasma marginale* as the type species, *A. phagocytophilum* the agent of human granulocytic ehrlichiosis (formerly *Ehrlichia phagocytophila* and *E. equi*), and the unclassified agent of human granulocytic ehrlichiosis *A. platys*, and *A. bovis*. *Haemobartonella* and *Eperythrozoon* are now considered most closely related to the mycoplasmas.

*Anaplasma* species are transmitted either mechanically or biologically by arthropod vectors. Reviews based on careful study of reported transmission experiments list up to 19 different ticks as capable of transmitting *A. marginale* (Kocan et al., 2004). These are: Argas persicus, Ornithodoros lahorensis, *Rhipicephalus annulatus* (formerly *Boophilus annulatus*), *R. calcarius*, *R. decoloratus*, *R. microplus*, *Dermacentor albipictus*, *D. andersoni*, *D. hunteri*, *D. occidentalis*, *D. variabilis*, *Hyalomma excavatum*, *H. ruifpes*, *Ixodes ricinus*, *I. scapularis*, *R. bursa*, *R. evertsi*, *R. Sanguineus* and *R. simus*. However, the classification of several ticks in these reports has been questioned. Intrastadial or transstadial transmission is the usual mode, even in the one-host *Rhipicephalus Boophilus*-species. Male ticks may be particularly important as vectors; they can become persistently infected and serve as a reservoir for infection. Experimental demonstration of vector competence does not necessarily imply a role in transmission in the field. However, *Rhipicephalus Boophilus*-species are clearly important vectors of anaplasmosis in countries such as Australia and countries in Africa, and Latin America, and some species of *Dermacentor* are efficient vectors in the United States of America (USA).

Various other biting arthropods have been implicated as mechanical vectors, particularly in the USA. Experimental transmission has been demonstrated with a number of species of *Tabanus* (horseflies), and with mosquitoes of the genus *Psorophora* (Kocan et al., 2004). The importance of biting insects in the natural transmission of anaplasmosis appears to vary greatly from region to region. Anaplasma marginale also can be readily transmitted during vaccination against other diseases unless a fresh or sterilised needle is used for injecting each animal. Similar transmission by means of unsterilised surgical instruments has been described (Reinbold et al., 2010a).
Chapter 2.4.1. – Bovine anaplasmosis

The main biological vectors of *A. centrale* appear to be multihost ticks peculiar to Africa, including *R. simus*. The common cattle tick (*R. microplus*) has not been shown to be a vector. This is of relevance where *A. centrale* is used as a vaccine in *R. microplus*-infested regions.

*Anaplasma marginale* infection has not been reported in humans. Thus, there is no risk of field or laboratory transmission to workers and laboratories working with *A. marginale* may operate at the lowest biosafety level, equivalent to BSL1.

### B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of bovine anaplasmosis and their purpose

<table>
<thead>
<tr>
<th>Method</th>
<th>Population freedom from infection</th>
<th>Individual animal freedom from infection prior to movement</th>
<th>Contribute to eradication policies</th>
<th>Confirmation of clinical cases</th>
<th>Prevalence of infection – surveillance</th>
<th>Immune status in individual animals or populations post-vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopic examination</td>
<td>=</td>
<td>=</td>
<td>+++</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>PCR</td>
<td>=</td>
<td>+++</td>
<td>=</td>
<td>+++</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>CAT</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ELISA</td>
<td>+++</td>
<td>=</td>
<td>+++</td>
<td>=</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>IFAT</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CFT</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.

Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Agent id. = agent identification; CAT = card agglutination test; CFT = complement fixation test; ELISA = enzyme-linked immunosorbent assay; IFAT = indirect fluorescent antibody test; PCR = polymerase chain reaction.

1. Identification of the agent

1.1. Microscopic examination

Samples from live cattle should include thin blood smears and blood collected into an anticoagulant. Air-dried thin blood smears will keep satisfactorily at room temperature for at least 1 week. The blood sample in anticoagulant should be held and transferred at 4°C, unless it can reach the laboratory within a few hours. This sample is useful for preparing fresh smears if those submitted are not satisfactory. In addition, a low packed cell volume and/or erythrocyte count can help to substantiate the involvement of *A. marginale* when only small numbers of the parasites are detected in smears, such as may occur in the recovery stage of the disease.

In contrast to *Babesia bovis*, *A. marginale* does not accumulate in capillaries, so blood drawn from the jugular or other large vessel is satisfactory. Because of the rather indistinctive morphology of *Anaplasma*, it is essential that smears be well prepared and free from foreign matter, as specks of debris can confuse diagnosis. Thick blood films as used for the diagnosis of babesiosis are not appropriate for the diagnosis of anaplasmosis, as *Anaplasma* are difficult to identify once they become dissociated from erythrocytes.

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1. A combination of agent identification methods applied on the same clinical sample is recommended.
2. One of the listed serological tests is sufficient.
Chapter 2.4.1. – Bovine anaplasmia

Samples from dead animals should include air-dried thin smears from the liver, kidney, heart and lungs and from a peripheral blood vessel. The latter is particularly recommended should there be a significant delay before post-mortem examination because, under these circumstances, bacterial contamination of organ smears often makes identification of *Anaplasma* equivocal. Brain smears, which are useful for the diagnosis of some forms of babesiosis, are of no direct value for diagnosing anaplasmosis, but should be included for differential diagnosis where appropriate.

Blood from organs, rather than organ tissues *per se*, is required for smear preparation, as the aim is to be able to examine microscopically intact erythrocytes for the presence of *Anaplasma*. Organ-derived blood smears will store satisfactorily at room temperature for several days.

Both blood and organ smears can be stained in 10% Giemsa stain for approximately 30 minutes after fixation in absolute methanol for 1 minute. After staining, the smears are rinsed three or four times with tap water to remove excess adhering stain, and are then air-dried. Conditions for Giemsa staining vary from laboratory to laboratory, but distilled water is not recommended for dilution of Giemsa stock. Water should be pH 7.2–7.4 to attain best resolution with Giemsa stain. Commercial stains that give very rapid staining of *Anaplasma* are available in some countries. Smears are examined under oil immersion at a magnification of ×700–1000.

*Anaplasma marginale* appear as dense, rounded and deeply stained intraerythrocytic bodies, approximately 0.3–1.0 µm in diameter. Most of these bodies are located on or near the margin of the erythrocyte. This feature distinguishes *A. marginale* from *A. centrale*, as in the latter most of the organisms have a more central location in the erythrocyte. However, particularly at low levels of rickettsaemia, differentiation of these two species in smears can be difficult. Appendages associated with the *Anaplasma* body have been described in some isolates of *A. marginale* (Kreier & Ristic, 1963; Stich et al., 2004).

The percentage of infected erythrocytes varies with the stage and severity of the disease. Maximum rickettsaemias in excess of 50% may occur with *A. marginale*. Multiple infections of individual erythrocytes are common during periods of high rickettsaemias.

The infection becomes visible microscopically 2–6 weeks following transmission. During the course of clinical disease, the rickettsaemia approximately doubles each day for up to about 10 days, and then decreases at a similar rate. Quite severe anaemia may persist for some weeks after the parasites have become virtually undetectable in blood smears. Following recovery from initial infection, cattle remain latentely infected for life.

The use of inoculation of a splenectomised calf with blood from a suspect carrier is no longer justified as presence of the rickettsia can be confirmed by either end point polymerase chain reaction (PCR) or quantitative PCR; this latter procedure is several-fold more sensitive and faster than examination of blood smears and inoculation.

An expensive procedure, but one that may occasionally be justified to confirm infection, particularly in persistently infected carrier cattle, is the inoculation of blood from the suspect animal into a splenectomised calf. A quantity (up to 500 ml) of the donor's blood in anticoagulant is inoculated intravenously into the splenectomised calf, which is then tested by blood smear examination at least every 2–3 days. If the donor is infected, *Anaplasmawill be observed in smears from the splenectomised calf generally within 4 weeks, but this period may extend up to 8 weeks.

1.2. Polymerase chain reaction

Nucleic-acid-based tests to detect *A. marginale* infection in carrier cattle have been developed although not yet fully validated. The analytical sensitivity of polymerase chain reaction (PCR)-based methods has been estimated at 0.0001% infected erythrocytes, but at this level only a proportion of carrier cattle would be detected. A nested PCR has been used to identify *A. marginale* carrier cattle with a capability of identifying as few as 30 infected erythrocytes per ml of blood, well below the lowest levels in carriers. However, nested PCR poses significant quality control and specificity problems for routine use (Torioni De Echaide et al., 1998), Real-time PCR has also been described for identification of *A. marginale* (Carelli et al., 2007; Decaro et al., 2008; Reinbold et al., 2010), and should be considered in place of the nested PCR. Two advantages of this technique, which uses a single closed tube for amplification and analysis, are reduced opportunity for amplicon contamination and a semi-quantitative assay result. Equipment needed for real-time PCR is expensive, requires preventive

3. Commercial stains include Cameo-Quik and Diff-Quik, Baxter Scientific Products, McGaw Park, Illinois, USA, and Hema 3 and Hema-Quik, Curtin Matheson, Houston, Texas, USA.
maintenance, and may be beyond the capabilities of some laboratories. Real-time PCR assays may target one of several genes (Carelli et al., 2007; Decaro et al., 2008), or 16S rRNA (Reinbold et al., 2010b), and are reported to achieve a level of analytical sensitivity equivalent to nested conventional PCR (Carelli et al., 2007; Decaro et al., 2008; Reinbold et al., 2010b).

2. Serological tests

In general, unless animals have been treated or are at a very early stage of infection (<14 days), serology using the competitive enzyme-linked immunosorbent assay (C-ELISA), indirect ELISA (I-ELISA) or card agglutination test (CAT) (see below) may be the preferred methods of identifying infected animals in most laboratories. Anaplasma infections usually persist for the life of the animal. However, except for occasional small recrudescences, Anaplasma cannot readily be detected in blood smears after acute rickettsiaemia and, even end-point PCR may not detect the presence of Anaplasma in blood samples from asymptomatic carriers. Thus, a number of serological tests have been developed with the aim of detecting persistently infected animals.

A feature of the serological diagnosis of anaplasmosis is the highly variable results with regard to both sensitivity and specificity reported for many of the tests from different laboratories. This is due at least in part to inadequate evaluation of the tests using significant numbers of known positive and negative animals. Importantly, the capacity of several assays to detect known infections of long-standing duration has been inadequately addressed. An exception is C-ELISA (see below), which has been validated using true positive and negative animals defined by nested PCR (Torioni De Echaide et al., 1998), and the card agglutination assay, for which relative sensitivity and specificity in comparison with the C-ELISA has been evaluated (Molloy et al., 1999). Therefore, while most of the tests described in this section are useful for obtaining broad-based epidemiological data, caution is advised on their use for disease certification. Both the C-ELISA and CAT are described in detail below.

It should be noted that there is a high degree of cross-reactivity between A. marginale and A. centrale, as well as cross-reactivity with both A. phagocytophilum and Ehrlichia spp. in serological tests (Al-Adhami et al., 2011; Dreher et al., 2005). While the infecting species can sometimes be identified using antigens from homologous and heterologous species, equivocal results are obtained on many occasions.

2.1. Competitive enzyme-linked immunosorbent assay

A C-ELISA using a recombinant antigen termed rMSP5 and MSP5-specific monoclonal antibody (MAb) has proven very sensitive and specific for detection of Anaplasma-infected animals (Hofmann-Lehmann et al., 2004; Reinbold et al., 2010b; Strik et al., 2007). All A. marginale strains tested, along with A. ovis and A. centrale, express the MSP5 antigen and induce antibodies against the immunodominant epitope recognised by the MSP5-specific MAb. A recent report suggests that antibodies from cattle experimentally infected with A. phagocytophilum will test positive in the C-ELISA (Dreher et al., 2005). However, in another study no cross-reactivity could be demonstrated, and the MAb used in the assay did not react with A. phagocytophilum MSP5 in direct binding assays (Strik et al., 2007). Recently, cross-reactivity has been demonstrated between A. marginale and Ehrlichia spp, in naturally and experimentally infected cattle (Al-Adhami et al., 2011). Earlier studies had shown that the C-ELISA was 100% specific using 261 known negative sera from a non-endemic region, detecting acutely infected cattle as early as 16 days after experimental tick or blood inoculation, and was demonstrated to detect cattle that have been experimentally infected as long as 6 years previously (Knowles et al., 1996). In detecting persistently infected cattle from an anaplasmosis-endemic region that were defined as true positive or negative using a nested PCR procedure, the rMSP5 C-ELISA had a sensitivity of 96% and a specificity of 95% (Torioni De Echaide et al., 1998).

Test results using the rMSP5 C-ELISA are available in less than 2.5 hours. A test kit available commercially contains specific instructions. In general, however, it is conducted as follows.

2.1.1. Kit reagents

A 96-well microtitre plate coated with rMSP5 antigen,

A 96-well coated adsorption/transfer plate for serum adsorption to reduce background binding,

10× MAb/peroxidase conjugate,

10× wash solution and ready-to-use conjugate-diluting buffer,

Ready-to-use substrate and stop solutions,

Positive and negative controls

2.1.2. Test procedure
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2.1.3. Test validation

The mean optical density (OD) of the negative control must range from 0.40 to 2.10. The percent inhibition of the positive control must be ≥30%.

2.1.4. Interpretation of the results

The % inhibition is calculated as follows:

\[
\frac{100 - \text{Sample OD} \times 100}{\text{Mean negative control OD}} = \text{Per cent inhibition}
\]

Samples with <30% inhibition are negative. Samples with ≥30% inhibition are positive.

Specificity of the MSP5 C-ELISA may be increased by using a higher percentage inhibition cut-off value (Bradway et al., 2001); however the effect of this change on sensitivity has not been thoroughly evaluated.

2.2. Indirect enzyme-linked immunosorbent assay

An I-ELISA was first developed using the CAT antigen (see below) and it can be implemented where the commercial C-ELISA is not available. Unlike the C-ELISA, most reagents, such as buffers and ready-to-dissolve substrates, are available commercially in many countries. Any laboratory can prepare the antigen using local strains of A. marginale I-ELISA uses small amounts of serum and antigen, and the sensitivity and specificity of the test standardised with true positive and negative sera is as good as for the C-ELISA. As it can be prepared in each laboratory, only the general procedure is described here (Barry et al., 1986). For commercial kits, the manufacturer’s instructions should be followed. In the case of home-made I-ELISA, refer to Barry et al. 1986). Initial bodies and membranes are obtained as for the Complement Fixation test (Rogers et al., 1964). This antigen is treated with 0.1% sodium dodecyl sulphate for 30 minutes prior to fixing the antigen to the microtitre plate. For each laboratory, the specific amount of antigen has to be adjusted to obtain the best reading and the least expenditure.

Test results using the I-ELISA are available in about 4 to 5 hours. It is conducted as follows:

2.2.1. Test reagents

A 96-well microtitre plate coated with crude A. marginale antigen, PBS/Tween buffer, (PBS 0.1 M, pH 7.2, Tween 20 0.05%), Blocking reagent this is usually commercial dry skim milk, Tris buffer 0.1M, MgCl2, 0.1M, NaCl 0.005 M, pH 9.8

Substrate p-Nitrophenyl phosphate disodium hexahydrate

Positive and negative controls.

2.2.2. Test procedure (this test is run in triplicates)
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278 i) Plates can be prepared ahead of time and kept under airtight conditions at –20°C

279 ii) Carefully remove the plastic packaging to use plates being careful not to touch the bottom
280 of them as it can distort the optical density reading.

281 iii) Remove the lid and deposit 200 µl PBST20 solution in each well and incubate for
282 5 minutes at room temperature (RT).

283 iv) For each plate, dissolve 1.1 g of skim milk in 22 ml of PBST20.

284 v) Remove the plate contents and deposit in each well, 200 µl of blocking solution put the lid
285 and incubate for 60 minutes at 37°C.

286 vi) Wash the plate three times for 5 minutes with PBST20.

287 vii) Dilute all serum samples including controls 1/100 in PBST20 solution;

288 viii) Remove the contents of the plate and deposit 200 µl of diluted serum in each of the three
289 wells for each dilution, starting with the positive and negative and blank controls.

290 ix) Incubate plate at 37°C covered for 60 minutes.

291 x) Wash three times as described in subsection vi.

292 xi) Dilute 1/1000 anti-IgG bovine alkaline phosphatase conjugate in PBST20 solution; Add
293 200 µl of the diluted conjugate per well; incubate the covered plate at 37°C for 60 minutes.

294 xii) Remove the lid and make three washes with PBST20.

295 xiii) Remove the contents of the plate and deposit 195 µl of 0.075% p-Nitrophenyl phosphate
296 disodium hexahydrate in Tris buffer and incubate for 60 minutes at 37°C

297 ix) The reaction is quantified by a microplate reader spectrophotometer, adjusted to 405 nm
298 wavelength. The data are expressed in optical density (OD).

2.2.3. Data analysis

Analysis of results should take into account the following parameters.

i) The mean value of the blank wells.

ii) The mean value of the positive wells with their respective standard deviations.

iii) The mean value of negative wells with their respective standard deviations.

iv) The mean value of the blank wells is subtracted from the mean of all the other samples if
305 not automatically subtracted by the ELISA reader.

v) Control sera are titrated to give optical density values ranging from 0.90 to 1.50 for the
307 positive and, 0.15 to 0.30 for the negative control

Positive values are those above the cut-off calculated value which is the sum of the average of
310 the negative and two times the standard deviation.

For purposes of assessing the consistency of the test operator, the error "E" must also be
311 estimated; this is calculated by determining the percentage represented by the standard
312 deviation of any against their mean serum.

2.3. Card agglutination test

The advantages of the CAT are that it is sensitive, may be undertaken either in the laboratory or in the
315 field, and gives a result within a few minutes. Nonspecific reactions may be a problem, and subjectivity
316 in interpreting assay reactions can result in variability in test interpretation. In addition, the CAT
317 antigen, which is a suspension of A. marginale particles, can be difficult to prepare and can vary from
318 batch to batch and laboratory to laboratory. Splenectomised calves are infected by intravenous
319 inoculation with blood containing Anaplasma-infected erythrocytes. When the rickettsaemia exceeds
320 50%, the animal is exsanguinated, the infected erythrocytes are washed, lysed, and the erythrocyte
321 ghosts and Anaplasma particles are pelleted. The pellets are sonicated, washed, and then
322 resuspended in a stain solution to produce the antigen suspension.

A test procedure that has been slightly modified from that originally described (Amerault & Roby, 1968;
324 Amerault et al., 1972) is as follows:
2.3.1. Test procedure

i) Ensure all test components are at a temperature of 25–26°C before use (this constant temperature is critical for the test).

ii) On each circle of the test card (a clear perspex/plastic or glass plate marked with circles that are 18 mm in diameter), place next to each other, but not touching, 10 µl of bovine serum factor (BSF), 10 µl of test serum, and 5 µl of CAT antigen. Negative and low positive control sera must be tested on each card.

BSF is serum from a selected animal with high known conglutinin level. If the conglutinin level is unknown, fresh serum from a healthy animal known to be free from *Anaplasma* can be used. The Jersey breed is often suitable. The BSF must be stored at −70°C in small aliquots, a fresh aliquot being used each time the tests are performed. The inclusion of BSF improves the sensitivity of the test.

iii) Mix well with a glass stirrer. After mixing each test, wipe the stirrer with clean tissue to prevent cross-contamination.

iv) Place the test card in a humid chamber and rock at 100–110 rpm for 7 minutes.

v) Read immediately against a backlight. Characteristic clumping of the antigen (graded from +1 to +3) is considered to be a positive result. The test is considered to give a negative result when there is no characteristic clumping.

2.4. Complement fixation test

The complement fixation (CF) test has been used extensively for many years; however, it shows variable sensitivity (ranging from 20 to 60%), possibly reflecting differences in techniques for antigen production, and poor reproducibility. In addition, it has been demonstrated that the CF assay fails to detect a significant proportion of carrier cattle (Bradway et al., 2001). It is also uncertain as to whether or not the CF test can identify antibodies in acutely infected animals prior to other assays (Coetzee et al., 2001; Molloy et al., 1999). Therefore, the CF test is no longer recommended as a reliable assay for detecting infected animals.

2.5. Indirect fluorescent antibody test

Because of the limitations on the number of indirect fluorescent antibody (IFA) tests that can be performed daily by one operator, other serological tests are generally preferred to the IFA test. The IFA test is performed as described for bovine babesiosis in chapter 2.4.2, except that *A. marginale* infected blood is used for the preparation of antigen smears. A serious problem encountered with the test is nonspecific fluorescence. Antigen made from blood collected as soon as adequate rickettsaemia (5–10%) occurs is most likely to be suitable. Nonspecific fluorescence due to antibodies adhering to infected erythrocytes can be reduced by washing the erythrocytes in an acidic glycine buffer before antigen smears are prepared. Infected erythrocytes are washed twice in 0.1 M glycine buffer (pH 3.0, centrifuged at 1000 g for 15 minutes at 4°C) and then once in PBS, pH 7.4. Recently published data show that the IFA, like the C-ELISA, can cross react with other members of the *Anaplasmataceae* family (Al-Adhami et al., 2011).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Background

Several immunisation methods have been used to protect cattle against anaplasmosis in countries where the disease is endemic, but none is ideal (McHardy, 1984). A review of *A. marginale* vaccines and antigens has been published (Kocan et al., 2003). Use of the less pathogenic *A. centrale*, which gives partial cross-protection against *A. marginale*, is the most widely accepted method, although not used in North America.

In this section, the production of live *A. centrale* vaccine is described. It involves infection of a susceptible, splenectomised calf and the use of its blood as a vaccine. Detailed accounts of the production procedure are available and reference should be made to these publications for details of the procedures outlined here (Bock et al., 2004; de Vos & Jorgensen, 1992; Pipano, 1995).

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4 The test as conducted in the USA and Mexico uses larger volumes of reagents: antigen (15 µl), serum (30 µl), and bovine serum factor (30 µl), and a 4-minute reaction time (see step iv).
Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

Anaplasma centrale vaccine can be provided in either frozen or chilled form depending on demand, transport networks, and the availability of liquid nitrogen or dry ice supplies. Frozen vaccine is recommended in most instances, as it allows for thorough post-production quality control of each batch. It is, however, more costly to produce and more difficult to transport than chilled vaccine. The risk of contamination makes post-production control essential, but may be prohibitively expensive.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Anaplasma centrale was isolated in 1911 in South Africa, and has been used as a vaccine in South America, Australia, Africa, the Middle East, and South-East Asia. It affords only partial, but adequate, protection in regions where the challenging strains are of moderate virulence (e.g. Australia) (Bock & de Vos, 2001). In the humid tropics where A. marginale appears to be a very virulent rickettsia, the protection afforded by A. centrale may be inadequate to prevent disease in some animals.

Anaplasma centrale usually causes benign infections, especially if used in calves under 9 months of age. Severe reactions following vaccination have been reported when adult cattle are inoculated. The suitability of an isolate of A. centrale as a vaccine can be determined by inoculating susceptible cattle, monitoring the subsequent reactions, and then challenging the animals and susceptible controls with a virulent local strain of A. marginale. Both safety and efficacy can be judged by monitoring rickettsaemias in stained blood films and the depression of packed cell volumes of inoculated cattle during the vaccination and challenge reaction periods.

Infective material for preparing the vaccine is readily stored as frozen stabilates of infected blood in liquid nitrogen or dry ice. Dimethyl sulphoxide (DMSO) and polyvinylpyrrolidone M.W. 40,000 (Bock et al., 2004) are the recommended cryopreservatives, as they allow for intravenous administration after thawing of the stabilate. A detailed account of the freezing technique using DMSO is reported elsewhere (Mellors et al., 1982), but briefly involves the following: infected blood is collected, chilled to 4°C, and cold cryoprotectant (4 M DMSO in PBS) is added slowly with stirring to a final blood:protectant ratio of 1:1, to give a final concentration of 2 M DMSO. The entire dilution procedure is carried out in an ice bath and the diluted blood is dispensed into suitable containers (e.g. 5 ml cryovials), and frozen, as soon as possible, in the vapour phase of a liquid nitrogen container.

2.1.2. Quality criteria

Evidence of purity of the A. centrale isolate can be determined by serological testing of paired sera from the cattle used in the safety test for possible contaminants that may be present (Bock et al., 2004; Pipano, 1997). Donor calves used to expand the seed for vaccine production should be examined for all blood-borne infections prevalent in the vaccine-producing country, including Babesia, Anaplasma, Ehrlichia, Theileria and Trypanosoma. This can be done by routine examination of stained blood films after splenectomy, and preferably also by serology. Any calves showing evidence of natural infections of any of these agents should be rejected.

The absence of other infective agents should also be confirmed. These may include the agents of enzootic bovine leukosis, mucosal disease, infectious bovine rhinotracheitis, ephemeral fever, Akabane disease, bluetongue, foot and mouth disease, and rinderpest. The testing procedures will depend on the diseases prevalent in the country and the availability of tests, but should involve serology of paired sera at the very least and, in some cases, virus isolation, antigen, or DNA/RNA detection (Bock et al., 2004; Pipano, 1981; 1997).

2.2. Method of manufacture

2.2.1. Procedure

i) Production of frozen vaccine
Quantities of the frozen stabilate (5–10 ml) are thawed by immersing the vials in water preheated to 40°C. The thawed material is kept on ice and used as soon as possible (within 30 minutes) to infect a susceptible, splenectomised calf by intravenous inoculation.

The rickettsaemia of the donor calf is monitored daily by examining stained films of jugular blood, and the blood is collected for vaccine production when suitable rickettsaemias are reached. A rickettsaemia of $1 \times 10^9$/ml (approximately 2% rickettsaemia in jugular blood) is the minimum required for production of vaccine as this is the dose to vaccinate a bovine. If a suitable rickettsaemia is not obtained, passage of the strain by subinoculation of 100–200 ml of blood to a second splenectomised calf may be necessary.

Blood from the donor is collected by aseptic jugular or carotid cannulation using heparin as an anticoagulant (5 International Units [IU] heparin/ml blood). The use of blood collection units for human use are also suitable and guarantee sterility and obviate the need to prepare glass flasks that make the procedure more cumbersome.

In the laboratory, the infective blood is mixed in equal volumes with 3 M glycerol in PBS supplemented with 5 mM glucose at 37°C (final concentration of glycerol 1.5 M). The mixture is then equilibrated at 37°C for 30 minutes and dispensed into suitable containers (e.g. 5 ml cryovials). The vials are cooled at approximately 10°C/minute in the vapour phase of liquid nitrogen and, when frozen, stored in the liquid phase (Bock et al., 2004).

DMSO can be used as a cryoprotectant in the place of glycerol. This is done in the same way as outlined for the preparation of seed stabulate (Mellors et al., 1982; Pipano, 1981).

If glycerolised vaccine is to be diluted, the diluent should consist of PBS with 1.5 M glycerol and 5 mM glucose (Jorgensen et al., 1989). Vaccine cryopreserved with DMSO should be diluted with diluent containing the same concentration of DMSO as in the original cryopreserved blood (Pipano et al., 1986).

ii) Production of chilled vaccine

Infected material for chilled vaccine is prepared in the same way as for frozen vaccine, but it must be issued and used as soon as possible after collection. The infective blood can be diluted to provide $1 \times 10^7$ parasites per dose of vaccine. A suitable diluent is 10% sterile bovine serum in a glucose/balanced salt solution containing the following quantities per litre: NaCl (7.00 g), MgCl₂·6H₂O (0.34 g), glucose (1.00 g), Na₂HPO₄·2H₂O (2.52 g), KH₂PO₄ (0.90 g), and NaHCO₃ (0.52 g).

If diluent is not available, acid citrate dextrose (20% [v/v]) or citrate phosphate dextrose (20% [v/v]) should be used as anticoagulant to provide the glucose necessary for survival of the organisms.

iii) Use of vaccine

In the case of frozen vaccine, vials should be thawed by immersion in water, preheated to 37°C to 40°C, and the contents mixed with suitable diluent to the required dilution. If glycerolised vaccine is prepared, it should be kept cool and used within 8 hours (Bock et al., 2004). If DMSO is used as a cryoprotectant, the prepared vaccine should be kept on ice and used within 15–30 minutes (Pipano, 1981). The vaccine is most commonly administered subcutaneously.

iv) Chilled vaccine should be kept refrigerated and used within 4–7 days of preparation.

The strain of *A. centrale* used in vaccine is of reduced virulence, but is not entirely safe. A practical recommendation is, therefore, to limit the use of vaccine to calves, where nonspecific immunity will minimise the risk of vaccine reactions. When older animals have to be vaccinated, there is a risk of severe reactions. These reactions occur infrequently, but valuable breeding stock or pregnant animals obviously warrant close attention, and should be observed daily for 3 weeks post-vaccination. Clinically sick animals should be treated with oxytetracycline or imidocarb at dosages recommended by the manufacturers. Protective immunity develops in 6–8 weeks and usually lasts for several years.

Anaplasmosis and babesiosis vaccines are often used concurrently, but it is not advisable to use any other vaccines at the same time (Bock et al., 2004).
2.2.2. Requirements for substrates and media

*Anaplasma centrale* cannot be cultured in vitro. No substrates or media other than buffers and diluents are used in vaccine production. DMSO or glycerol should be purchased from reputable companies.

2.2.3. In-process controls

i) Source and maintenance of vaccine donors

A source of calves free from natural infections of *Anaplasma* and other tick-borne diseases should be identified. If a suitable source is not available, it may be necessary to breed the calves under tick-free conditions specifically for the purpose of vaccine production.

The calves should be maintained under conditions that will prevent exposure to infectious diseases and to ticks and biting insects. In the absence of suitable facilities, the risk of contamination with the agents of infectious diseases present in the country involved should be estimated, and the benefits of local production of vaccine weighed against the possible adverse consequences of spreading disease (Bock *et al.*, 2004).

ii) Surgery

Donor calves should be splenectomised to allow maximum yield of organisms for production of vaccine. This is best carried out in young calves and under general anesthesia.

iii) Screening of vaccine donors before inoculation

As for preparation of seed stabilate, donor calves for vaccine production should be examined for all blood-borne infections prevalent in the vaccine-producing country, including *Babesia*, *Anaplasma*, *Cowdria*, *Theileria* and *Trypanosoma*. This can be done by routine examination of stained blood films after splenectomy, and preferably also by serology. Any calves showing evidence of natural infections of any of these agents should be rejected. The absence of other infective agents should also be confirmed. These may include the agents of enzootic bovine leukosis, mucosal disease, infectious bovine rhinotracheitis, ephemeral fever, Akabane disease, bluetongue, foot and mouth disease, and rinderpest. The testing procedures will depend on the diseases prevalent in the country and the availability of tests, but should involve serology of paired sera at the very least and, in some cases, virus isolation, antigen, or DNA/RNA detection (Bock *et al.*, 2004; Pipano, 1981; 1997).

iv) Monitoring of rickettsaemias following inoculation

It is necessary to determine the concentration of rickettsia in blood being collected for vaccine. The rickettsial concentration can be estimated from the erythrocyte count and the rickettsaemia (percentage of infected erythrocytes).

v) Collection of blood for vaccine

All equipment should be sterilised before use (e.g. by autoclaving). Once the required rickettsaemia is reached, the blood is collected in heparin using strict aseptic techniques. This is best done if the calf is sedated and with the use of a closed-circuit collection system.

Up to 3 litres of heavily infected blood can be collected from a 6-month-old calf. If the calf is to live, the transfusion of a similar amount of blood from a suitable donor is indicated. Alternatively, the calf should be killed immediately after collection of the blood.

vi) Dispensing of vaccine

All procedures are performed in a suitable environment, such as a laminar flow cabinet, using standard sterile techniques. Use of a mechanical or magnetic stirrer will ensure thorough mixing of blood and diluent throughout the dispensing process. Penicillin (500,000 IU/litre) and streptomycin (370,000 µg/litre) are added to the vaccine at the time of dispensing.
2.2.4. Final product batch tests

The potency, safety and sterility of vaccine batches cannot be determined in the case of chilled vaccine, and specifications for frozen vaccine depend on the country involved. The following are the specifications for frozen vaccine produced in Australia.

i) Sterility and purity

Standard tests for sterility are employed for each batch of vaccine and diluent (see Chapter 1.1.7 Tests for sterility and freedom from contamination of biological materials).

The absence of contaminants is determined by doing appropriate serological testing of donor cattle, by inoculating donor lymphocytes into sheep and then monitoring them for evidence of viral infection, and by inoculating cattle and monitoring them serologically for infectious agents that could potentially contaminate the vaccine. Cattle inoculated during the test for potency (see Section C.2.2.4.iii) are suitable for the purpose. Depending on the country of origin of the vaccine, these agents include the causative organisms of enzootic bovine leukosis, infectious bovine rhinotracheitis, mucosal disease, ephemerah fever, Akabane disease, Aino virus, bluetongue, parainfluenza, foot and mouth disease, lumpy skin disease, rabies, Rift Valley fever, rinderpest, contagious bovine pleuropneumonia, Jembrana disease, heartwater, pathogenic Theileria and Trypanosoma spp., Brucella abortus, Coxiella, and Leptospira (Bock et al., 2004; Pipano, 1981; 1997). Other pathogens to consider include the causal agents of bovine tuberculosis and brucellosis as they may spread through contaminated blood used for vaccine production. Most of these agents can be tested by means of specific PCR and there are many publications describing primers, and assay conditions for any particular disease.

ii) Safety

Vaccine reactions of the cattle inoculated in the test for potency (see Chapter 1.1.6 Principles of veterinary vaccine production Section C.2.2.4.iii) are monitored by measuring rickettsaemia and depression of packed cell volume. Only batches with pathogenicity levels equal to or lower than a predetermined standard are released for use.

iii) Potency

Vaccine is thawed and diluted 1/5 with a suitable diluent (Bock et al., 2004). The diluted vaccine is then incubated for 8 hours at 4°C, and five cattle are inoculated subcutaneously with 2 ml doses. The inoculated cattle are monitored for the presence of infections by examination of stained blood smears. All should become infected for a batch to be accepted. A batch proving to be infective is recommended for use at a dilution of 1/5 with isotonic diluent.

2.3. Requirements for authorisation

2.3.1. Safety

The strain of A. centrale used in vaccine is of reduced virulence, but is not entirely safe. A practical recommendation is, therefore, to limit the use of vaccine to calves, where nonspecific immunity will minimise the risk of vaccine reactions. When older animals have to be vaccinated, there is a risk of severe reactions. These reactions occur infrequently, but valuable breeding stock or pregnant animals obviously warrant close attention, and should be observed daily for 3 weeks post-vaccination. Clinically sick animals should be treated with oxytetracycline or imidocarb at dosages recommended by the manufacturers.

Anaplasma centrale is not infective to other species, and the vaccine is not considered to have other adverse environmental effects. The vaccine is not infective for humans. When the product is stored in liquid nitrogen, the usual precautions pertaining to the storage, transportation and handling of deep-frozen material applies.

2.3.2. Efficacy requirements

Partial but long-lasting immunity results from one inoculation. There is no evidence that repeated vaccination will have a boosting effect. The vaccine is used for control of clinical anaplasmosis in endemic areas. It will not provide sterile immunity, and should not be used for eradication of A. marginale.
2.3.3. Stability

The vaccine can be kept for 5 years when stored in liquid nitrogen. Once thawed, it rapidly loses its potency. Thawed vaccine cannot be refrozen.

3. Vaccines based on biotechnology

There are no vaccines based on biotechnology available for anaplasmosis.

REFERENCES


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Chapter 2.4.1. – Bovine anaplasmosis

STIK N.I., ALLEMAN A.R., BARBET A.F., SORENSON H.L., WANSLEY H.L., GASCHEN F.P., LUCKSCHANDER N., WONG S.,
phagocytophilum major surface protein 5 and the extent of its cross-reactivity with A. marginale. Clin. Vaccine
Immunol., 14, 262–268.

Detection of cattle naturally infected with Anaplasma marginale in a region of endemicity by nested PCR and a
36, 777–782.

NB: There is an OIE Reference Laboratory for Anaplasma sp.
(see Table in Part 4 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date
Please contact the OIE Reference Laboratory for any further information on
diagnostic tests, reagents and vaccines for bovine anaplasmosis
This chapter has been extensively revised and updated. Although some portions of the existing text have been incorporated, new text and deleted text have not been marked, in the interest of clarity.

NB: Last adopted by the World Assembly of Delegates of the OIE in May 2008

CHAPTER 2.8.7.

BOVINE VIRAL DIARRHOEA

SUMMARY

Cattle of all ages are susceptible to infection with bovine viral diarrhoea viruses (BVDV). Distribution is world-wide although some countries have recently eradicated the virus. BVDV infection results in a wide variety of clinical manifestations, including enteric and respiratory disease in any class of cattle or reproductive and fetal disease following infection of a susceptible breeding female. Infection may be subclinical or extend to severe fatal disease. Animals that survive in-utero infection in the first trimester of gestation are almost always persistently infected (PI). PI animals provide the main reservoir of the virus in a population and excrete large amounts of virus in urine, faeces, discharges, milk and semen. Identification of such PI cattle is a key element in controlling the infection. It is important to avoid the trade of such animals. They may appear clinically healthy, or weak and unthrifty. Many PI animals die before reaching maturity. They may infrequently develop mucosal disease with anorexia, gastrointestinal erosions, and profuse diarrhoea, invariably leading to death. Mucosal disease can arise only in PI animals. Latent infections generally do not occur following recovery from acute infection. However bulls may rarely have a persistent testicular infection and excrete virus in semen for prolonged periods.

Identification of the agent: BVDV is a pestivirus in the family Flaviviridae and is closely related to classical swine fever and ovine border disease viruses. The two genotypes (types 1 and 2) are classified as separate species in the genus Pestivirus. Although both cytopathic and non-cytopathic biotypes exist within each species, non-cytopathic strains are usually encountered in field infections and are the main focus of diagnostic virus isolation in cell cultures. PI animals can be readily identified by a variety of methods aimed to detect viral antigens or viral RNA directly in blood and tissues. Virus can also be isolated by inoculation of specimens onto susceptible cell cultures followed by immune-labelling methods to detect the replication of the virus in the cultures. Persistence of virus infection should be confirmed by resampling after an interval of at least 3 weeks, when virus will again be detected. PI animals are usually seronegative. Viraemia in acute cases is transient and difficult to detect. Virus isolation in semen from bulls that are acutely or persistently infected requires special attention to specimen transport and testing. RNA detection assays are particularly useful because they are rapid, have very high sensitivity and do not depend on the use of cell cultures.

Serological tests: Acute infection with BVDV is best confirmed by demonstrating seroconversion using sequential paired samples, ideally from several animals in the group. The testing of paired (acute and convalescent samples) should be done a minimum of 21 days apart and samples should be tested concurrently in the same assay. Enzyme-linked immunosorbent assays and the virus neutralisation test are the most widely used.

Requirements for vaccines: There is no standard vaccine for BVD, but a number of commercial preparations are available. An ideal vaccine should be able to prevent transplacental infection in pregnant cows. Modified live virus vaccine should not be administered to pregnant cattle (or to their sucking calves) due to the risk of transplacental infection. Live vaccines that contain cytopathic strains of BVDV present a risk of inducing mucosal disease in PI animals. Inactivated viral vaccines are safe and can be given to any class of animal but generally require booster vaccinations. BVDV is a particularly important hazard to the manufacture of vaccines and biological products for other diseases due to the high frequency of contamination of batches of fetal calf serum used as a culture medium supplement.
1. Impact of the disease

Cattle of all ages are susceptible to infection with bovine viral diarrhoea viruses (BVDV). Distribution of the virus is world-wide although some countries have recently eradicated the virus. BVDV infection results in a wide variety of clinical manifestations, including enteric and respiratory disease in any class of cattle or reproductive and fetal disease following infection of a susceptible breeding female. Infection may be subclinical or extend to severe fatal disease. Clinical presentations and severity of disease may vary with different strains of virus. BVDV viruses also cause immune suppression which can render infected animals more susceptible to infection with other viruses and bacteria. The clinical impact may be more apparent in intensively managed livestock. Animals that survive infection in the first trimester of gestation are almost always persistently infected (PI). PI animals provide the main reservoir of the virus in a population and excrete large amounts of virus in urine, faeces, discharges, milk and semen. The virus spreads mainly by close contact between PI animals and other cattle. Virus shedding by acutely infected animals is usually less important. This virus may also persist in the environment for short periods or be transmitted with contaminated reproductive materials. Vertical transmission plays an important role in its epidemiology and pathogenesis.

Infections of the breeding female may result in conception failure or embryonic and fetal infection which results in abortions, stillbirths, teratogenic abnormalities or the birth of PI calves. Persistently viraemic animals may be born as weak, unthrifty calves or may appear as normal healthy calves and be unrecognised clinically for a long time. However, PI animals have a markedly reduced life expectancy, with a high proportion dying before reaching maturity. Infrequently, some of these animals may later develop mucosal disease with anorexia, gastrointestinal erosions, and profuse diarrhoea, invariably leading to death. Mucosal disease can arise only in PI animals. It is important to avoid the trade of viraemic animals. It is generally considered that serologically positive, non-viraemic cattle are ‘safe’, providing that they are not pregnant. However, a small proportion of persistently viraemic animals may produce antibodies to some of the viral proteins if they are exposed to another strain of BVDV that is antigenically different to the persisting virus. Consequently, seropositivity cannot be completely equated with ‘safety’. Detection of PI animals must be specifically directed at detection of the virus or its components (RNA or antigens). Latent infections generally do not occur following recovery from acute infection. However, semen collected from bulls during an acute infection is likely to contain virus during the viraemic period and often for a short time afterwards. Although extremely rare, some recovered bulls may have a persistent testicular infection and excrete virus in semen, perhaps indefinitely.

While BVDV strains are predominantly pathogens of cattle, interspecies transmission can occur following close contact with sheep, goats or pigs. Infection of pregnant small ruminants or pigs with BVDV can result in reproductive loss and the birth of PI animals. BVDV infections have been reported in both New World and Old World camels. Additionally, strains of border disease virus (BDV) have infected cattle, resulting in clinical presentations indistinguishable from BVDV infection. The birth of cattle PI with BDV and the subsequent development of mucosal disease have also been described. Whilst BVDV and BDV have been reported as natural infections in pigs, the related virus of classical swine fever does not naturally infect ruminants.

Although ubiquitous, control of BVDV can be achieved at the herd level, and even at the national level, as evidenced by the progress towards eradication made in many European countries (Moennig et al., 2005).

2. The causal agent

Bovine viral diarrhoea virus (BVDV) is a single linear positive-stranded RNA virus in the genus Pestivirus of the family Flaviviridae. The genus contains a number of species including the two genotypes of bovine viral diarrhoea virus (BVDV) (types 1 and 2) and the closely related classical swine fever and ovine border disease viruses. Viruses in these genotypes show considerable antigenic difference from each other and, within the type 1 and type 2 species, BVDV isolates exhibit considerable biological and antigenic diversity. Within the two BVDV genotypes, further subdivisions are discernible by genetic analysis (Vilcek et al., 2001). The two genotypes may be differentiated from each other, and from other pestiviruses, by monoclonal antibodies (MAbs) directed against the major glycoproteins E2 and E1 or by genetic analysis. Reverse-transcription polymerase chain reaction (RT-PCR) assays enable virus typing direct from blood samples (Letellier & Kerhofs, 2003; McGoldrick et al., 1999). Type 1 viruses are generally more common although the prevalence of type 2 strains can be high in North America. BVDV of both genotypes may occur in non-cytopathic and cytopathic forms (biotypes), classified according to whether or not microscopically apparent cytopathology is induced during infection of cell cultures. Usually, it is the non-cytopathic biotype that circulates freely in cattle populations. Non-cytopathic strains are most frequently responsible for disease in cattle and are associated with enteric and respiratory disease in any class of cattle or reproductive and fetal disease following infection of a susceptible breeding female. Infection may be subclinical or extend to severe fatal disease (Brownlie, 1985). Cytopathic viruses are encountered in cases of mucosal disease, a clinical syndrome that is relatively uncommon and involves the ‘super-infection’ of an animal that is PI with a non-cytopathic virus by a closely related cytopathic strain. The two virus biotypes found in a mucosal disease case are usually antigenically closely related if not identical. Type 2 viruses are usually non-
cytopathic and have been associated with outbreaks of severe acute infection and a haemorrhagic syndrome. However some type 2 viruses have also been associated with a disease indistinguishable from that seen with the more frequently isolated type 1 viruses. Further, some type 1 isolates have been associated with particularly severe and fatal disease outbreaks in adult cattle. Clinically mild and inapparent infections are common following infection of non-pregnant animals with either genotype.

3. Pathogenesis

3.1. Acute infections

Acute infections with BVDV are encountered more frequently in young animals, and may be clinically inapparent or associated with fever, diarrhoea (Baker 1995), respiratory disease and sometimes sudden death. The severity of disease may vary with virus strain and the involvement of other pathogens (Brownlie, 1990). In particular, outbreaks of a severe form of acute disease with haemorrhagic lesions, thrombocytopenia and high mortality have been reported sporadically from some countries (Baker, 1995; Bolin & Ridpath, 1992). Infection with type 2 viruses in particular has been demonstrated to cause altered platelet function. During acute infections there is a brief viraemia for 7–10 days and shedding of virus can be detected in nasal and ocular discharges. There may also be a transient leukopenia, thrombocytopenia or temperature response, but these can vary greatly among animals. Affected animals may be predisposed to secondary infections with other viruses and bacteria. Although BVDV may cause a primary respiratory disease on its own, the immunosuppressive effects of the virus exacerbate the impact of this virus. BVDV is one of the major pathogens of the bovine respiratory disease complex in feedlot cattle and in other intensive management systems such as calf raising units.

Infection of breeding females immediately prior to ovulation and in the first few days after insemination can result in conception failure and early embryonic loss (McGowan & Kirkland, 1995). Cows may also suffer from infertility, associated with changes in ovarian function and secretions of gonadotropin and progesterone (Fray et al., 2002). Bulls may excrete virus in semen for a short period during and immediately after infection and may suffer a temporary reduction of fertility. Although the virus level in this semen is generally low it can result in reduced conception rates and be a potential source of introduction of virus into a naive herd (McGowan & Kirkland, 1995).

3.2. In-utero infections

Infection of a breeding female can result in a range of different outcomes, depending on the stage of gestation at which infection occurred. Before about 25 days of gestation, infection of the developing conceptus will usually result in embryo-fetal death, although abortion may be delayed for a considerable time (McGowan & Kirkland, 1995). Surviving fetuses are normal and uninfected. However, infection of the female between about 30–90 days will invariably result in fetal infection, with all surviving progeny PI and sero-negative. Infection at later stages and up to about day 150 can result in a range of congenital defects including hydranencephaly, cerebellar hypoplasia, optic defects, skeletal defects such as arthrogryposis and hypotrichosis. Growth retardation may also occur, perhaps as a result of pituitary dysfunction. Fetal infection can result in abortion, stillbirth or the delivery of weak calves that may die soon after birth (Baker, 1995; Brownlie, 1990; Duffell & Harkness, 1985; Moennig & Liess, 1995). Some PI calves may appear to be normal at birth but fail to grow normally. They remain PI for life and are usually sero-negative. The onset of the fetal immune response and production of antibodies occurs between approximately day 90–120, with an increasing proportion of infected calves having detectable antibodies while the proportion in which virus may be detected declines rapidly. Infection of the bovine fetus after day 180 usually results in the birth of a normal seropositive calf.

3.3. Persistent infections

Persistently viraemic animals are a continual source of infective virus to other cattle and are the main reservoir of BVDV in a population. In a population without a rigorous BVDV control programme, approximately 1–2% of cattle are PI. During outbreaks in a naive herd or breeding group, if exposure has occurred in the first trimester of pregnancy, a very high proportion of surviving calves will be PI. If a PI animal dies, there are no pathognomonic lesions due to BVDV and the pathology is often complicated by secondary infections with other agents. Some PI animals will survive to sexual maturity and may breed successfully but their progeny will also always be PI. Animals being traded or used for artificial breeding should first be screened to ensure that they are not PI.

3.4. Mucosal disease

Persistently viraemic animals may later succumb to mucosal disease (Brownlie, 1985). However, cases are rare. This syndrome has been shown to be the outcome of the infection of a PI animal with an antigenically similar cytopathic virus, which can arise either through superinfection, recombination...
between non-cytopathic biotypes, or mutation of the persistent biotype (Brownlie, 1990). There is
usually little need to specifically confirm that a PI animal has succumbed to mucosal disease as this is
largely a scientific curiosity and of little practical significance, other than that the animal is PI with
BVDV. However, cases of mucosal disease may be the first indication in a herd that BVDV infection is
present, and should lead to more in depth investigation and intervention.

3.5. Semen and embryos

Bulls that are PI usually have poor quality, highly infective semen and reduced fertility (McGowan &
Kirkland, 1995). All bulls used for natural or artificial insemination should be screened for both acute
and persistent BVDV infection. A rare event, possibly brought about by acute infection during
pubescence, can result in persistent infection of the testes and thus strongly seropositive bulls that
intermittently excrete virus in semen (Voges et al., 1998). This phenomenon has also been observed
following vaccination with an attenuated virus (Givens et al., 2007). Embryo donor cows that are PI with
BVDV also represent a potential source of infection, particularly as there are extremely high
concentrations of BVDV in uterine and vaginal fluids. While oocysts without an intact zona pellucida
have been shown to be susceptible to infection in vitro, the majority of oocysts remain uninfected with
BVDV. Normal uninfected progeny have also been ‘rescued’ from PI animals by the use of extensive
washing of embryos and in vitro fertilisation. Female cattle used as embryo recipients should always be
screened to confirm that they are not PI, and ideally, are sero-positive or were vaccinated at least
4 weeks before first use.

Biological materials used for in-vitro fertilisation techniques (bovine serum, bovine cell cultures) have a
high risk of contamination and should be screened for BVDV. Incidents of apparent introduction of virus
via such techniques have highlighted this risk. It is considered essential that serum supplements used
in media should be free of contaminants as detailed in Chapter 1.1.7 Tests for sterility and freedom
from contamination of biological materials, using techniques described in Section B.3.1 of this chapter.

4. Approaches to diagnosis and sample collection

The diagnosis of BVDV infection can sometimes be complex because of the delay between infection and clinical
expression. While detection of PI animals should be readily accomplished using current diagnostic methods, the
recognition of acute infections and detection of BVDV in reproductive materials can be more difficult.

4.1. Acute infections

Unlike PI animals, acutely infected animals excrete relatively low levels of virus and for a short period
time (usually about 7–10 days) but the clinical signs may occur during the later stages of viraemia,
reducing the time to detect the virus even further. In cases of respiratory or enteric disease, samples
should be collected from a number of affected animals, preferentially selecting the most recently
affected. Swabs should be collected from the nares and conjunctiva of animals with respiratory disease
or from rectum and faeces if there are enteric signs. Lung and spleen are preferred from dead animals.
Viral RNA may be detected by real-time RT-PCR assays and have the advantages of high sensitivity
and being able to detect genome from non-infectious virus. As the virus levels are very low, it is not
usually practical to undertake virus isolation unless there is a need to characterise the strain of BVDV
involved. Serology undertaken on paired acute and convalescent sera (collected at least 21 days after
the acute sample and from 8–10 animals) is worthwhile and gives a high probability of incriminating or
excluding BVDV infection.

Confirmation that an abortion, stillbirth or perinatal death is caused by BVDV is often difficult to
establish because there can be a long delay between initial infection and death or expulsion of the
fetus. Sampling should take into consideration the need to detect either viral components or antibodies.
Spleen and lung are preferred samples for virus detection while pericardial or pleural fluids are ideal
samples for serology. The stomach of newborn calves should be checked to confirm that sucking has
not occurred. While virus may be isolated from fetal tissue in some cases, emphasis should be placed
on the detection of viral antigen by enzyme-linked immunosorbent assay (ELISA) or RNA by real-time
RT-PCR. For serology, both ELISAs and virus neutralisation test (VNT) are suitable though sample
quality and bacterial contamination may compromise the ability to detect antibodies by VNT. Maternal
serology, especially on a group of animals, can be of value, with the aim of determining whether there
has been recent infection in the group. A high antibody titre (>1/1000) to BVDV in maternal serum is
suggestive of fetal infection and is probably due to the fetus providing the dam with an extended
exposure to virus.

4.2. Persistent infections

In the past, identification of PI animals relied heavily on the use of virus isolation in cell cultures.
However, antigen detection ELISAs and real-time RT-PCR assays, each with relatively high sensitivity,
are widely used for the detection of viral antigens or RNA in both live and dead animals. Virus isolation aimed at the detection of non-cytopathic BVDV in blood is also used, while in some countries, the virus has been identified by immunohistochemistry (IHC). Skin samples have been collected from live animals while a wide range of tissues from dead animals are suitable. Both virus isolation and IHC are labour intensive and costly and can be technically demanding. Virus isolation from blood can be confounded by the presence of maternal antibody to BVDV in calves less than 4–5 months of age. In older animals with persistent viraemia infection, low levels of antibody may be present due to their ability to seroconvert to strains of BVDV (including vaccines) antigenically different to the persisting virus (Brownlie, 1990). Bulk (tank) or individual milk samples have been used to monitor dairy herds for the presence of a PI animal. Antigen ELISA, real-time PCR and virus isolation have all been used. To confirm a diagnosis of persistent infection, animals should be retested after an interval of at least 3 weeks by testing of blood samples for the presence of the virus and for evidence of seroconversion. Care should be taken with retesting of skin samples as it has been shown that, in some acute cases, viral antigen may persist for many weeks in skin (Cornish et al., 2005).

4.3. Mucosal disease

Although not undertaken for routine diagnostic purposes, for laboratory confirmation of a diagnosis of mucosal disease it is necessary to isolate the cytopathic virus. This biotype may sometimes be isolated from blood, but it can be recovered more consistently from a variety of other tissues, in particular spleen, intestine and Peyer’s patch tissue. Virus isolation is readily accomplished from spleen which is easy to collect and is seldom toxic for cell culture.

4.4. Reproductive materials

Semen donor bulls should be sampled for testing for freedom from BVDV infection prior to collection of semen, in accordance with the Terrestrial Animal Health Code. It is necessary to confirm that these bulls are not PI, are not undergoing an acute infection and to establish their serological status. This initial testing should be carried out on whole blood or serum samples. To establish that a seropositive bull does not have a persistent testicular infection (PTI), samples of semen should be collected on at least three separate occasions at intervals of not less than 7 days due to the possibility of intermittent low level virus excretion, especially during the early stages of infection. There is also a need to submit a number of straws from each collection, or an appropriate volume of raw semen. Particular care should be taken to ensure that sample transport recommendations are adhered to and that the laboratory documents the condition of the samples on arrival at the laboratory. Further details of collection, transport and test requirements are provided in sections that follow.

### B. Diagnostic Techniques

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<td>Population freedom from infection</td>
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<td>Individual animal freedom from infection prior to movement</td>
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<td>Confirmation of clinical cases</td>
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<td>Prevalence of infection – surveillance</td>
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<td>Immune status in individual animals or populations post-vaccination</td>
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**Table 1. Test methods available for diagnosis of bovine viral diarrhea and their purpose**

A combination of agent identification methods applied on the same clinical sample is recommended.
Chapter 2.4.8. – Bovine viral diarrhoea

### Method

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<th>Individual animal freedom from infection prior to movement</th>
<th>Contribution to eradication policies</th>
<th>Confirmation of clinical cases</th>
<th>Prevalence of infection – surveillance</th>
<th>Immune status in individual animals or populations post-vaccination</th>
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<td><strong>NA detection by real-time RT-PCR</strong></td>
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### Detection of immune response

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<th>Contribution to eradication policies</th>
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<td><strong>ELISA</strong></td>
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Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose; n/a = not applicable.

Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

ELISA = enzyme-linked immunosorbent assay; IHC = immunohistochemistry method; NA = nucleic acid; RT-PCR = reverse-transcription polymerase chain reaction; ISH = in-situ hybridisation; VN = virus neutralisation.

### 1. Detection of the agent

To prevent the shipment of either animals or animal derivatives (especially semen and embryos) that are infected with BVDV, it is necessary to test for the presence of the infectious virus (virus isolation), viral antigens (antigen detection ELISA or rtRNA (real-time RT-PCR) in the blood of the animal being shipped, or the donor of the germplasm (semen or embryos). The exception is for seropositive bulls where semen must be tested rather than the donor bull. Serology only plays a role for establishing that seronegative animals are not undergoing an acute infection or, to establish the serological status of donor bulls. Due to their variable sensitivity without prior virus amplification, procedures such as IHC or in-situ hybridisation (ISH) directly on tissues are not considered to be suitable for certification for freedom from BVDV for international trade purposes. In contrast, immune-staining is an essential component of virus isolation in cell culture to detect the presence of non-cytopathic strains of BVDV which predominate in field infections.

All test methods must be extensively validated by testing on known uninfected and infected populations of cattle, including animals with low- and high-titre viraemias. Methods based on polyclonal or MAb-binding assays (ELISA or IHC), immune labelling (VI) or on nucleic acid recognition (PCR) must be shown to detect the full range of antigenic and genetic diversity found among BVD viruses. There are three designated OIE Reference Laboratories for BVDV that can assist with relevant information (see list in Part 4 of this Terrestrial Manual); the reference laboratories for classical swine fever could also be approached to offer some advice.

#### 1.1. Virus isolation (a prescribed test for international trade)

When performed to a high standard, BVDV isolation is very reliable. However, it does have very exacting requirements to ensure that the cell cultures and medium components give a system that is very sensitive and are not compromised by the presence of either low levels of BVDV specific antibody or virus. Virus isolation only has the capacity to detect infectious virus which imposes certain limits on sample quality. Further, to detect low levels of virus that may be present in some samples, particularly semen, it may be necessary to examine larger volumes of specimen than is usual. Some of these limitations can be overcome by the use of antigen detection ELISAs with proven high analytical sensitivity, or the use of real-time RT-PCR.

The virus may be isolated in a number of bovine monolayer cell cultures (e.g. kidney, lung, testis or turbinate). In some instances, ovine cells are also suitable. Primary or secondary cultures can be frozen as cell suspensions in liquid nitrogen. These can then be tested over a series of passages, or seeded to other susceptible cells and checked for freedom from contaminants and to evaluate their sensitivity compared to an approved batch of cells before routine use. Such problems may be reduced by the use of continuous cell lines, which can be obtained BVD-free, however, their BVD-free status and susceptibility must be monitored regularly. Continuous cells should be used under a ‘seed lot’ system where they are only used over a limited passage range, within which they have been shown to

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2 One of the listed serological tests is sufficient.
have acceptable sensitivity to BVDV infection. Although particular continuous cell lines are considered to be appropriate for use for BVDV isolation, there can be significant variation in batches of cells from different sources due to differing passage histories so their suitability must still be confirmed before routine use.

Non-cytopathic BVDV is a common contaminant of bovine tissues, and cell cultures must be checked for freedom from adventitious virus by regular testing. Cells must be grown in proven cell culture medium components and a large area of cells must be examined. It is not appropriate to screen a few wells of a 96 well plate – examining all wells of a 96 well plate will be more convincing evidence of freedom. The fetal bovine serum that is selected for use in cell culture must also be free not only from virus, but also and of equal or perhaps even greater importance, from BVDV neutralising antibody. Heat treatment (56°C for 30–45 minutes) is inadequate for the destruction of BVDV in contaminated serum; irradiation with a dose of at least 25 kiloGrays (2.5 Mrad) is more certain. Commercial batches of fetal bovine serum mostly test positive by real-time RT-PCR even after the virus has been inactivated by irradiation. Further, most commercially collected batches of fetal bovine serum contain antibodies to pestiviruses, sometimes at levels that are barely detectable but sufficient to inhibit virus isolation. To overcome this, serum can be obtained from BVD virus and antibody free donor animals and used with confidence. Testing of donors for both virus and antibody occurs on an individual animal basis. Although horse serum has been substituted for bovine fetal serum, it is often found to have poorer cell-growth-promoting characteristics. Further there has sometimes been cross contamination with fetal bovine serum during processing, negating the objective of obtaining a BVDV-free product.

Buffy coat cells, whole blood, washed leukocytes or serum are suitable for isolation of the virus from live animals. Maternal antibody may interfere with isolation from serum in young calves. Tissue suspensions from post-mortem cases should be prepared by standard methods. Confirmation that a bull is not PI with BVDV is most readily achieved by testing of a blood sample. However, persistent testicular infections (PTI) have been detected in some bulls that have recovered from acute infection, are no longer viraemic and are now seropositive (Voges et al., 1998). Virus may be detected in most but not all collections of semen from these bulls. Although still considered to be uncommon, to exclude the potential for a PTI it is essential to screen semen from all seropositive bulls. To be confident that a bull does not have a PTI, batches of semen collected over several weeks should be screened. Once a series of collections have been screened, further testing of semen from a seropositive bull is not warranted. Raw semen, and occasionally extended semen, is cytotoxic and must be diluted in culture medium. For these reasons, it is important to monitor the health of the cells by microscopic examination at intervals during the incubation. These problems are largely overcome by the use of real-time RT-PCR which has several advantages over virus isolation, including higher sensitivity and the potential to be completed within a few hours rather than weeks for virus isolation.

There are many variations of procedure in use for virus isolation. All should be optimised to give maximum sensitivity of detection of a standard virus preparation. All biological components used for cell culture should be screened and shown to be free of both BVDV and antibodies to BVDV. Cell cultures (whether primary or continuous lines) should be regularly checked to confirm that they maintain maximum susceptibility to virus infection. Depending on the specimen type and purpose for testing, virus isolation is likely to require one or more passages in cell cultures. While PI animals can be readily identified by screening blood or serum with one passage, semen should be routinely cultured for three passages and biological products such as fetal bovine serum during processing, negating the objective of obtaining a BVDV-free product.

1.1.1. Microplate immunoperoxidase method for mass screening for virus detection in serum samples (Meyling, 1984)

i) 10–25 μl of the serum sample is placed into each of four wells of a 96-well tissue-culture-grade microplate. This is repeated for each sample. Known positive and negative controls are included.

ii) 100 μl of a cell suspension at the appropriate concentration (usually about 150,000 cells/ml) in medium without fetal calf serum (FCS) is added to all wells. Note: the sample itself acts as the cell-growth supplement. If testing samples other than serum, use medium with 10% FCS that is free of antibodies to ruminant pestiviruses.

iii) The plate is incubated at 37°C for 4 days, either in a 5% CO₂ atmosphere or with the plate sealed.
iv) Each well is examined microscopically for evidence of cytopathology (cytopathic effect or CPE), or signs of cytotoxicity.

v) The cultures are frozen briefly at approximately –80°C and 50 µl of the culture supernatant is passaged to new cell cultures, repeating steps 3.1.1.i to iv above.

vi) The cells are then fixed and stained by one of two methods:

- Paraformaldehyde:
  a) Add 200 µl of a 1/10 dilution of formaldehyde solution (approximately 3% concentration) to the plate and leave at room temperature for 10 minutes.
  b) The contents of the plate are then discarded and the plate is washed.
  c) Wash plates 5 times with 0.05%Tween 20 in water (an automatic microplate washer can be used with a low pressure and speed setting).
  d) To each well add 50µl of an antiviral antibody at the appropriate dilution (prepared in phosphate buffered saline/ PBS containing 1% gelatin) and incubate for 60–90 minutes at 37°C in a humidified chamber.
  e) Wash plates five times as in step c).
  f) Dilute the appropriate peroxidase conjugated antiserum to the optimum dilution in 1% gelatin/PBS (e.g. peroxidase conjugated rabbit anti-mouse immunoglobulin when the antiviral antibody is a mouse monoclonal). The optimum concentration should be determined for each batch of conjugate by "checkerboard" titration against reference positive and negative controls.
  g) To each well of the microplate add 50µl of the diluted peroxidase conjugate and incubate for 90 minutes at 37°C in a humidified chamber.
  h) Wash plates five times as in step c).
  i) "Develop" the plate by adding 3-amino-9-ethyl carbazole (AEC) substrate (100 µl/well) and allowing to react for 30 minutes at room temperature.
  j) Add 100µl of PBS to each well and add a lid to each plate.
  k) Examine the wells by light microscopy, starting with the negative and positive control wells. There should be no or minimal staining apparent in the cells that were uninfected (negative control). The infected (positive control) cells should show a reddish- brown colour in the cytoplasm.

- Acetone
  a) The plate is emptied by gentle inversion and rinsed in PBS.
  b) The cells are fixed as follows: the plate is dipped into a bath of 20% acetone in PBS, emptied immediately and then transferred to a fresh bath of 20% acetone in PBS for 10 minutes. The plate is drained thoroughly and as much fluid as possible is removed by tapping and blotting. The plate is dried thoroughly for at least 3 hours at a temperature of 25–30°C (e.g. using radiant heat from a bench lamp). NB: the drying is part of the fixation process.
  c) The fixed cells are rinsed by adding PBS to all wells.
  d) The wells are drained and the BVD antibody (50 µl) is added to all wells at a predetermined dilution in PBS containing 1% Tween 80 (PBST) and 5% horse serum or 1% gelatin. (Horse serum or gelatin may be added to reduce nonspecific staining.)
  e) Incubate at 37°C for 15 minutes.
  f) Empty the plate and wash three times in PBST.
  g) Drain and add the appropriate anti-species serum conjugated to peroxidase at a predetermined dilution in PBST (50 µl per well) for 15 minutes at 37°C.
  h) Empty the plate and wash three times in PBST.
  i) Rinse the plate in distilled water. All fluid is tapped out from the plate.
  j) Add freshly prepared hydrogen peroxide substrate with a suitable chromogen, e.g. 3-amino-9-ethyl carbazole (AEC).
  k) An alternative substrate can be made, consisting of 9 mg dianinobenzidine tetrahydrochloride and 6 mg sodium perborate tetrahydrate dissolved in 15 ml of
PBS. Though the staining is not quite so intense, these chemicals have the advantage that they can be shipped by air.

i) The plate is examined microscopically. Virus-positive cells show red-brown cytoplasmic staining.

Alternative methods for fixation of the cells may be used and include the use of heat (see Chapter 2.8.3 Classical swine fever, Section B.2.2.1.viii). These should be first evaluated to ensure that the capacity to detect viral antigen is not compromised.

1.1.2. Tube method for tissue or buffy coat suspensions

NB: this method can also be conveniently adapted to 24-well plastic dishes. Note that a minimum of 2 and preferably 3 passages (including primary inoculation) are required.

i) Tissue samples are ground up and a 10% suspension in culture medium is made. This is then centrifuged to remove the debris.

ii) Test tube cultures with newly confluent or subconfluent monolayers of susceptible bovine cells are inoculated with 0.1 ml of the sample. The culture is left to adsorb for 1 hour at 37°C.

iii) The culture is washed with 1 ml of medium; this is then discarded and 1 ml of culture maintenance medium is added.

iv) The culture is incubated for 4–5 days at 37°C, and examined microscopically for evidence of CPE or signs of cytotoxicity.

v) The culture should then be frozen and thawed for passage to fresh cultures for one or preferably two more passages (including the culture inoculated for the final immunostaining). At the final passage, after freeze–thaw the tissue culture fluid is harvested and passaged on to microtitre plates for culture and staining by the immunoperoxidase method (see section B.3.1.1 above) or by the immunofluorescent method. For immunofluorescence, cover-slips are included in the tubes and used to support cultured cells. At the end of the culture period, the cover slips are removed, fixed in 100% acetone and stained with an immunofluorescent conjugate to BVDV. Examine the cover slips under a fluorescent microscope for diffuse, cytoplasmic fluorescence characteristic of pestiviruses. Alternatively, culture supernatant from the final passage can be screened by real-time RT-PCR (see below).

1.1.3. Virus isolation from semen

The samples used for the test are, typically, extended bovine semen or occasionally raw semen. Semen samples should be transported to the laboratory in liquid nitrogen, or on dry ice. The samples should be stored in liquid nitrogen or at lower than –70°C (for long-term storage) or 4°C (for short-term storage of not more than 1–2 days). The receiving laboratory should document the condition under which samples are received. Raw semen is generally cytotoxic and should be prediluted (e.g. 1/10 in BVDV free bovine serum) before being added to cell cultures. At least 0.1 ml of raw semen should be tested with three passages in cell culture. Toxicity may also be encountered with extended semen. For extended semen, an approximation should be made to ensure that the equivalent of a minimum of 0.1 ml raw semen is examined (e.g. a minimum of 1.0 ml extended semen). If toxicity is encountered, multiple diluted samples may need to be tested to reach a volume equivalent to 0.1 ml raw semen (e.g. 5 × 1 ml of a sample of extended semen that has been diluted 1/5 to reduce toxicity). A suggested method is as follows:

i) Dilute 200 μl fresh semen in 1.8 ml bovine serum containing antibiotics. This can be the same serum as is being used for supplementing the cell cultures, and must be shown to be free from antibodies against BVDV.

ii) Mix vigorously and leave for 30 minutes at room temperature.

iii) Inoculate 1 ml of the semen/serum mixture into a monolayer of susceptible cells (see virus isolation from tissue above) in cell culture tubes or a six-well tissue culture plate.

iv) Incubate the cultures for 1 hour at 37°C.

v) Remove the mixture, wash the monolayer several times with maintenance medium and then add new maintenance medium to the cultures.
vi) Include BVDV negative and positive controls in the test. Special caution must be taken to avoid accidental contamination of test wells by the positive control, for example always handling the positive control last.

vii) Observe plates microscopically to ensure freedom from contamination and cytopathicity. No cytopathology is expected as a result of BVDV infection but other viruses such as BHV-1 could be inadvertently isolated.

viii) After 5–7 days, the cultures are frozen at or below approximately −70°C and thawed, confirmed by centrifugation, and the supernatant used to inoculate fresh monolayers.

ix) At the end of the second passage, the supernatant from the freeze-thaw preparation should be passaged onto cultures in a suitable system for immunoperoxidase staining or other antigen detection or by real-time RT-PCR after 5 days of culture. This is most readily achieved in 96 well microplates. The sample is considered to be negative, if there is no evidence of viral antigen or BVDV RNA detected.

1.2. Nucleic acid detection

Conventional gel based RT-PCR has in the past been used for the detection of BVD viral RNA for diagnostic purposes. A multiplex RT-PCR has been used for the simultaneous amplification and typing of virus from cell culture, or directly from blood samples. However, gel based RT-PCR has the disadvantage that it is relatively labour intensive, expensive and prone to cross contamination. These problems have been markedly reduced following the introduction of probe-based real-time or quantitative RT-PCR methods. Nevertheless, stringent precautions should still be taken to avoid nucleic acid contamination in the test system and general laboratory areas where samples are handled and prepared (see Chapter 1.1.5 Principles and methods of validation of diagnostic assays for infectious diseases and Validation Guideline 3.6.3 Development and optimisation of nucleic acid assays). These assays have even higher sensitivity than gel based RT-PCR and can be completed in a few hours. They are in widespread use for the diagnosis of infectious diseases, allowing the direct detection of viral RNA from a wide range of specimens including serum, whole blood, tissues, milk and semen. The high analytical sensitivity allows the adoption of strategies to screen pools of individual samples or testing of bulk tank milk. By using this approach the presence of one or more PI animals can be identified in herds containing several hundred cows. Although slightly more expensive than gel-based RT-PCR, real-time RT-PCR is a quick and reliable method that can also be used to screen culture supernatant from the final passage of cell cultures. While real-time RT-PCR has very high sensitivity and can be applied to the screening of biological materials used for vaccine manufacture, caution is needed in the interpretation of results, as the detection of viral RNA does not imply per se that infective virus is present. Real-time RT-PCR assays based on fluorescent-labelled DNA probes can also be used to differentiate pestiviruses (e.g. McGoldrick et al., 1999).

Primers for the assay should be selected in highly conserved regions of the genome, ideally the 5'-noncoding region, or the NS3 (p80 gene). There are published assays that are broadly reactive across the pestivirus genus, detecting all BVDV types, CSFV and most of the ‘atypical’ pestiviruses (e.g. Hoffman et al., 2006). A sensitive broadly reactive assay is recommended for diagnostic applications because interspecies transfer of different pestiviruses is occasionally encountered. When further identification of the specific virus is required, pestivirus species-specific assays can be applied to further type the virus. It is important to thoroughly optimise all aspects of the real-time RT-PCR assay, including the nucleic acid extraction and purification. Optimal concentrations of Mg²⁺, primers, probe and polymerase, and the cycling parameters need to be determined. However, fully formulated and optimised ‘ready to use’ ‘mastermixes’ are now available commercially and only require addition of optimised concentrations of primers and probe. Optimised cycling conditions are often recommended for a particular mastermix.

A variety of commercially available nucleic acid purification systems are available in kit form and several can be semi-automated. Systems based on the capture and purification of RNA using magnetic beads are in widespread use and allow rapid processing of large numbers of samples. Specific products should be evaluated to determine the optimal kit for a particular sample type and whether any preliminary sample processing is required. For whole blood samples, the type of anticoagulant and volume of blood in a specimen tube is important. More problems with inhibitors of the PCR reaction are encountered with samples collected into heparin treated blood than EDTA. These differences are also exacerbated if the tube does not contain the recommended volume of blood, thereby increasing the concentration of anticoagulant in the sample. To identify possible false-negative results, it is recommended to spike an exogenous (‘internal control’) RNA template into the specimen prior to RNA extraction (e.g. Hoffman et al., 2006). By the inclusion of PCR primers and probe specific to the exogenous sequence, the efficiency of both the RNA extraction and also the presence of any PCR inhibitors can be monitored. While valuable for all sample types, the inclusion of an internal control is
particularly desirable when testing semen and whole blood. When using an internal control, extensive
testing is necessary to ensure that PCR amplification of the internal control does not compete with the
diagnostic PCR and thus lower the analytical sensitivity (see also chapter 1.1.5).

When it is suspected that a sample may contain substances that are adversely affecting either the
efficiency of RNA extraction or the real-time RT-PCR assay, modest dilution of the sample in saline,
cell culture medium or a buffer solution (e.g. PBGS) will usually overcome the problem. Dilution of a
semen sample by 1/4 and whole unclotted blood at 1/10 is usually adequate. As the real-time RT-PCR
has extremely high analytical sensitivity, dilution of the sample rarely has a significant impact on the
capacity of the assay to detect viral RNA when present.

1.2.1. Real-time polymerase chain reaction for BVDV detection in semen

Real-time RT-PCR has been shown to be extremely useful to screen semen samples to
demonstrate freedom from BVDV and, apart from speed, often gives superior results to virus
isolation in cell culture, especially when low virus levels are present, such as may be found in bulls with a PTI. The real-time RT-PCR described here uses a pair of sequence-specific primers
for amplification of target DNA and a 5'-nuclease oligoprobe for the detection of amplified
products. The oligoprobe is a single, sequence-specific oligonucleotide, labelled with two
different fluorophores. The primers and probe are available commercially and several different
fluorophores options are available. This pan-pestivirus real-time RT-PCR assay is designed to
detect viral DNA of all strains of BVDV1 & BVDV2 as well as BDV, CSFV and most atypical
pestiviruses. The assay selectively amplifies a 208 base pair sequence of the 5' non-translated
region (5' NTR) of the pestivirus genome. Details of the primers and probes are given in the
protocol outlined below.

i) Sample preparation, equipment and reagents

a) The samples used for the test are, typically, extended bovine semen or occasionally
raw semen. The semen samples should be transported to the laboratory in liquid
nitrogen, or on dry ice. The samples should be stored in liquid nitrogen or at lower
than −70°C (for long-term storage) or 4°C (for short-term storage of up to 7 days).
Note however that samples for virus isolation should not be stored at 4°C for more
than 1–2 days.

b) Due to the very high analytical sensitivity of real-time RT-PCR, much smaller
volumes of semen may be used. However, at least three straws (minimum 250 µl
each) from each collection batch of semen should be processed. The semen in the
three straws should be pooled and mixed thoroughly before taking a sample for
nucleic acid extraction.

c) A real-time PCR detection system, and the associated data analysis software, is
required to perform the assay. A number of real-time PCR detection systems are
available from various manufacturers. Other equipment required for the test includes
a micro-centrifuge, a chilling block, a micro-vortex, and micropipettes. As real-time
RT-PCR assays are able to detect very small amounts of target nucleic acid
molecules, appropriate measures are required to avoid contamination, including
dedicated and physically separated 'clean' areas for reagent preparation (where no
samples or materials used for PCR are handled), a dedicated sample processing
area and an isolated area for the PCR thermocycler and associated equipment. Each
area should have dedicated reagents and equipment. Furthermore, a minimum of
one negative sample should be processed in parallel to monitor the possibility of low
level contamination. Sources of contamination may include product carry-over from
positive samples or, more commonly, from cross contamination by PCR products
from earlier work.

d) The real-time RT-PCR assay involves two separate procedures.

1) Firstly, BVDV RNA is extracted from semen using an appropriate validated
nucleic acid extraction method. Systems using magnetic beads for the capture
and purification of the nucleic acid are recommended. It is also preferable that
the beads are handled by a semi-automated magnetic particle handling system.

2) The second procedure is the RT-PCR analysis of the extracted RNA template in
a real-time RT-PCR system.

ii) Extraction of RNA

RNA or total nucleic acid is extracted from the pooled (three straws collected at the same
time from the same animal) semen sample. Use of a commercially available magnetic
bead based extraction kit is recommended. However, the preferred kit should be one that has been evaluated to ensure optimal extraction of difficult samples (semen and whole blood). Some systems and kit protocols are sufficiently refined that it is not necessary to remove cells from the semen sample. Prior to extraction dilute the pooled semen sample 1/4 in phosphate buffered gelatin saline (PBGS) or a similar buffered solution. Complete the RNA extraction by taking 50 µl of the diluted, pooled sample and add it to the sample lysis buffer. Some commercial extraction kits may require the use of a larger volume. It has also been found that satisfactory results are obtained by adding 25 µl of undiluted pooled sample to sample lysis buffer. Complete the extraction by following the kit manufacturer's instructions.

iii) Real-time RT-PCR assay procedure

a) Reaction mixture: There are a number of commercial real-time PCR amplification kits available from various sources and the particular kits selected need to be compatible with the real-time PCR platform selected. The required primers and probes can be synthesised by various commercial companies. The OIE Reference Laboratories for BVDV can provide information on suitable suppliers.

b) Supply and storage of reagents: The real-time PCR reaction mixture is normally provided as a 2 × concentration ready for use. The manufacturer's instructions should be followed for application and storage. Working stock solutions for primers and probe are made with nuclease-free water at the concentration of 20 µM and 3 µM, respectively. The stock solutions are stored at −20°C and the probe solution should be kept in the dark. Single-use or limited use aliquots can be prepared to limit freeze–thawing of primers and probes and extend their shelf life.

c) Primers and probe sequences

Selection of the primers and probe are outlined in Hoffmann et al. (2006) and summarised below.

Forward: BVD 190-F 5’-GRA-GTC-GTC-ART-GGT-TCG-AC
Reverse: V326 5’-TCA-ACT-CCA-TGT-GCC-ATG-TAC
Probe: TQ-pesti 5’-FAM-TGC-YAY-GTG-GAC-GAG-GGC-ATG-C-TAMRA-3’

d) Preparation of reaction mixtures

The PCR reaction mixtures are prepared in a separate room that is isolated from other PCR activities and sample handling. For each PCR test, appropriate controls should be included. As a minimum, a no template control (NTC), appropriate negative control (NC) two positive controls (PC1, PC2) should be included. The positive and negative controls are included in all steps of the assay from extraction onwards while the NTC is added after completion of the extraction. The PCR amplifications are carried out in a volume of 25 µl. The protocol described is based on use of a 96 well microplate based system but other options using microtubes are also suitable. Each well of the PCR plate should contain 20 µl of reaction mix and 5 µl of sample as follows:

12.5 µl 2× RT buffer – from a commercial kit.
1 µl BVD 190-F Forward primer (20 µM)
1 µl V326 Reverse primer (20 µM)
1 µl TQ-pesti Probe (3 µM)
2 µl tRNA (40 ng/µl)
1.5 µl water
1 µl 25× enzyme mix
5 µl sample (or controls – NTC, NC, PC1, PC2)

e) Selection of controls

NTC: usually consists of tRNA in nuclease free water that is added in place of a sample when the PCR reaction is set up.
NC: In practice, many laboratories use PBGS or a similar buffer. Ideally the controls for testing of semen samples should be negative semen, from sero-negative bulls. However, as a minimum, the assay in use should have been extensively validated with negative and positive samples to confirm that it gives reliable extraction and amplification with semen.

PCs: There are two positive controls (PC1=moderate – [Ct 29-32] and PC2=weak [Ct 32–35] positive). Positive semen from naturally infected bulls is preferable as a positive control. However, this is likely to be difficult to obtain. Further, semen from a PI bull is not considered suitable because the virus loads are usually very high and would not give a reliable indication of any moderate reduction in extraction or assay performance. Negative semen spiked with defined quantities of BVDV virus could be used as an alternative. If other samples are used as a routine PC, as a minimum the entire extraction process and PCR assay in use must have been extensively validated using known positive semen from bulls with a PTI or from bulls undergoing an acute infection. If these samples are not available and spiked samples are used for validation purposes, a number of samples spiked with very low levels of virus should be included. On a day to day basis, the inclusion of an exogenous control with each test sample will largely compensate for not using positive semen as a control and will give additional benefits by monitoring the efficiency of the assay on each individual sample. Positive control samples should be prepared carefully to avoid cross contamination from high titred virus stocks and should be prepared in advance and frozen at a ‘ready to use’ concentration and ideally ‘single use’ volume.

f) Extracted samples are added to the PCR mix in a separate room. The controls should be added last, in a consistent sequence in the following order: NTC, negative and then the two positive controls.

g) Real-time polymerase chain reaction

The PCR plate or tubes are placed in the real-time PCR detection system in a separate, designated PCR room. Some mastermixes have uniform reaction conditions that are suitable for many different assays. As an example, the PCR detection system is programmed for the test as follows:

1× 48°C 10 minutes
1× 95°C 10 minutes
45 × (95°C 15 seconds, 60°C 1 minute)

h) Analysis of real-time PCR data

The software program is usually set to automatically adjust results by compensating for any background signal and the threshold level is usually set according to the manufacturer’s instructions for the selected analysis software used. In this instance, a threshold is set at 0.05.

i) Interpretation of results

a) Test controls – all controls should give the expected results with positive controls PC1 and PC2 falling within the designated range and both the negative control NC and no template control NTC should have no Ct values.

b) Test samples

1) Positive result: Any sample that has a cycle threshold (Ct) value less than 40 is regarded as positive.

2) Negative result: Any sample that shows no Ct value is regarded as negative. However, before reporting a negative result for a sample, the performance of the exogenous internal control should be checked and shown to give a result within the accepted range for that control (for example, a Ct value no more than 2–3 Ct units higher than the NTC).

1.3. Enzyme-linked immunosorbsent assay for antigen detection

Antigen detection by ELISA has become a widely adopted method for the detection of individual PI animals. These assays are not intended for the detection of acutely infected animals (though from time this may be achieved). Importantly, these assays are not designed for screening of semen or
biological materials used in assays or vaccine manufacture. Several methods for the ELISA for antigen
detection have been published and a number of commercial kits are available. Most are based on the
sandwich ELISA principle, with a capture antibody bound to the solid phase, and a detector antibody
coujugated to a signal system, such as peroxidase. Amplification steps such as the use of biotin and
streptavidin in the detection system are sometimes used to increase assay sensitivity. Both
monoclonal- and polyclonal-based systems are described. The test measures BVD antigen (NS2-3 or
Erens) in lysates of peripheral blood leukocytes; the new generation of antigen-capture ELISAs (Erens
capture ELISAs) are able to detect BVD antigen in blood as well as in plasma or serum samples. The
best of the methods gives a sensitivity similar to virus isolation, and may be preferred in those rare
cases where persistent infection is combined with sero-positivity. Due to transient viraemia, the antigen
ELISA is less useful for virus detection in acute BVD infections.

The NS2-3 ELISA may be less effective in young calves that have had colostrum due to the presence
of BVDV maternal antibodies. The real-time RT-PCR is probably the most sensitive detection method
for this circumstance, but the Erens ELISA has also been shown to be a sensitive and reliable test,
particularly when used with skin biopsy (ear-notch) samples (Cornish et al., 2005).

1.4. Immunohistochemistry

Enzyme-labelled methods are useful to detect BVDV antigen in tissue sections, particularly where
suitable MAbS are available. However, these assays are not appropriate to certify animals for
international trade and use should be limited to diagnostic investigations. It is important that the
reagents and procedures used be fully validated, and that nonspecific reactivity be eliminated. For PI
cattle almost any tissue can be used, but particularly good success has been found with lymph nodes,
thyroid gland, skin, brain, abomasum and placenta. Skin biopsies, such as ear-notch samples, have
shown to be useful for in-vivo diagnosis of persistent BDV infection.

2. Serological tests

Antibody to BVDV can be detected in cattle sera by a standard VNT or by ELISA, using one of several published
methods or with commercial kits (e.g. Edwards, 1990). Serology is used to identify levels of herd immunity, for the
detection of the presence of PI animals in a herd, to assist with investigation of reproductive disease and possible
involvement of BVDV and to establish the serological status of bulls being used for semen collection and to
identify whether there has been a recent infection. ELISA for antibody in bulk milk samples can give a useful
indication of the BVD status of a herd (Niskanen, 1993). A high ELISA value (0.8 or more absorbance units) in an
unvaccinated herd indicates a high probability of the herd having been exposed to BVDV in the recent past, most
likely through one or more persistently viraemic animals being present. In contrast, a very low or negative value
(≤0.2) indicates that is unlikely that persistently viraemic animals are present. However, ELISA values are not
always a reliable indicator of the presence of PI animals on farms, due to differing husbandry (Zimmer
al., 2002), recent administration of vaccine and also due to the presence of viral antigen in bulk milk, which may
interfere with the antibody assay itself. Determination of the antibody status of a small number of young stock (9–
18 months) has also been utilised as an indicator of recent transmission of BVDV in the herd (Houe
et al., 1995), but this approach is also dependent on the degree of contact between different groups of animals in the herd and
the potential for exposure from neighbouring herds. VN tests are more frequently used for regulatory purposes
(e.g. testing of semen donors) while ELISAs (usually in the form of commercially prepared kits) are commonly
used for diagnostic applications. Whether ELISA or VNT, control positive and negative standard sera must be
included in every test. These should give results within predetermined limits for the test to be considered valid. In
the VNT, a ‘serum control’ to monitor sample toxicity should also be included for each test sample.

2.1. Virus neutralisation test

Selection of the virus strain to include in a VNT is very important. No single strain is likely to be ideal for
all circumstances, but in practice one should be selected that detects the highest proportion of
serological reactions in the local cattle population. Low levels of antibody to BVD type 2 virus may not
be detectable by a neutralisation test that uses type 1 strain of the virus, and vice versa (Fulton
et al., 1997). It is important that BVD type 1 and BVD type 2 be used in the test and not just the one that the
diagnostician thinks is present, as this can lead to under reporting. Because it makes the test easier to
read, most laboratories use highly cytopathic, laboratory-adapted strains of BVDV for VN tests. Two
widely used cytopathic strains are ‘Oregon C24V’ and ‘NADL’. However immune-labelling techniques
are now available that allow simple detection of the growth or neutralisation of non-cytopathic strains
where this is considered desirable, especially to support the inclusion of a locally relevant virus strain.
An outline protocol for a microtitre VN test is given below (Edwards, 1990):

2.1.1. Test procedure

i) The test sera are heat-inactivated for 30 minutes at 56°C.
ii) From a starting dilution of 1/4, serial twofold dilutions of the test sera are made in a cell-culture grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For each sample, three or four wells are used at each dilution depending on the degree of precision required. At each dilution of serum, for each sample one well is left without virus to monitor for evidence of sample toxicity that could mimic viral cytopathology or interfere with virus replication. Control positive and negative sera should also be included in each batch of tests.

iii) An equal volume (e.g. 50 μl) of a stock of cytopathic strain of BVDV containing 100 TCID$_{50}$ (50%) tissue culture infective dose is added to each well. A back titration of virus stock is also done in some spare wells to check the potency of the virus (acceptance limits 30–300 TCID$_{50}$).

iv) The plate is incubated for 1 hour at 37°C.

v) A flask of suitable cells (e.g. bovine turbinate, bovine testis) is trypsinized and the cell concentration is adjusted to 1.5 × 10$^5$/ml. 100 μl of the cell suspension is added to each well of the microtitre plate.

vi) The plate is incubated at 37°C for 4–5 days, either in a 5% CO$_2$ atmosphere or with the plate sealed.

vii) The wells are examined microscopically for CPE or fixed and stained by immunoperoxidase staining using an appropriate monoclonal antibody. The VN titre for each serum is the dilution at which the virus is neutralised in 50% of the wells. This can be calculated by the Spearman–Kärber or Reed Muench methods. A sero-negative animal will show no neutralisation at the lowest dilution (1/4), equivalent to a final dilution of 1/8. For accurate comparison of antibody titres, and particularly to demonstrate significant (more than fourfold) changes in titre, samples should be tested in parallel in the same test.

### 2.2. Enzyme-linked immunosorbent assay

Both indirect and blocking types of test can be used. A number of commercial kits are available. As with the virus neutralisation test, ELISAs configured using antigen from one genotype of BVD may not efficiently detect antibody induced by another genotype. Tests should therefore be selected for their ability to detect antibody to the spectrum of genotypes and strains circulating in the country where the test is to be performed.

The chief difficulty in setting up the test lies in the preparation of a viral antigen of sufficient potency. The virus must be grown under optimal culture conditions using a highly permissive cell type. Any serum used in the medium must not inhibit growth of BVDV. The optimal time for harvest should be determined experimentally for the individual culture system. The virus can be concentrated and purified by density gradient centrifugation. Alternatively, a potent antigen can be prepared by treatment of infected cell cultures with detergents, such as Nonidet P40, N-decanoyl-N-methylglucamine (Mega 10), Triton X-100 or 1-octylbeta-D-glucopyranoside (OGP). Some workers have used fixed, infected whole cells as antigen. In the future, increasing use may be made of artificial antigens manufactured by expressing specific viral genes in bacterial or eukaryotic systems. Such systems should be validated by testing sera specific to a wide range of different virus strains. In the future, this technology should enable the production of serological tests complementary to subunit or marker vaccines, thus enabling differentiation between vaccinated and naturally infected cattle. An example outline protocol for an indirect ELISA is given below (Edwards, 1990).

#### 2.2.1. Test procedure

i) Roller cultures of secondary calf testis cells with a high multiplicity of infection (about one), are inoculated with BVDV strain Oregon C24V, overlaid with serum-free medium and incubated for 24 hours at 37°C.

ii) The cells are scraped off and pelleted. The supernatant medium is discarded. The pellet is treated with two volumes of 2% OGP in PBS for 15 minutes at 4°C, and centrifuged to remove the cell debris. The supernatant antigen is stored in small aliquots at –70°C, or freeze-dried. Non-infected cells are processed in parallel to make a control antigen.

iii) The antigen is diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6. Alternate rows of an ELISA-grade microtitre plate are coated with virus and control antigens overnight at 4°C. The plates are then washed in PBS with 0.05% Tween 20 or Tween 80 (PBST) before use in the test.
iv) Test sera are diluted 1/50 in serum diluent (0.5 M NaCl; 0.01 M phosphate buffer; 0.05% Tween 20; 0.001 M ethylene diamine tetra-acetic acid; 1% polyvinyl pyrrolidone, pH 7.2) and added to virus- and control-coated wells for 1 hour at 37°C. The plates are then washed five times in PBST.

v) Rabbit anti-bovine IgG peroxidase conjugate is added at a predetermined dilution (in serum diluent) for 1 hour at 37°C, then the plates are again washed five times in PBST.

vi) A suitable enzyme substrate is added, such as hydrogen peroxide/tetramethyl benzidine. After colour development, the reaction is stopped with sulphuric acid and the absorbance is read on an ELISA plate reader. The value obtained with control antigen is subtracted from the test reaction to give a net absorbance value for each serum.

vii) It is recommended to convert net absorbance values to sample:positive ratio (or percentage positivity) by dividing net absorbance by the net absorbance on that test of a standard positive serum that has a net absorbance of about 1.0. This normalisation procedure leads to more consistent and reproducible results.

C. REQUIREMENTS FOR VACCINES

1. Background

BVDV vaccines are used primarily for disease control purposes although they can convey production advantages especially in intensively managed cattle such as in feedlots. In some countries where BVDV eradication is being undertaken, PI animals are removed and remaining cattle are vaccinated to maintain a high level of infection and prevent the generation of further PI animals. Vaccination to control BVDV infections can be challenging due in part to the antigenic variability of the virus and the occurrence of persistent infections that arise as a result of fetal infection. On-going maintenance of the virus in nature is predominantly sustained by PI animals that are the product of in-utero infection. The goal for a vaccine should be to prevent systemic viraemia and the virus crossing the placenta, if this is successfully achieved it is likely that the vaccine will prevent the wide range of other clinical manifestations, including reproductive, respiratory and enteric diseases and immunosuppression with its secondary sequelae. There are many different vaccines available in different countries. Traditionally, BVD vaccines fall into two classes: modified live virus or inactivated vaccines. Experimental recombinant subunit vaccines based on BVD viral glycoprotein E2 expressed with baculovirus or transgenic plants and BVDV E2 DNA vaccines have been described but few if any are in commercial production. They offer a future prospect of ‘marker vaccines’ when used in connection with a complementary serological test.

1.1. Characteristics of a target product profile

Traditionally, BVD vaccines fall into two classes: modified live or inactivated virus vaccines. The essential requirement for both types is to afford a high level of fetal infection. Many of the live vaccines have been based on a cytopathic strain of the virus which is considered to be unable to cross the placenta. However, it is important to ensure that the vaccine virus does not cause fetal infection. In general vaccination of breeding animals should be completed well before insemination to ensure optimal protection and avoid any risk of fetal infection. Live virus vaccine may also be immunosuppressive and precipitate other infections. On the other hand, modified live virus vaccines may only require a single dose. Use of a live product containing a cytopathic strain of BVDV may precipitate mucosal disease by superinfection of persistently viraemic animals. Properly formulated inactivated vaccines are very safe to use but, to obtain satisfactory levels of immunity, they usually require booster vaccinations, which may be inconvenient. A combined vaccination protocol using inactivated followed by live vaccine may reduce the risk of adverse reaction to the live strain. Whether live or inactivated, because of the propensity for antigenic variability, the vaccine should contain strains of BVDV that are closely matched to viruses found in the area in which they are used. For example, in countries where strains of BVDV type 2 are found, it is important for the vaccine to contain a suitable type 2 strain. For optimal immunity against type 1 strains, antigens from the dominant subtypes (e.g. 1a and 1b) should be included. Due to the need to customise vaccines for the most commonly encountered strains within a country or region, it is not feasible to produce a vaccine antigen bank that can be drawn upon globally.

Guidance for the production of veterinary vaccines is given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.
2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

For optimal efficacy, it is considered that there should be a close antigenic match between viruses included in a vaccine and those circulating in the target population. BVDV type 2 strains should be included as appropriate. Due to the regional variations in genotypes and subtypes of BVDV, many vaccines contain more than one strain of BVDV to give acceptable protection. A good appreciation of the antigenic characteristics of individual strains can be obtained by screening with panels of MAbs (Paton et al., 1995).

2.1.1. Biological characteristics of the master seed

Isolates of cytopathic virus are often mixed with the noncytopathic biotype. The separation and purification of the two biotypes from an initial mixed culture is important to maintain the expected characteristics of the seed and depends on several cycles of a limiting dilution technique for the noncytopathic virus, or plaque selection for the cytopathic virus. Purity of the cytopathic virus should be confirmed by at least one additional passage at limiting dilution. When isolates have been cloned, their identity and key antigenic characteristics should be confirmed. The identity of the seed virus should be confirmed by sequencing. Where there are multiple isolates included in the vaccine, each has to be prepared separately.

While retaining the desirable antigenic characteristics, the strains selected for the seed should not show any signs of disease when susceptible animals are vaccinated. Live attenuated vaccines should not be transmissible to unvaccinated 'in-contact' animals and should not be able to infect the fetus. Ideally seeds prepared for the production of inactivated vaccines should grow to high titre to minimise the need to concentrate the antigens and there should be a minimal amount of protein from the cell cultures incorporated into the final product. Master stocks for either live or inactivated vaccines should be prepared under a seed lot system involving master and working stocks that can be used for production in such a manner that the number of passages can be limited and minimise antigenic drift. While there are no absolute criteria for this purpose, as a general guide, the seed used for production should not be passaged more than 20 times beyond the master seed and the master seed should be of the lowest passage from the original isolate as is practical.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

It is crucial to ensure that all materials used in the preparation of the bulk antigens have been extensively screened to ensure freedom from extraneous agents. This should include master and working seeds, the cell cultures and all medium supplements such as bovine serum. It is particularly important to ensure that any serum used that is of bovine origin is free of both adventitious BVDV and antibodies against BVDV strains because low levels of either virus or antibody can mask the presence of the other. Materials and vaccine seeds should be tested for sterility and freedom from contamination with other agents, especially viruses as described in the chapter 1.1.6 and chapter 1.1.7.

2.1.3. Validation as a vaccine strain

All vaccines should pass standard tests for efficacy. Tests should include as a minimum the demonstration of a neutralising antibody response following vaccination, a reduction in virus shedding after challenge in vaccinated cattle and ideally a prevention of viraemia. Efficacy tests of BVD vaccines by assessing clinical parameters in non-pregnant cattle can be limited by the difficulty of consistently establishing clinical signs but, when employed, clinical parameters such as a reduction in the rectal temperature response and leukopenia should be monitored. Although it can be difficult by using virus isolation in cell culture to consistently demonstrate the low levels of viraemia associated with an acute infection, real-time PCR could be considered as an alternative method to establish the levels of circulating virus.

If a vaccine passes basic tests, the efficacy of vaccination should ultimately be measured by the capacity to prevent transplacental transmission. If there is a substantial reduction and ideally complete prevention of fetal infection, a vaccine would be expected to be highly effective in other situations (for example prevention of respiratory disease). A suitable challenge system can be established by intranasal inoculation of noncytopathic virus into pregnant cows between 60 and 90 days of gestation (Brownlie et al., 1995). Usually this system will reliably produce persistently viraemic offspring in non-immune cows. In countries where BVDV type 2 viruses

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are commonly encountered, efficacy in protecting against BVDV2 infections should be measured.

2.2. Method of manufacture

2.2.1. Procedure

Both cytopathic and noncytopathic biotypes will grow in a variety of cell cultures of bovine origin. Standard procedures may be used, with the expectation for harvesting noncytopathic virus on days 4–7 and cytopathic virus on days 2–4. The optimal yield of infectious virus will depend on several factors, including the cell culture, isolate used and the initial seeding rate of virus. These factors should be taken into consideration and virus replication kinetics investigated to establish the optimal conditions for large scale virus production. Whether a live or inactivated vaccine, the essential aim will be to produce a high-titred virus stock. This bulk antigen preparation can subsequently be prepared according to the type of vaccine being considered.

2.2.2. Requirements for ingredients

Most BVDV vaccines are grown in cell cultures of bovine origin that are frequently supplemented with medium components of animal origin. The material of greatest concern is bovine serum due to the potential for contamination with BVD viruses and antibodies to these viruses. These adventitious contaminants not only affect the efficiency of production but also may mask the presence of low levels of infectious BVDV that may have undesirable characteristics. In addition to the virus seeds, all materials should be tested for sterility and freedom from contamination with other agents, especially viruses as described in chapters 1.1.6 and 1.1.7. Further, materials of bovine or ovine origin should originate from a country with negligible risk for transmissible spongiform encephalopathies [TSEs] (see chapter 1.1.7).

2.2.3. In-process controls

In-process controls are part of the manufacturing process. Cultures should be inspected regularly to ensure that they remain free from contamination, and to monitor the health of the cells and the development or absence of CPE, as appropriate. While the basic requirement for efficacy is the capacity to induce an acceptable neutralising antibody response, during production, target concentrations of antigen required to achieve an acceptable response may be monitored indirectly by assessment of the quantity of infectious virus or antigen mass that is produced. Rapid diagnostic assays such as the ELISA are useful to monitor BVDV antigen production. Alternatively, the quality of a batch of antigen may be determined by titration of the quantity of infectious virus present, although this may underestimate the quantity of antigen. For inactivated vaccines, infectivity is evaluated before inactivation. For inactivated vaccines the inactivation kinetics should be established so that a suitable safety margin can be determined and incorporated into the routine production processes. At the end of production, in vitro cell culture assays should be undertaken to confirm that inactivation has been complete. These innocuity tests should include a sufficient number of passages and volume of inoculum to ensure that very low levels of infectious virus would be detected if present.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.7.

ii) Identity

Identity tests should demonstrate that no other strain of BVDV is present when several strains are propagated in a facility producing multivalent vaccines.

iii) Safety

Safety tests shall consist of detecting any abnormal local or systemic adverse reactions to the vaccine by all vaccination route(s). Batch-to-batch safety tests are required unless safety of the product is demonstrated and APPROVED in the registration dossier and production is consistent with that described in chapter 1.1.6. The safety test is different to the inocuity test (see above).

Live vaccines must either be demonstrated to be safe in pregnant cattle (i.e. no transmission to the fetus), or should be licensed with a warning not to use them in
Chapter 2.4.8. – Bovine viral diarrhoea

pregnant animals. Live vaccines containing cytopathic strains should have an appropriate
warning of the risk of inducing mucosal disease in PI cattle.

iv) Batch potency
BVD vaccines must be demonstrated to produce adequate immune responses, when used
in their final formulation according to the manufacturer’s published instructions. The
minimum quantity of infectious virus and/or antigen required to produce an acceptable
immune response should be determined. In-vitro assays should be used to monitor
individual batches during production.

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process
For registration of a vaccine, all relevant details concerning manufacture of the vaccine and
quality control testing should be submitted to the relevant authorities. Unless otherwise
specified by the authorities, information should be provided from three consecutive vaccine
batches with a volume not less than 1/3 of the typical industrial batch volume.

There is no standard method for the manufacture of a BVD vaccine, but conventional laboratory
techniques with stationary, rolled or suspension (micro-carriers) cell cultures may be used.
Inactivated vaccines can be prepared by conventional methods, such as binary ethylenimine or
beta-propiolactone inactivation (Park & Bolin, 1987). A variety of adjuvants may be used.

2.3.2. Safety requirements
In-vivo tests should be undertaken using a single dose, overdose (for live vaccines only) and
repeat doses (taking into account the maximum number of doses for primary vaccination and, if
appropriate, the first revaccination/booster vaccination) and contain the maximum permitted
antigen load and, depending on the formulation of the vaccine, the maximum number of vaccine
strains.

i) Target and non-target animal safety
The safety of the final product formulation of both live and inactivated vaccines should be
assessed in susceptible young calves that are free of maternally derived antibodies and in
pregnant cattle. They should be checked for any local reactions following administration,
and, in pregnant cattle, for any effects on the unborn calf. Live attenuated vaccines may
contribute to immunosuppression that might increase mortality. It may also contribute to
the development of mucosal disease in PI animals that is an animal welfare concern.
Therefore vaccination of PI animals with live attenuated vaccines containing cytopathic
BVDV should be avoided. Live attenuated vaccines must not be capable of being
transmitted to other unvaccinated animals that are in close contact.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations
Virus seeds that have been passaged at least up to and preferably beyond the passage
limit specified for the seed should be inoculated into young calves to confirm that there is
no evidence of disease. If a live attenuated vaccine has been registered for use in
pregnant animals, reversion to virulence tests should also include pregnant animals. Live
attenuated vaccines should not be transmissible to unvaccinated ‘in-contact’ animals.

iii) Precautions (hazards)
BVDV is not considered to be a human health hazard. Standard good microbiological
practice should be adequate for handling the virus in the laboratory. A live virus vaccine
should be identified as harmless for people administering the product however adjuvants
included in either live or inactivated vaccines may cause injury to people. Manufacturers
should provide adequate warnings that medical advice should be sought in the case of
self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with
warnings included on the product label/leaflet so that the vaccinator is aware of any
danger.

2.3.3. Efficacy requirements
The potency of the vaccine should be determined by inoculation into seronegative and virus
negative calves, followed by monitoring of the antibody response. Antigen content can be
assayed by ELISA and adjusted as required to a standard level for the particular vaccine.
Standardised assay protocols applicable to all vaccines do not exist. Live vaccine batches may be assayed by infectivity titration. Each production batch of vaccine should undergo potency and safety testing as batch release criteria. BVD vaccines must be demonstrated to produce adequate immune responses, as outlined above, when used in their final formulation according to the manufacturer’s published instructions.

### 2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

To date, there are no commercially available vaccines for BVDV that support use of a true DIVA strategy. Experimental subunit vaccines based on baculovirus-expressed BVD viral glycoprotein E2 have been described but are not available commercially. They offer a future prospect of ‘marker vaccines’ when used in connection with a complementary serological test. Experimental BVDV E2 DNA vaccines and BVDV E2 subunit vaccines expressed using transgenic plants and alphavirus replicon have also been described.

### 2.3.5. Duration of immunity

There are few published data on the duration of antibody following vaccination with a commercial product. Protocols for their use usually recommend a primary course of two inoculations and boosters at yearly intervals. Only limited data are available on the antibody levels that correlate with protection against respiratory infections (Bolin & Ridpath, 1995; Howard et al., 1989) or in-utero infection (Brownlie et al., 1995). However, there are many different commercial formulations and these involve a range of adjuvants that may support different periods of efficacy. Consequently, duration of immunity data must be generated separately for each commercially available product by undertaking challenge tests at the end of the period for which immunity has been claimed.

### 2.3.6. Stability

There are no accepted guidelines for the stability of BVD vaccines, but it can be assumed that attenuated virus vaccine (freeze-dried) should remain potent for at least 1 year if kept at 4°C. Inactivated virus vaccine could have a longer shelf life at 4°C. Lower temperatures could prolong shelf life for either type, but adjuvants in killed vaccine may preclude this. Bulk antigens that have not been formulated into finished vaccine can be reliably stored frozen at low temperatures but the antigen quality should be monitored with in vitro assays prior to incorporation into a batch of vaccine.

### REFERENCES


Chapter 2.4.8. – Bovine viral diarrhoea


* NB: There are OIE Reference Laboratories for Bovine viral diarrhoea (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for bovine viral diarrhoea.
CHAPTER 2.5.9.

EQUINE RHINOPNEUMONITIS
(EQUINE HERPESVIRUS 1 AND 4)

SUMMARY

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

Primary infection by either EHV-1 or EHV-4 is characterised by upper respiratory tract disease of varying severity that is related to the age and immunological status of the infected animal. EHV-1 also causes the more serious complications of abortion, perinatal foal death, or paralytic neurological disease (equine herpesvirus myeloencephalopathy). EHV-4 has been associated with rare sporadic cases of abortion, but not the large outbreaks associated with EHV-1. Like other herpesviruses, EHV-1 and 4 induce long-lasting latent infections and can reactivate following stress or pregnancy. Most horses will be infected during their lifetime, detection of viral DNA or anti-EHV antibodies should therefore be interpreted with care. Infections by EHV-1 in particular are capable of progression beyond the respiratory mucosa to cause the more serious disease manifestations of abortion, perinatal foal death, or neurological dysfunction.

Identification of the agent: The standard method of identification of EHV-1 and EHV-4 the herpesviral agents of ER continues to be laboratory isolation of the virus from appropriate clinical or necropsy material is polymerase chain reaction (PCR), followed by seroconfirmation of its identity. laboratory isolation of the virus in cell culture. Positive identification of viral isolates as EHV-1 or EHV-4 can be achieved by type-specific PCR. Viruses can be isolated in equine cell culture from nasal or nasopharyngeal swab extracts samples taken from horses during the febrile stage of respiratory tract infection, from liver, lung, spleen, or thymus of aborted fetuses and early foal deaths, and from the leukocyte fraction of the blood of animals with acute EHV-1 disease. Unlike EHV-4, EHV-1 will also grow on other cell types such as RK-13s and this property can be used to distinguish between the two viruses. Positive identification of viral isolates as EHV-1 or EHV-4 can be achieved by immunofluorescence with type-specific monoclonal antibodies.

A rapid presumptive diagnosis of EHV-1/4 induced rhinopneumonitis abortion can be achieved by direct immunofluorescent detection of viral antigen in cryostat sections of tissues from aborted fetuses, using conjugated polyclonal antiserum.

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

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

Serological tests: Because Most horses will possess some level of antibody to EHV-1/4, the demonstration of specific antibody in the serum collected from a single blood sample is therefore not sufficient for a positive diagnosis of recent infection acute ER. Paired, acute and convalescent sera from animals suspected of being infected with EHV-1 or EHV-4 should be tested for a four-fold or greater rise in virus-specific antibody titre by either virus neutralisation (VN) or enzyme-linked immunosorbent assay, or complement fixation (CF). Neither of these assays is type-specific but both have proven useful for diagnostic purposes as VN and CF antibodies are relatively short-
A. INTRODUCTION

Equine rhinopneumonitis (ER) is an historically derived term that describes a constellation of several disease entities of horses that may include respiratory disease, abortion, neonatal foal pneumonitis, or myeloencephalopathy (Allen & Bryans, 1986; Allen et al., 1999; Bryans & Allen, 1988; Crabb & Studdert, 1995).

The disease has been recognised for over 60 years as a threat to the international horse industry, and is caused by either of two members of the Herpesviridae family, equid herpesvirus-1 and -4 (EHV-1 and EHV-4). EHV-1 and EHV-4 are closely related alphaherpesviruses of horses with nucleotide sequence identity within individual homologous genes ranging from 55% to 84%, and amino acid sequence identity from 55% to 96% (Telford et al., 1992; 1998). The two herpesviruses are enzootic in all countries in which large populations of horses are maintained as part of the cultural tradition or agricultural economy. There is no recorded evidence that the two herpesviruses of ER pose any health risks to humans working with the agents. Infection with EHV-1 is listed by the OIE.

ER is highly contagious among susceptible horses, with viral transmission to cohort animals occurring by inhalation of aerosols of virus-laden respiratory secretions. Aborted tissue from infected mares can contain extremely high levels of live virus and represents a major source of infection. Extensive use of vaccines has not eliminated EHV infections, and the world-wide annual financial burden from these equine pathogens is immense.

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

Neurological disease, also known equine herpesvirus myeloencephalopathy, remains an infrequent but serious complication of EHV-1 infection. A single mutation in the DNA polymerase gene (ORF30) has been associated with increased risk of neurological disease, however strains without this marker can also cause paralysis (Nugent et al., 2006; Goodman et al., 2007). Strain typing techniques have been employed to identify viruses carrying the neuropathic marker, and it can be useful to be aware of an increased risk of neurological complications. However, for practical purposes strain-typing does not influence the requirement for strict management practices during an outbreak of EHV-1.
Both EHV-1 and EHV-4 are because ER is a highly contagious disease with viruses and the former has the potential for causing explosive outbreaks with high mortality from abortigenic or neurological sequelae. Rapid diagnostic methods are therefore useful for managing the disease. Polymerase chain reaction (PCR) and quantitative PCR (qPCR) assays are widely used by diagnostic laboratories and are both rapid and sensitive. qPCR assays that allow simultaneous testing for EHV-1 and EHV-4 have also been developed. Virus isolation can also be useful, particularly for the detection of viraemia. Immunohistochemical or immunofluorescent approaches can be extremely useful for rapid diagnosis of EHV-induced abortion from fresh or embedded tissue and are relatively straightforward. Several other techniques based on enzyme-linked immunosorbent assay (ELISA) or nucleic acid hybridisation probes have also been described, however their use is often restricted to specialised laboratories and they are not included here. Important. Although several and innovative diagnostic techniques based on enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), immunohistochemical staining with peroxidase, or nucleic acid hybridisation probes have been recently described, their use is often restricted to specialised reference laboratories, and thus the method of choice for diagnosis of ER by diagnostic virology laboratories handling many routine samples continues to be the traditional methodology of cell culture isolation followed by sero-identification of the isolated viruses. Successful laboratory isolation of EHV-1/4 depends on strict adherence to proper methods for both sample collection and laboratory processing.

### Table 1. Test methods available and their purpose

<table>
<thead>
<tr>
<th>Method</th>
<th>Population freedom from infection</th>
<th>Individual animal freedom from infection prior to movement</th>
<th>Contribution to eradication policies</th>
<th>Confirmation of clinical cases</th>
<th>Prevalence of infection - surveillance</th>
<th>Immune status in individual animals or populations post-vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus isolation</td>
<td>=</td>
<td>+++</td>
<td>=</td>
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<td>PCR</td>
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<td>+++</td>
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<td>AGID</td>
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<td>CFT</td>
<td>=</td>
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<td>+++</td>
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</tr>
</tbody>
</table>

Detection of immune response

| VN | ++ | +++ | ++ | +++ | +++ | +++ |
| ELISA | + | = | = | +++ | = | = |

**Key:** +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose. Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable. PCR = polymerase chain reaction; AGID = agar gel immunodiffusion; CFT = complement fixation test; VN = virus neutralisation; ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent (Allen et al., 2004)

### 1.1. Collection and preparation of samples

Samples of nasopharyngeal exudate for virus isolation. Nasal/nasopharyngeal swabs: swab extract can be used for DNA extraction and subsequent virus detection by PCR using one of a variety of published techniques or commercially available kits (see below). Virus isolation can also be attempted from the swab extracts. To increase the chances of isolating live virus, swabs are best obtained from horses during the very early, febrile stages of the respiratory disease, and are collected via the nares.
by swabbing the nasopharyngeal area with a 5 × 5 cm gauze sponge attached to the end of a 50 cm length of flexible, stainless steel wire encased in latex rubber tubing. A guarded uterine swab device can also be used sampling the area with a swab of an appropriate size and length for horses. After collection, the swab should be removed from the wire and transported immediately to the virology laboratory in 3 ml of cold (not frozen) fluid transport medium (e.g. PBS or serum-free MEM [minimal essential medium] with antibiotics. Virus infectivity can be prolonged by the addition of bovine serum albumin, fetal calf serum or gelatine to 0.1% (w/v).

Virological examination of fetal Tissue samples: total DNA can be extracted using a number of commercially available kits and used in PCR to detect viral DNA (described below Section B.1.2.i). Virus isolation from fetal tissues from suspect cases of EHV-1 abortion is most successful when performed on aseptically collected samples of liver, lung, thymus and spleen. The tissue samples should be transported to the laboratory and held at 4°C until inoculated into tissue culture. Samples that cannot be processed within a few hours should be stored at -70°C. In ante mortem cases of EHV-1 neurological disease, the virus can often be isolated from the leukocyte fraction of the blood of acutely infected horses or, less often, from the nasopharynx of the affected animal or cohort animals. For attempts at virus isolation from blood leukocytes, a 20 ml sample of sterile blood, collected in citrate, or heparin anticoagulant (EDTA [ethylene diamine tetra-acetic acid] should not be used as it can destroy the cell cultures). The samples should be transported without delay to the laboratory on ice, but not frozen. Although the Virus has, on occasion, been isolated from post-mortem cases of EHV-1 neurological disease by culture of samples of brain and spinal cord, such attempts to isolate virus are often unsuccessful; however, they may be useful for PCR examination – pathological examination. Tissue samples should be transported to the laboratory and held at 4°C until inoculated into tissue culture. Samples that cannot be processed within a few hours should be stored at -70°C.

Blood: for attempts at virus isolation from blood leukocytes, take a 20 ml sample of sterile blood, collected in citrate, or heparin anticoagulant. EDTA [ethylene diamine tetra-acetic acid] should not be used as it can destroy cell cultures. The samples should be transported without delay to the laboratory on ice, but not frozen.

1.2. Virus detection by polymerase chain reaction

PCR has become the primary diagnostic method for the detection of EHV-1 and -4 in clinical specimens, paraffin-embedded archival tissue, or inoculated cell cultures (Borchers & Slater, 1993; Lawrence et al., 1994; O’Keefe et al., 1994; Varrasso et al., 2001). A variety of type-specific PCR primers have been designed to distinguish between the presence of EHV-1 and EHV-4. The correlation between PCR and virus isolation techniques for diagnosis of EHV-1 or EHV-4 is high (Varrasso et al., 2001). Diagnosis by PCR is rapid, sensitive, and does not depend on the presence of infectious virus in the clinical sample.

For diagnosis of active infection by EHV, PCR methods are most reliable with tissue samples from aborted fetuses and placental tissue or from nasopharyngeal swabs of foals and yearlings. They are useful in explosive epizootics of abortion or respiratory tract disease in which a rapid identification of the virus is critical for guiding management strategies. PCR examinations of spinal cord and brain tissue, as well as PBMC, are important in seeking a diagnosis on a horse with neurological signs. However, the interpretation of the amplification by PCR of genomic fragments of EHV-1 or EHV-4 in lymph nodes or trigeminal ganglia from adult horses is complicated by the high prevalence of latent EHV-1 and EHV-4 DNA in such tissues (Welch et al., 1992).

PCR technology is evolving rapidly and a variety of assays have been published. The OIE reference laboratories have used quantitative PCR assays such as those targeting the major glycoproteins to distinguish between EHV-1 and 4. PCR protocols have been developed that can differentiate between EHV-1 strains carrying the ORF30 neuropathic marker, using both restriction enzyme digestion of PCR products (Fritsche & Borchers, 2011) or by quantitative PCR (Allen et al., 2007; Smith et al., 2012). Methods have also been developed to type strains for epidemiological purposes, based on the ORF58 gene (Nugent et al. 2007, Smith et al. 2015). The OIE reference laboratories employ in-house methods for strain typing, however these protocols have not been validated between different laboratories at an international level.

A simple nested PCR procedure can be used to distinguish between EHV-1 and EHV-4. A sensitive protocol suitable for clinical or pathological specimens (nasal secretions, blood leukocytes, brain and spinal cord, fetal tissues, etc.) is described here (Borchers & Slater, 1993).

1.2.1. Test procedure
i) Prepare template DNA from test specimens: following sample homogenisation and lysis in the presence of a chaotropic salt, nucleic acids bind selectively to silica or cationic resin substrates. Substrate-bound nucleic acids are purified in a series of rapid wash steps followed by recovery with low-salt elution. The reagents for performing such steps for rapid nucleic acid isolation are available in kit format from a number of commercial sources.

ii) Nested primer sequences specific for EHV-1

BS-1-P1 = 5’-TCT-ACC-CCT-ACG-ACT-CCT-TC-3’ (917–936)
gB1-R-2 = 5’-ACG-CTG-TCG-ATG-TCG-TAA-AAC-CTG-AGA-G-3’ (2390–2363)
BS-1-P3 = 5’-CTT-TAG-CGG-TGA-TGT-GGA-AT-3’ (1377–1396)
gB1-R-a = 5’-AAG-TAG-CGC-TTC-TGA-TTG-AGG-3’ (2147–2127)

iii) Nested primer sequences specific for EHV-4

BS-4-P1 = 5’-TCT-ATT-GAG-TTT-GCT-ATG-CT-3’ (1705–1724)
BS-4-P2 = 5’-TCC-TGG TTG-TTA-TTG-GGT-AT-3’ (2656–2637)
BS-4-P3 = 5’-TGT-TTC-CGC-CAC-TCT-TGA-CG-3’ (1857–1876)
BS-4-P4 = 5’-ACT-GCC-TCT-CCC-ACC-TTA-CC-3’ (2456–2437)

iv) PCR conditions for first stage amplification: specimen template DNA (1–2 µg in 2 µl) is added to a PCR mixture (total volume of 50 µl) containing 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, 0.1% Triton X-100), 200 µM of each deoxynucleotide triphosphate (dNTP), 2.5 mM MgCl2, 2.0 µM of each outer primer (BS-1-P1 and gB1-R-2 for EHV-1 detection and, in a separate reaction mixture, BS-4-P1 and BS-4-P2 for EHV-4 detection) and 0.5 u Taq DNA polymerase. Cycling parameters are: initial denaturation at 94°C for 4 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds; with a final extension at 72°C for 10 minutes. Separate reaction mixtures containing either known viral DNA or no DNA (water) should be prepared and amplified as positive and negative controls.

v) PCR conditions for second stage (nested) amplification: two µl of a 1/10 dilution of the first amplification product is added to a fresh PCR mixture (total volume of 50 µl) containing 1 × PCR buffer, 200 µM of each dNTP, 2.5 mM MgCl2, 2.0 µM of each nested primer (BS-1-P3 and gB1-R-a for EHV-1 detection and, in a separate reaction mixture, BS-4-P3 and BS-4-P4 for EHV-4 detection) and 0.5 u Taq DNA polymerase. Cycling parameters are: initial denaturation at 94°C for 4 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute; with a final extension at 72°C for 10 minutes.

vi) Gel analysis of amplified products: 10 µl of each final amplified product, including controls, is mixed with 2 µl of 6 × loading dye and electrophoresed on a 1.5% agarose gel in Tris/acetate or Tris-Borate running buffer, along with a 100 base pairs (bp) DNA ladder. Amplified products are detected using a suitable DNA stain, of either 770 bp for EHV-1 or 580 bp for EHV-4.

1.3. Virus isolation

For efficient primary isolation of EHV-4 from horses with respiratory disease, equine-derived cell cultures must be used. Both EHV-1 and EHV-4 may be isolated from nasopharyngeal samples using primary equine fetal kidney cells or cell strains of equine fibroblasts derived from dermal (E-Derm) or lung tissue. EHV-1 can be isolated on other cell types, as will be discussed later. The nasopharyngeal swab and its accompanying 3 ml of transport medium are transferred into the barrel of a sterile 10 ml syringe. Using the syringe plunger, the fluid is squeezed from the swab into a sterile tube. A portion of the expressed fluid can be then filtered through a sterile, 0.45 µm membrane syringe filter unit into a second sterile tube. Filtration will decrease if heavy bacterial contamination is expected, but this may also lower virus titre. Recently prepared cell monolayers in 25 cm² tissue culture flasks are inoculated with 0.5 ml of the filtered, as well as the unfiltered, nasopharyngeal swab extract. Cell monolayers in multiwell plates incubated in a 5% CO₂ environment may also be used. Virus is allowed to attach by incubating the inoculated monolayers at 37°C on a platform rocker for 1.5–2 hours. Monolayers of uninoculated control cells should be incubated in parallel with sterile transport medium only.
At the end of the attachment period, the inocula are removed and the monolayers are rinsed twice with phosphate buffered saline (PBS) to remove virus-neutralising antibody that may be present in the nasopharyngeal secretions. After addition of 5 ml of supplemented maintenance medium (MEM containing 2% fetal calf serum [FCS] and twice the standard concentrations of antibiotics [penicillin, streptomycin, gentamicin, and amphotericin B]), the flasks are incubated at 37°C. The use of positive control virus samples to validate the isolation procedure carries the risk that this may lead to eventual contamination of diagnostic specimens. This risk can be minimised by using routine precautions and good laboratory technique, including the use of biosafety cabinets, inoculating positive controls after the diagnostic specimens, decontaminating the surfaces of the hood while the inocum is adsorbing and using a positive control of relatively low titre. Inoculated flasks should be inspected daily by microscopy for the appearance of characteristic herpesvirus cytopathic effect (CPE) (focal rounding, increase in refractility, and detachment of cells). Cultures exhibiting no evidence of viral CPE after 1 week of incubation should be blind-passaged into freshly prepared monolayers of cells, using small aliquots of both media and cells as the inoculum. Further blind passage is usually not productive.

A number of cell types may be used for isolation of EHV-1 from the tissues of aborted fetuses or from post-mortem cases of neurological disease (e.g. rabbit kidney [RK-13 [AATC–CCL37]], baby hamster kidney [BHK-21], Madin–Darby bovine kidney [MDBK], pig kidney [PK-15], etc.). It can be useful to inoculate samples into both non-equine and equine cells in parallel to distinguish between EHV-1 and EHV-4, which causes but equine-derived cell cultures are most sensitive and must be used if the infrequent cases of EHV-4 abortion are to be detected. Around 10% (w/v) pooled tissue homogenates of liver, lung, thymus, and spleen (from aborted fetuses) or of CNS tissue (from cases of neurological disease) are used for virus isolation. These are prepared by first mincing small samples of tissue into 1 mm cubes in a sterile Petri dish with dissecting scissors, followed by macerating the tissue cubes further in serum-free culture medium with antibiotics using a homogeniser or mechanical tissue grinder (e.g. Ten-Broeck or Stomacher). After centrifugation at 1200 g for 10 minutes, the supernatant is removed and 0.5 ml is inoculated into duplicate cell monolayers in 25 cm² tissue culture flasks. Following incubation of the inoculated cells at 37°C for 1.5–2 hours, the inocula are removed and the monolayers are rinsed twice with PBS or media. After addition of 5 ml of supplemented maintenance medium, the flask is incubated at 37°C for up to 1 week or until viral CPE is observed.

Culture of peripheral blood leukocytes (PBMC) for the presence of EHV-1 can be attempted from horses during the early stages of myeloencephalomyelopathy or neurological disease. Buffy coats may be prepared from unclotted blood by centrifugation at 600 g for 15 minutes, and the buffy coat is taken after the plasma has been carefully removed. The buffy coat is then layered onto Ficol 1,077 g/ml commercially available) and centrifuged at 400 g for 20 minutes and the leukocyte-rich interface is then layered onto Ficol 1,077 and centrifuged in the same way. The PBMC interface (without most granulocytes) is washed twice in PBS (300 g for 10 minutes) and resuspended in 1 ml of MEM containing 2% FCS. Then, 0.5 ml As a quicker alternative method, PBMC may be collected by centrifugation directly from plasma. An aliquot of the rinsed cell suspension is added to each of the duplicate monolayers of equine fibroblast, equine fetal or RK-13 cell monolayers in 25 cm² flasks containing 8–10 ml freshly added maintenance medium. The flasks are incubated at 37°C for 7 days; either with or without removal of the inoculum. If PBMCs are not removed prior to incubation, CPE may be difficult to detect in the presence of the massive inoculum of leukocytes: each flask of cells is freeze-thawed after 7 days of incubation and the contents centrifuged at 300 g for 10 minutes. Finally, 0.5 ml of the cell-free, culture medium supernatant is transferred to freshly made cell monolayers that are just subconfluent. These are incubated and observed for viral CPE for at least 5–6 days before discarding as negative.

Virus isolates from positive cultures should be submitted to an OIE reference laboratory to maintain a geographically diverse archive. Further strain characterisation for surveillance purposes or detection of the neurological marker can be completed at some laboratories.

c. Seroconfirmation of virus identity

The basis for identification of any herpesvirus isolate recovered from specimens submitted from suspected cases of ER is its immunoreactivity with specific antisera. Specific identification of an isolate as EHV-1 or EHV-4 can be quickly and simply accomplished by immunofluorescent (FA) detection of viral antigen in the infected cell culture using type-specific monoclonal antibodies (MAbs), which are available from OIE Reference Laboratories for equine rhinopneumonitis. The test, which is type-specific and accurate, can be performed on a small aliquot of infected cells from the same container inoculated with clinical or post-mortem material. An isolate made in a laboratory that lacks MAbs or FA capability can be confirmed as EHV-1/4 by virus neutralization using a virus-specific polyclonal antiserum or by the PCR (see section B.1.f).

Cell monolayers infected with the isolate are removed from the culture medium and resuspended in 0.5 ml of PBS. 50 µl of the cell
1.4. Virus detection by direct immunofluorescence

Direct immunofluorescent detection of EHV-4 antigens in samples of post-mortem tissues collected from aborted equine fetuses and placentas provides an indispensable method to the veterinary diagnostic laboratory for making a rapid preliminary diagnosis of herpesvirus abortion (Gunn, 1992). Side-by-side comparisons of the immunofluorescent and cell culture isolation techniques on more than 100 cases of equine abortion have provided evidence that the diagnostic reliability of direct immunofluorescent staining of fetal tissues obtained at necropsy approaches that of virus isolation attempts from the same tissues.

In the United States of America (USA), specific and potent polyclonal antiserum to EHV-1, prepared in swine and conjugated with FITC, is provided to veterinary diagnostic laboratories for this purpose by the National Veterinary Services Laboratories of the United States Department of Agriculture (USDA). The antiserum cross-reacts with EHV-4 and hence is not useful for serotyping, however, this can be conducted on any virus positive specimens by PCR.

Freshly dissected samples (5 × 5 mm pieces) of fetal tissue (lung, liver, thymus, and spleen) are frozen, sectioned on a cryostat at −20°C, mounted on to microscope slides, and fixed with 100% acetone. After air-drying, the sections are incubated at 37°C in a humid atmosphere for 30 minutes with an appropriate dilution of the conjugated swine antibody to EHV-1. Unreacted antibody is removed by two washes with PBS, and the tissue sections are then covered with aqueous mounting media and a cover-slip, and examined for fluorescent cells indicating the presence of EHV antigen. Each test should include a positive and negative control consisting of sections from known EHV-1 infected and uninfected fetal tissue.

1.5. Virus detection by immunoperoxidase staining

Enzyme—Immunohistochemical (IH) staining methods, such as immunoperoxidase, have been developed recently as procedures for detecting EHV-1 antigen in paraffin-embedded fixed tissues of aborted equine fetuses, placentae, or neurologically affected horses (Schultheiss et al., 1993; Whitwell et al., 1992). Such ancillary IH techniques for antigen detection may facilitate identification of the virus in; can be used as an alternative to immunofluorescence described above and can also be readily applied to archival tissue samples or in clinical cases in which traditional laboratory methods for EHV-1 detection have been unsuccessful. Immunoenzymatic immunohistochemical staining for EHV-1 is particularly useful for the simultaneous evaluation of morphological lesions and the identification of the virus infectious agent. Immunoperoxidase staining for EHV-1/4 may also be carried out on infected cell monolayers (van Maanen et al., 2000). Adequate controls must be included with each immunoperoxidase test run for evaluation of both the method specificity and antibody specificity. In one OIE reference laboratory, this method is used routinely for frozen or fixed tissue, using rabbit polyclonal sera raised against EHV-1. This staining method is not type-specific and therefore needs to be combined with virus isolation or PCR to discriminate between EHV-1 and 4, however it provides a useful method for rapid diagnosis of EHV-induced abortion.

f) Virus detection by polymerase chain reaction

The PCR can be used for rapid amplification and diagnostic detection of nucleic acids of EHV-1 and 4 in clinical specimens, paraffin-embedded archival tissue, or inoculated cell cultures (Borchers & Slater, 1993; Lawrence et al., 1991; O’Keefe et al., 1994; Varrasso et al., 2001; Wagner et al., 1992). A variety of type-specific PCR primers have been designed to distinguish between the presence of EHV-1 and EHV-4. The correlation between PCR and virus isolation techniques for diagnosis of EHV-1 or EHV-4 is high (Varrasso et al., 2001). Diagnosis of ER by PCR is rapid, sensitive, and does not depend on the presence of infectious virus in the clinical sample. It now forms an integral part of a range of diagnostic tests currently available for ER, each with its own advantages and limitations.
For diagnosis of active infection by EHV, PCR methods are most reliable with samples from aborted fetuses or from nasopharyngeal swabs and peripheral blood leukocytes of foals and yearlings; they are most useful in explosive epizootics of abortion or respiratory tract disease in which a rapid identification of the virus is critical for guiding management strategies. PCR examinations of spinal cord and brain tissue, as well as PBMC, are important in seeking a diagnosis on a horse with neurological signs. However, the interpretation of the amplification by PCR of genomic fragments of EHV-1 or EHV-4 in lymph nodes or trigeminal ganglia from adult horses is complicated by the high prevalence of latent EHV-1 and EHV-4 DNA in such tissues (Welch et al., 1992).

A simple multiplex PCR assay for simultaneous detection of both EHV-1 and EHV-4 has been described (Wagner et al., 1992). A more sensitive protocol for nested PCR detection of EHV-1 or EHV-4 in clinical or pathological specimens (nasal secretions, blood leukocytes, brain and spinal cord, fetal tissues, etc.) is described here (Borchers & Slater, 1993). This procedure has been used successfully; however, the technology in this area is changing rapidly and other simpler more sensitive techniques are becoming available.

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

ii) Nested primer sequences specific for EHV-1 (based on those described in Borchers & Slater, 1993):

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS-1-P1</td>
<td>5’-TCT-ACC-CCT-ACG-3’</td>
<td>917–936</td>
</tr>
<tr>
<td>BS-1-P2</td>
<td>5’-ACG-CTG-TCG-ATG-3’</td>
<td>2390–2363</td>
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<td>BS-1-P3</td>
<td>5’-CTT-TAG-CGG-TGA-3’</td>
<td>1377–1396</td>
</tr>
<tr>
<td>BS-1-P4</td>
<td>5’-AAC-TAG-CCC-ACC-3’</td>
<td>2147–2127</td>
</tr>
</tbody>
</table>

iii) Nested primer sequences specific for EHV-4 (Borchers & Slater, 1993):

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS-4-P1</td>
<td>5’-TCT-ATT-GAG-TTT-3’</td>
<td>1705–1724</td>
</tr>
<tr>
<td>BS-4-P2</td>
<td>5’-TCC-TGG-TTG-TTA-3’</td>
<td>2656–2637</td>
</tr>
<tr>
<td>BS-4-P3</td>
<td>5’-TGT-TTC-CGC-CAC-3’</td>
<td>1857–1876</td>
</tr>
<tr>
<td>BS-4-P4</td>
<td>5’-ACT-GCC-TCT-CCC-3’</td>
<td>2456–2437</td>
</tr>
</tbody>
</table>

iv) PCR conditions for first stage amplification: Specimen template DNA (1–2 µg in 2 µl) is added to a PCR mixture (total volume of 50 µl) containing 1× PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, 0.1% Triton X-100), 200 µM of each deoxynucleotide triphosphate (dNTP), 2.5 mM MgCl₂, 2.0 µM of each outer primer (BS-1-P1 and gB1-R-2 for EHV-1 detection, and, in a separate reaction mixture, BS-4-P1 and BS-4-P2 for EHV-4 detection) and 0.5 u Taq DNA polymerase. Cycling parameters are: initial denaturation at 94°C for 4 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds; with a final extension at 72°C for 10 minutes. Separate reaction mixtures containing either known viral DNA or no DNA (water) should be prepared and amplified as positive and negative controls.

v) PCR conditions for second stage (nested) amplification: Two µl of a 1/10 dilution of the first amplification product is added to a fresh PCR mixture (total volume of 50 µl) containing 1× PCR buffer, 200 µM of each dNTP, 2.5 mM MgCl₂, 2.0 µM of each nested primer (BS-1-P3 and gB1-R-a for EHV-1 detection, and, in a separate reaction mixture, BS-4-P3 and BS-4-P4 for EHV-4 detection) and 0.5 u Taq DNA polymerase. Cycling parameters are: initial denaturation at 94°C for 4 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds; with a final extension at 72°C for 10 minutes.

vi) Gel analysis of amplified products: 10 µl of each final amplified product, including controls, is mixed with 2 µl of 6 × loading dye and electrophoresed on a 1.5% agarose gel in Tris/acetate or Tris-Borate running buffer, along with a 100 base pairs (bp) DNA ladder. The gel is stained with ethidium bromide and viewed by UV transillumination for amplified products of either 770 bp for EHV-1 or 580 bp for EHV-4.
1.6. Histopathology

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

2. Serological tests

Because of the ubiquity of the viral agents of ER and the high seroprevalence among horses in most parts of the world, the demonstration of a negative antibody titre to EHV-1/4 by serological testing of horses designated for export is not part of present veterinary regulations that seek to prevent international spread of infectious diseases of horses. Serodiagnosis of ER, EHV-1 and 4 are endemic in most parts of the World and seroprevalence is high, however serological testing of paired sera can be useful for diagnosis of ER in horses. A positive diagnosis is based on the demonstration of significant increases (four-fold or greater) in antibody titres in paired sera taken during the acute and convalescent stages of the disease. The results of tests performed on sera from a single collection date are, in most cases, impossible to interpret with any degree of confidence. The initial (acute phase) serum sample should be taken as soon as possible after the onset of clinical signs, and the second (convalescent phase) serum sample should be taken 2-4 weeks later.

‘Acute phase’ sera from mares after abortion or from horses with EHV-1 neurological disease may already contain maximal titres of EHV-1 antibody, with no increase in titres detectable in sera collected at later dates. In such cases, serological testing of paired serum samples from clinically unaffected cohort members of the herd for rising antibody titres against EHV-1/4 may provide information useful for retrospective diagnosis of ER within the herd.

Finally, the serological detection of antibodies to EHV-1 in heart or umbilical cord blood or other fluids of equine fetuses can be of diagnostic value in rare cases of virologically negative fetuses aborted as a result of EHV-1 infection; in some cases, the EHV 1/4 nucleic acid genome may be identified from these tissues by PCR.

Serum antibody levels to EHV-1/4 may be determined by ELISA (Dutta et al., 1983), virus neutralisation (VN) (Thomson et al., 1976), complement fixation (CF) tests (Thomson et al., 1976) or ELISA (Dutta et al., 1983). There are no internationally recognised reagents or standardised techniques for performing any of the serological tests for detection of EHV-1/4 antibody; antibody titre determinations on the same serum may differ from one laboratory to another. Furthermore, all of the serological tests mentioned detect antibodies that are cross-reactive between EHV-1 and EHV-4. Nonetheless, the demonstration by any of the tests, of a four-fold or greater rise in antibody titre to EHV-1 or EHV-4 during the course of a clinical illness provides serological confirmation of recent infection with one of the viruses. The ELISA and CF test have the advantage that they provide results faster and do not require cell culture facilities. Recently, a type-specific ELISA that can distinguish between antibodies to EHV-1 and EHV-4 was developed and made commercially available (Crabb et al., 1995). The microneutralisation test is a widely used and sensitive serological assay for detecting EHV-1/4 antibody and will thus be described here.

The microneutralisation test is a widely used and sensitive serological assay for detecting EHV-1/4 antibody and will thus be described here.

2.1. Virus neutralisation test

This serological test is most commonly performed in flat-bottom 96-well microtitre plates (tissue culture grade) using a constant dose of virus and doubling dilutions of equine test sera. At least two replicate wells for each serum dilution are required. Serum-free MEM is used throughout as a diluent. Virus stocks of known titre are diluted just before use to contain 100 TCID(50) (50% tissue culture infective dose) in 25 µl. Monolayers of E-Derm or RK-13 cells are monodispersed with EDTA/trypsin and resuspended at a concentration of 5 × 10⁴/ml. Note that RK-13 cells can be used with EHV-1 but do not give clear CPE with EHV-4. Antibody positive and negative control equine sera and controls for cell viability, virus infectivity, and test serum cytotoxicity, must be included in each assay. End-point VN titres of antibody are calculated by determining the reciprocal of the highest serum dilution that protects 100% of the cell monolayer from virus destruction in both of the replicate wells.
2.1. Test procedure

A suitable test procedure is as follows:

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

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

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

iv) Add 25 µl of the appropriately diluted EHV-1 or EHV-4 virus stock to each well (100 TCID₅₀/well) except those of row A, which are the serum control wells for monitoring serum toxicity for the indicator cells. Note that the final serum dilutions, after addition of virus, run from 1/4 to 1/256.

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

vi) Incubate the plates for 1 hour at 37°C in 5% CO₂ atmosphere.

vii) Add 50 µl of the prepared E-Derm or RK-13 cell suspension (5 × 10⁵ cells/ml) in MEM/10% FCS to each well.

viii) Incubate the plates for 4–5 days at 37°C in an atmosphere of 5% CO₂ in air.

ix) Examine the plates microscopically for CPE and record the results on a worksheet. Alternatively, the cell monolayers can be scored for CPE after fixing and staining as follows: after removal of the culture fluid, immerse the plates for 15 minutes in a solution containing 2 mg/ml crystal violet, 10% formalin, 45% methanol, and 45% water. Then, rinse the plates vigorously under a stream of running tap water.

x) Wells containing intact cell monolayers stain blue, while monolayers destroyed by virus do not stain. Verify that the cell control, positive serum control, and serum cytotoxicity control wells stain blue, that the virus control and negative serum control wells are not stained, and that the actual amount of virus added to each well is between 10¹⁻⁵ and 10²⁻⁵ TCID₅₀. Wells are scored as positive for neutralisation of virus if 100% of the cell monolayer remains intact. The highest dilution of serum resulting in complete neutralisation of virus (no CPE) in both duplicate wells is the end-point titre for that serum.

xi) Calculate the neutralisation titre for each test serum, and compare acute and convalescent phase serum titres from each animal for a four-fold or greater increase.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

NB: SECTION C IS “UNDER STUDY”. THIS IS THE LAST ADOPTED VERSION PUBLISHED IN 2008

Both live attenuated and inactivated vaccines are available as licensed, commercially prepared products for use as prophylactic aids in reducing the burden of disease in horses caused by EHV-1/4 infection. Clinical experience has demonstrated that none of the vaccine preparations should be relied on to provide an absolute degree of protection from ER. Multiple doses repeated annually, of each of the currently marketed ER vaccines are recommended by their respective manufacturers. Vaccination schedules vary with the particular vaccine.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

At least sixteen vaccine products for ER, each containing different permutations of EHV-1, EHV-4, and the two subtypes of equine influenza virus, are currently marketed by five veterinary biologicals manufacturers.

The clinical indications stated on the product label for use of the several available vaccines for ER are either herpesvirus-associated respiratory disease, abortion, or both. Only four vaccine products have met the regulatory requirements for claiming efficacy in providing protection from herpesvirus abortion as a result of successful
vaccination and challenge experiments in pregnant mares. None of the vaccine products has been conclusively
demonstrated to prevent the occurrence of neurological disease sometimes associated with EHV-1 infection.

1. Seed management

1.1. Characteristics and culture

The master seed virus (MSV) for ER vaccines must be prepared from strains of EHV-1 and/or EHV-4
that have been positively and unequivocally identified by both serological and genetic tests. Seed virus
must be propagated in a cell line approved for equine vaccine production by the appropriate regulatory
agency. A complete record of original source, passage history, medium used for propagation, etc.,
shall be kept for the master seed preparations of both the virus(es) and cell stock(s) intended for use in
vaccine production. Permanently stored stocks of both MSV and master cell stock (MCS) used for
vaccine production must be demonstrated to be pure, safe and, in the case of MSV, also immunogenic.

Generally, the fifth passage from the MSV and the twentieth passage from the MCS are the highest
allowed for vaccine production. Results of all quality control tests on master seeds must be recorded
and made a part of the licensee’s permanent records.

1.2. Validation as a vaccine

1.2.1. Purity

Tests for master seed purity include prescribed procedures that demonstrate the virus and cell
seed stocks to be free from bacteria, fungi, mycoplasmas, and extraneous viruses. Special tests
must be performed to confirm the absence of equine arteritis virus, equine infectious anaemia
virus, equine influenza virus, equine herpesvirus-2, -3, and -5, equine rhinovirus, the
alphaviruses of equine encephalomyelitis, bovine viral diarrhoea virus (BVDV – common
contaminant of bovine serum), and porcine parvovirus (PPV – potential contaminant of porcine
tryptsin). The purity check should also include the exclusion of the presence of EHV-1 from
EHV-4 MSV and vice versa.

1.2.2. Safety

Samples of each lot of MSV to be used for preparation of live attenuated ER vaccines must be
tested for safety in horses determined to be susceptible to the virulent wild-type virus, including
pregnant mares in the last 4 months of gestation. Vaccine safety must be demonstrated in a
’safety field trial’ in horses of various ages from three different geographical areas. The safety
trial should be conducted by independent veterinarians using a prelicensing batch of vaccine.

EHV-1 vaccines making a claim for efficacy in controlling abortion must be tested for safety in a
significant number of late gestation pregnant mares, using the vaccination schedule that will be
recommended by the manufacturer for the final vaccine product.

1.2.3. Immunogenicity

Tests for immunogenicity of the EHV-1/4 MSV stocks should be performed in horses on an
experimental test vaccine prepared from the highest passage level of the MSV allowed for use
in vaccine production. The test for MSV immunogenicity consists of vaccination of horses with
low antibody titres to EHV-1/4, with doses of the test vaccine that will be recommended on the
final product label. Second serum samples should be obtained and tested for significant
increases in neutralising antibody titre against the virus, 21 days after the final dose.

1.2.4. Efficacy

An important part of the validation process is the capacity of a prelicensing lot of the ER vaccine
to provide a significant level of clinical protection in horses from the particular disease
manifestation of EHV-1/4 infection for which the vaccine is offered, when used under the
conditions recommended by the manufacturer’s product label. Serological data are not
acceptable for establishing the efficacy of vaccines for ER. Efficacy studies must be designed to
ensure appropriate randomisation of test animals to treatment groups, blinding of the recording
of clinical observations, and the use of sufficient numbers of animals to permit statistical
evaluation for effectiveness in prevention or reduction of the specified clinical disease. The
studies should be performed on fully formulated experimental vaccine products (a) produced in
accordance with, (b) at or below the minimum antigenic potency specified in, and, (c) produced
with the highest passage of MSV and MCS allowed by the approved ‘Outline of Production’ (see
Section C.2). Vaccine efficacy is demonstrated by vaccinating a minimum of 20 EHV-1/4-
susceptible horses possessing serum neutralising antibody titres ≤32, followed by challenge of
the vaccinates and ten nonvaccinated control horses with virulent virus. A significant difference
in the clinical signs of ER must be demonstrated between vaccinates and nonvaccinated control horses. The vaccination and challenge study must be performed on an identical number of pregnant mares and scored for abortion if the vaccine product will make a label usage claim ‘for prevention of’ or ‘as an aid in the prevention of’ abortion caused by EHV-1.

2. Method of manufacture

A detailed protocol of the methods of manufacture to be followed in the preparation of vaccines for ER must be compiled, approved, and filed as an Outline of Production with the appropriate licensing agency. Specifics of the methods of manufacture for ER vaccines will differ with the type (live or inactivated) and composition (EHV-1 only, EHV-1 and EHV-4, EHV-4 and equine influenza viruses, etc.) of each individual product, and also with the manufacturer.

3. In-process control

Cells, virus, culture medium, and medium supplements of animal origin that are used for the preparation of production lots of vaccine must be derived from bulk stocks that have passed the prescribed tests for bacterial, fungal, and mycoplasma sterility; nontumorgenicity; and absence of extraneous viral agents.

4. Batch control

Each bulk production lot of ER vaccine must pass tests for sterility, safety, and immunogenic potency.

4.1. Sterility

Samples taken from each batch of completed vaccine are tested for bacteria, fungi, and mycoplasma contamination. Procedures to establish that the vaccine is free from extraneous viruses are also required; such tests should include inoculation of cell cultures that allow detection of the common equine viruses, as well as techniques for the detection of BVDV and PPV in ingredients of animal origin used in the production of the batch of vaccine.

4.2. Safety

Tests to assure safety of each production batch of ER vaccine must demonstrate complete inactivation of virus (for inactivated vaccines) as well as a level of residual virus-killing agent that does not exceed the maximal allowable limit (e.g. 0.2% for formaldehyde). Safety testing in laboratory animals is also required.

4.3. Potency

Batch control of antigenic potency for EHV-1 vaccines only may be tested by measuring the ability of dilutions of the vaccine to protect hamsters from challenge with a lethal dose of hamster-adapted EHV-1 virus. Although potency testing on production batches of ER vaccine may also be performed by vaccination of susceptible horses followed by either viral challenge or assay for seroconversion the recent availability of virus type-specific MAbs has permitted development of less costly and more rapid in-vitro immunoassays for antigenic potency. The basis for such in-vitro assays for ER vaccine potency is the determination, by use of the specific MAb, of the presence of at least the minimal amount of viral antigen within each batch of vaccine that correlates with the required level of protection (or seroconversion rate) in a standard animal test for potency.

4.4. Duration of immunity

Tests to establish the duration of immunity to EHV-1/4 achieved by immunisation with each batch of vaccine are not required. The results of many reported observations indicate that vaccination-induced immunity to EHV-1/4 is not more than a few months in duration; these observations are reflected in the frequency of revaccination recommended on ER vaccine product labels.

4.5. Stability

At least three production batches of vaccine should be tested for shelf life before reaching a conclusion on the vaccine’s stability. When stored at 4°C, inactivated vaccine products generally maintain their original antigenic potency for at least 1 year. Lyophilised preparations of the live virus vaccine are also...
stable during storage for 1 year at 4°C. Following reconstitution, live virus vaccine is unstable and cannot be stored without loss of potency.

5. Tests on the final product

Before release for labelling, packaging, and commercial distribution, randomly selected filled vials of the final vaccine product must be tested by prescribed methods for freedom from contamination and safety in laboratory test animals.

5.1. Safety

See Section C.4.2.

5.2. Potency

See Section C.4.3.

REFERENCES


Chapter 2.5.9. – Equine rhinopneumonitis


* * *

NB: There are OIE Reference Laboratories for Equine rhinopneumonitis
(see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for equine rhinopneumonitis and to submit strains for further characterisation.
CHAPTER 2.5.11.

GLANDERS

SUMMARY

Glanders is a contagious and fatal disease of horses, donkeys, and mules, caused by infection with the bacterium Burkholderia mallei (previously named Pseudomonas mallei). The pathogen 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. As B. mallei can be transmitted to humans, all infected/contaminated or potentially infected/contaminated material must be handled in a laboratory with appropriate biosafety and biosecurity controls following a biorisk analysis.

Identification of the agent: Smears from fresh material may reveal Gram-negative nonsporulating, nonencapsulated rods. The presence of a capsule-like cover has been demonstrated by electron microscopy. The bacteria grow aerobically and prefer media that contain glycerol. Unlike the Pseudomonas species and the closely related bacterium B. pseudomallei, B. mallei is nonmotile. Guinea-pigs are highly susceptible, and males can be used, if strictly necessary, to recover the organism from a heavily contaminated sample. Commercially available biochemical identification kits lack diagnostic sensitivity. Specific monoclonal antibodies and polymerase chain reaction (PCR) as well as real-time PCR assays are available.

Serological tests: CFT is an accurate and reliable serological method for diagnostic use. Enzyme-linked immunosorbent assays show promise once their validation is complete. A Rose Bengal plate agglutination test has been developed. The immunoblot test based on a crude formalin preparation of B. mallei antigens from isolates of different geographical regions is also a sensitive and specific assay.

Mallein test: The mallein test is a hypersensitivity test against B. mallei. The test is not generally recommended because of animal welfare concerns, however it can be useful in remote endemic areas where sample transport or proper cooling of samples is not possible. Mallein, a water soluble protein fraction of the organism, is injected intradermo-palpebrally. In infected animals, the eyelid swells markedly within 1–2 days.

Requirements for vaccines and diagnostic biologicals: There are no vaccines. Mallein purified protein derivative is currently available commercially.

A. INTRODUCTION

Glanders is a bacterial disease of perissodactyls or odd-toed ungulates. It has zoonotic potential and has been known since ancient times. It is caused by the bacterium Burkholderia mallei (previously known as Pseudomonas mallei, Yabuuchi et al., 1992) and has been classified in the past as Pfeifferella, Loefflerella, Malleomyces or Actinobacillus. It is a serious contagious disease in equids and outbreaks may also occur in felids living in the wild or in zoological gardens. Susceptibility to glanders has been proved in camels, bears, wolves and dogs. Carnivores may become infected by eating infected meat, but cattle and pigs are resistant. Small ruminants may be infected if kept in close contact with glanderous horses (Wittig et al., 2006). Glanders generally takes an acute form in donkeys and mules with high fever and respiratory signs (swollen nostrils, dyspnoea, and pneumonia) and death occurs within a few days. In horses, glanders generally takes a more chronic course and horses may survive for several years. Chronic and subclinical ‘occult’ cases are dangerous sources of infection due to the permanent or intermittent shedding of bacteria (Wittig et al., 2006). Kahn et al. (2012) reviewed the disease, its epidemiology, diagnosis and control.

In horses, inflammatory pustules and ulcers develop in the nasal conchae and nasal septae, which give rise to a sticky yellow discharge, accompanied by enlarged firm submaxillary lymph nodes. Stellate scarring follows upon healing of the ulcers. The formation of reddish nodular abscesses with a central grey necrotic zone 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 0.5–2.5 cm sized nodular abscesses ('buds') develop, which ulcerate and discharge yellow oily pus. Dry ulcers may also develop. Pyogranulomatous nodules are sometimes found in the liver and spleen (Wernery et al., 2012). Discharges from the respiratory tract and skin are infective, and transmission between animals, which is facilitated by close contact, inhalation, ingestion of contaminated material (e.g. from infected feed and water troughs), or by inoculation (e.g. via a harness) is common. The incubation period can range from a few days to many months (Wittig et al., 2006).

Glanders is transmissible to humans by direct contact with diseased animals or with infected/contaminated material. In the untreated acute disease, the mortality rate can reach 95% within 3 weeks (Neubauer et al., 1997). However, survival is possible if the infected person is treated early and aggressively with multiple systemic antibiotic therapies. A chronic form with abscessation can occur (Neubauer et al., 1997). When handling suspect or known infected animals or fomites, stringent precautions must be taken to prevent self-infection or transmission of the bacterium. Laboratory samples must be securely packaged, kept cool (not frozen) and shipped as outlined in Chapter 1.1.2 Transport of specimens of animal origin. All manipulations with potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

Glanders has been eradicated from many countries by statutory testing, culling of infected animals, and import restrictions. It persists in numerous Asian, African and South American countries and can be considered a re-emerging disease. Glanders can be introduced into glanders-free areas by leisure or racing equids (Neubauer et al., 2005).

1. Diagnostic pathway to confirm a case of glanders

   i) *Burkholderia mallei* has been isolated and identified in a sample from an equid or a product derived from that equid; or

   ii) antigen or genetic material specific to *B. mallei* has been identified in samples from an equid, whether showing clinical or pathological signs consistent with glanders or not, or is epidemiologically linked to a confirmed or suspected outbreak of glanders, or is giving cause for suspicion of previous contact with *B. mallei*; or

   iii) antibodies have been identified by an appropriate testing regime:

      a) a horse, whether showing clinical or pathological signs or not, subjected with positive result at a serum dilution of 1 in 5 to a complement fixation test (CFT), confirmed by a second test with equal or higher sensitivity and higher specificity, e.g. *B. mallei*-specific lipopolysaccharide (LPS)-western blot, I-ELISA (indirect enzyme-linked immunosorbent assay) (based on a recombinant protein from type VI secretion system) or C-ELISA (competitive ELISA) (based on *B. mallei*-specific monoclonal antibodies);

      b) a mule, hinny or donkey, whether showing clinical or pathological signs or not, by one of the following tests: *B. mallei*-specific LPS-western blot, I-ELISA (based on a recombinant protein from type VI secretion system) or C-ELISA (based on *B. mallei*-specific monoclonal antibodies).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of glanders and their purpose

<table>
<thead>
<tr>
<th>Method</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Population freedom from infection</td>
</tr>
<tr>
<td>Agent identification</td>
<td>–</td>
</tr>
<tr>
<td>PCR</td>
<td>–</td>
</tr>
<tr>
<td>Culture</td>
<td>–</td>
</tr>
<tr>
<td>Animal inoculation</td>
<td>–</td>
</tr>
</tbody>
</table>

1 A combination of agent identification methods applied on the same clinical sample is recommended.
**Chapter 2.5.11. – Glanders**

### Detection of immune response

<table>
<thead>
<tr>
<th>Method</th>
<th>Population freedom from infection</th>
<th>Individual animal freedom from infection</th>
<th>Contribute to eradication policies</th>
<th>Confirmation of clinical cases</th>
<th>Prevalence of infection – surveillance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement fixation</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>ELISA</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Malleinisation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Western blotting</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

2 **Detection of immune response**

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose; n/a = not applicable.

Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

ELISA = enzyme-linked immunosorbert assay; PCR = polymerase chain reaction.

### 1. Identification of the agent

Cases for specific glanders investigation should be differentiated on clinical grounds from other chronic infections affecting the nasal mucous membranes or sinuses. Among these are strangles (Streptococcus equi), ulcerative lymphangitis (Corynebacterium pseudotuberculosis), pseudotuberculosis (Yersinia pseudotuberculosis) and sporotrichosis (Sporotrichum spp.). Glanders should be unmistakably excluded from suspected cases of epizootic lymphangitis (Histoplasma farciminosum), with which it has many clinical similarities. In humans in particular, glanders should be distinguished from melioidosis, caused by B. pseudomallei a bacterium closely related to B. mallei.

#### 1.1. Morphology of *Burkholderia mallei*

The organisms are fairly numerous in smears from fresh lesions, but scarce in older lesions. Smears should be stained with methylene blue or Gram stain. The Gram-negative rods have rounded ends, are 2–5 µm long and 0.3–0.8 µm wide with granular inclusions of various size. The bacteria are generally located extracellularly and frequently stain irregularly and poorly when Gram stain is used. They do not have a readily visible capsule under the light microscope and do not form spores. The presence of a capsule-like cover has been verified by electron microscopy. This capsule is composed of neutral carbohydrates and serves to protect the cell from unfavourable environmental factors. Unlike other organisms in the *Pseudomonas* group and its close relative *B. pseudomallei*, *B. mallei* has no flagellae and is therefore nonmotile (Sprague & Neubauer, 2004). Nonmotility is the most important phenotypic characteristic diagnostically and must be demonstrated when pure culture is available. The organisms are difficult to detect in tissue sections, where they may have a beaded appearance. 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 (Neubauer et al., 2005).

#### 1.2. Cultural characteristics

It is preferable to attempt isolation from unopened, uncontaminated lesions. The organism is aerobic and facultative anaerobic only in the presence of nitrate, growing optimally at 37°C. It grows well, but slowly, on ordinary culture media, including sheep blood agar. 72-hour incubation of cultures is recommended; glycerol enrichment is particularly useful. The tiny greyish shiny colonies of *B. mallei* on sheep blood agar can be easily overgrown by other bacteria; hence careful observation is needed not to overlook the bacteria after 72 hours of incubation. After a few days on glycerol agar, a confluent, smooth, moist and slightly viscous cream coloured growth can be observed. On continued incubation, the growth thickens and becomes dark brown and tough. *Burkholderia mallei* 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 effusive, and growth is poor on gelatine. Various commercially available

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2 One of the listed serological tests is sufficient.

3 Horse samples only – care needed with interpretation of test on donkey samples.
**1.3. Identification of *Burkholderia* by polymerase chain reaction and real-time PCR**

In the past few years, several PCR and real-time PCR assays for the identification of *B. mallei* have been developed (Lee et al., 2005; Sprague et al., 2002; Thibault et al., 2004; Ulrich et al., 2006; U'Ren et al., 2005), but only one conventional PCR and one real-time PCR assay were evaluated using samples from a recent outbreak of glanders in horses (Scholz et al., 2006; Tomas et al., 2006). These two assays will therefore be described in more detail, but inter-laboratory studies are needed to confirm the robustness of these assays. The guidelines and precautions outlined in Chapter 1.1.5 *Principles and methods of validation of diagnostic assays for infectious diseases* should be observed.

**1.3.1. DNA preparation**

Single colonies are transferred from an agar plate to 200 µl deionised water. After heat inactivation (for example 99°C for 30 minutes), the DNA isolation can be performed using commercial DNA preparation kits for gram negative bacteria (see Scholz et al., 2006 and Tomas et al., 2006). Alternatively, heat-inactivated bacteria from pure cultures (99°C, 10 minutes) can be used directly for PCR reaction.

Tissue samples from horses (skin, lung, mucous membrane of the nasal conchae and septae, liver and spleen) that have been inactivated and preserved in formalin (48 hours, 10% v/v) are cut with a scalpel into pieces of 0.5 × 0.5 cm (approximately 500 mg). The specimens are washed twice in deionised water (10 ml), incubated overnight in sterile saline at 4°C, and minced by freezing in liquid nitrogen, followed by grinding with a mortar and pestle. Total DNA is prepared from 50 mg tissue using a commercial extraction kit according to the manufacturer's
instructions. DNA is eluted with 80 µl dH₂O or as appropriate for the kit used 4 µl eluate is used as template.

1.3.2. PCR assay (Scholz et al., 2006)

The assay may have to be adapted to the PCR instrument used with minor modifications to the cycle conditions and the concentration of the reagents used.

The oligonucleotides used by Scholz et al., (2006) are based on the differences between the fliP sequences from *B. mallei* ATCC 23344\(^T\) (accession numbers NC_006350, NC_006351) and *B. pseudomallei* K96243 (accession numbers NC_006348, NC_006349). Primers Bma-IS407-flip-f (5’-TCA-GGT-TTG-TAT-GTC-GCT-CGG-3’) and Bma- IS407-flip-r (5’-CTA-GGT-GAA-GCT-CTG-CGC-GAG-3’) are used to amplify a 989 bp fragment. The PCR uses 50 µl ready-to-go master mix and 15 pmol of each primer. Thermal cycling conditions are 94°C for 30 seconds and 35 cycles at 65°C for 30 seconds and 72°C for 60 seconds and succeeded by a final elongation step at 72°C for 7 minutes. Visualisation of the products takes place under UV light after agarose gel (1% w/v in TAE buffer) electrophoresis and staining with ethidium bromide. No template controls containing PCR-grade water instead of template and positive controls containing *B. mallei* DNA have to be included in each run to detect contamination by amplicons of former runs or amplification failure.

The lower detection limit of this assay is 10 fg or 2 genome equivalents.

1.3.3. Real-time PCR assay (Tomaso et al., 2006)

The assay should be adapted to the real-time PCR instrument used, e.g. the cycling vials should be chosen according to the manufacturer’s recommendations, the concentration of the oligonucleotides may have to be increased, or the labelling of the probes altered.

The oligonucleotides used in Tomaso et al. (2006) are based on differences in the fliP sequences of *B. mallei* ATCC 23344\(^T\) (accession numbers NC_006350, NC_006351) and *B. pseudomallei* K96243 (accession numbers NC_006348, NC_006349). The fluorogenic probe is synthesised with 6-carboxy-fluorescein (FAM) at the 5’-end and black hole quencher 1 (BHQ1) at the 3’-end. Oligonucleotides used were Bma-flip-f (5’-CCC-ATT-GGC-CCT-ATC-GAA-G-3’), Bma-flip-r (5’-GCC-CGA-CGA-GCA-CCT-GAT-T-3’), and probe Bma-flip (5’-6FAM-CAG-GTC-AAC-GAG-CTT-CAC-GCG-GAT-C-BHQ1-3’).

The 25 µl reaction mixture consists of 12.5 µl 2× master mix, 0.1 µl of each primer (10 pmol/µl), 0.1 µl of the probe (10 pmol/µl) and 4 µl sample. Thermal cycling conditions are 50°C for 2 minutes; 95°C for 10 minutes; 45 cycles at 95°C for 25 seconds and 63°C for 1 minute. Possible contaminations with amplification products from former reactions are inactivated by an initial incubation step using uracil N’-glycosilase.

The authors suggest including an internal inhibition control based on a bacteriophage lambda gene target (Lambda-F [5’-ATG-CCA-CGT-AAG-CCA-AA-A-3’] Lambda-R [5’-GCA-TAA-AAC-AAC-TGG-TAA-5’], Lambda-YAK [5’-YAK-ACC-TTA-CCG-AAA-TTG-GTA-CCG-ATA-CGC-C-DB-3’]), which can be titrated to provide reproducible cycle threshold values. However, depending on the sample material a house keeping gene targeting PCR may be used additionally or as an alternative. No template controls containing 4 µl of PCR-grade water instead and positive controls containing DNA of *B. mallei* have to be included in each run to detect amplion contamination or amplification failure.

The linear range of the assay was determined to cover concentrations from 240 pg to 70 fg bacterial DNA/reaction. The lower limit of detection defined as the lowest amount of DNA that was consistently detectable in three runs with eight measurements each is 60 fg DNA or four genome equivalents (95% probability). The intra-assay variability of the fliP PCR assay for 35 pg DNA/reaction is 0.68% (based on Ct values) and for 875 fg 1.34%, respectively. The inter-assay variability for 35 pg DNA/reaction is 0.89% (based on Ct values) and for 875 fg DNA 2.76%, respectively.

To date, a positive result confirms the diagnosis ‘*Burkholderia mallei*’ for an isolate and the diagnosis ‘glanders’ in clinical cases. It has to be kept in mind, however, that future genetic evolution may well result in *B. mallei* clones that can no longer be detected by these standard PCRs. The sensitivity of the PCR assays for clinical samples is unknown. A negative result therefore, is no proof of the absence of *B. mallei* in the sample and other diagnostic means must be applied for confirmation.
1.4. Laboratory animal inoculation

Animal inoculation is not recommended, because of welfare concerns. If isolation in a laboratory animal is considered unavoidable, suspected material is inoculated intraperitoneally into male guinea-pigs. As this technique has a sensitivity of only 20%, the inoculation of at least five animals is recommended (Neubauer et al., 1997). Positive material will cause a severe localised peritonitis and orchitis (the Strauss reaction). The number of organisms and their virulence determines the severity of lesions. Additional pre-treatment steps have to be used if the test material is heavily contaminated. The Strauss reaction is not specific for glanders and can be provoked by other organisms, therefore B. mallei must be cultured from the infected testes.

1.5. Other methods

Molecular typing techniques such as PCR-restriction fragment length polymorphism (Tanpiboonsak et al., 2004), pulsed field gel electrophoresis (Chantratita et al., 2006), ribotyping (Harvey & Minter, 2005) or multilocus sequence typing (MLST) (Godoy et al., 2003) are only appropriate for use in specialised laboratories.

2. Serological tests

2.1. Complement fixation test in horses, donkeys, and mules (a prescribed test for international trade)

The CFT is an accurate serological test that has been used for many years for diagnosing glanders. It will deliver positive results within 1 week post-infection and will also recognise sera from exacerbated chronic cases. Application of rigorous quality control in the formulation of CFT antigens, complement and haemolytic systems are crucial for the performance of this test as its specificity and sensitivity are critically dependent on the antigen used (Elschner et al., 2011; Khan et al., 2011). Recently, however, the specificity of CF testing has been questioned (Neubauer et al., 2005). The CFT is valid for horses, mules and camels; if used in donkeys particular care is needed to avoid misdiagnosis.

2.1.1. Antigen preparation

i) The stock culture strain of B.mallei (Dubai 7) stored at −80°C is revived by plating onto sheep blood agar and incubated at 37°C for 48 hours to get a confluent growth.

ii) From this 48 hours culture, a loopful (0.5 mm diameter) is inoculated to 5 ml of brain–heart infusion (BHI) broth with 3% glycerol and incubated at 37°C for 24 hours.

iii) 1 ml from the above culture broth is further inoculated to 100 ml BHI broth with 3% glycerol and incubated at 37°C for 48 hours with gentle agitation.

iv) The cultures are inactivated by exposing the flasks to flowing steam (100°C) for 60 minutes.

v The clear supernatant is decanted and filtered. The filtrate is heated again by exposure to live steam for 1 hour, and clarified by centrifugation at 3000 rpm for 10 minutes.

vi) The clarified product is stored as concentrated antigen in brown glass bottles to shield from light and stored at 4°C. Antigen has been shown to be stable for at least 10 years in this concentrated state.

vii) Aliquots of antigen are prepared by diluting the concentrated antigen 1/20 with sterile physiological saline containing 0.5% phenol. The diluted antigen is dispensed into brown-glass vials and stored at 4°C. The final working dilution is determined by a block titration. The final working dilution for the CFT is prepared when performing the test.

The resulting antigen consists primarily of lipopolysaccharides (LPS). An alternative procedure is to use young cultures by growing the organism on glycerol–agar slopes for up to 48 hours and washing them off with normal saline. A suspension of the culture is heated for 1 hour at 70°C and the heat-treated bacterial suspension is used as antigen. The disadvantage of this antigen preparation method is that the antigen contains all the bacterial cell components. The antigen should be safety tested by inoculating blood agar plates.
2.1.2. CFT procedure

i) Serum is diluted 1/5 in veronal (barbiturate) buffered saline containing 0.1% gelatine (VBSG) or CFD (complement fixation diluent – available as tablets) without gelatine or other commercially provided CFT buffers.

ii) Diluted serum is inactivated for 30 minutes at 58–60°C. Serum of equidae other than horses should be inactivated at 63°C for 30 minutes. Camel serum is inactivated for 30 minutes at 56°C.

iii) Twofold dilutions of the sera are prepared using veronal buffer or alternative commercially available CFT buffers in 96-well round-bottom microtitre plates.

iv) Guinea-pig complement is diluted in the chosen buffer and 5 (or optionally 4) complement haemolytic units-50% (CH50) are used.

v) Sera, complement and antigen are mixed in the plates and incubated for 1 hour at 37°C. An alternative procedure is overnight incubation at 4°C.

vi) A 3% suspension of sensitised washed sheep red blood cells is added.

vii) Plates are incubated for 45 minutes at 37°C, and then centrifuged for 5 minutes at 600 g.

When using commercially available CFT-antigens and ready-to-use CFT reagents, the manufacturers’ instructions should be applied.

Recommended controls to verify test conditions:

Positive control: a control serum that gives a positive reaction;

Negative control serum: a control serum that gives a negative reaction;

Anti-complementary control (serum control): diluent + inactivated test serum + haemolytic system;

Antigen control: diluent + antigen + complement + haemolytic system;

Haemolytic system control: diluent + haemolytic system;

Complement control: diluent + complement titration + antigen + haemolytic system.

2.1.3. Reading the results

The absence of anti-complementary activity must be checked for each serum; anti-complementary sera must be excluded from analyses. A sample that produces 100% haemolysis at the 1/5 dilution is negative, 25–75% haemolysis is suspicious, and no haemolysis (100% fixation) is positive. Unfortunately, false-positive results can occur, and animals can remain positive for months. Moreover, B. pseudomallei and B. mallei cross react and cannot be differentiated by serology (Neubauer et al., 1997). It must also be kept in mind that healthy non-glanderous equids can show a false positive CF reaction for a variable period of time following a mallein intradermal test.

2.2. Enzyme-linked immunosorbent assays

Both plate and membrane based ELISAs have been used for the serodiagnosis of glanders, but none of these procedures has been able to differentiate between B. mallei and B. pseudomallei. An avidin–biotin dot ELISA has been described, but has not yet been widely used or validated. The antigen used is a concentrated and purified heat-inactivated bacterial culture. A spot of this antigen is placed on a nitrocellulose dipstick. Using antigen-dotted, pre-blocked dipsticks, the test can be completed in approximately 1 hour. An I-ELISA was shown to be of limited value for the serological diagnosis of glanders (Sprague et al., 2009). An I-ELISA based on recombinant Burkholderia intracellular motility A protein (rBimA) showed a promising sensitivity of 100% and a specificity of 98.88% (Kumar et al., 2011). A C-ELISA that makes use of an uncharacterised anti-LPS MAb has also been developed and found to be similar to the CF test in performance (Katz et al., 2000). The C-ELISA was used again on a panel of horse sera originating mainly from Middle Eastern countries (Sprague et al., 2009). A commercially available C-ELISA has recently been developed using anti-s B. mallei LPS MAb along with antigen prepared from a regional B. mallei isolate. This showed higher sensitivity than CFT in identifying field cases. The C-ELISA has been evaluated on donkey sera and reliable results obtained in an infection trial (Altemann, in preparation). Continuing development of monoclonal antibody reagents specific for B. mallei antigenic components will offer the possibility to develop more specific ELISAs that will help to resolve questionable test results of quarantined imported horses (Neubauer et al., 1997).
None of these tests has been fully validated to date. However, a C-ELISA based on one or more B. mallei-specific antibodies and an I-ELISA making use of (recombinant) B. mallei-specific antigens have the potential to be used as alternative tests after their validations have been completed.

### 2.3. Immunoblot assays

An immunoblot assay was developed for the serodiagnosis of glanders, but further validation was impossible because of the lack of a positive serum control panel (Katz et al., 1999). Recently, the development of an immunoblot using B. mallei LPS antigen was reinitiated. The aim was to obtain a more sensitive test than the CFT in order to retest false positive CFT sera in non-endemic areas (Elschner et al., 2011). The developed assay is based on crude antigen preparations of the B. mallei strains Bogor, Zagreb and Mukteswar, which are also the basis of most CFT antigen formulations. The antigens are separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and subsequently transferred to nitrocellulose membranes. Anti-B. mallei LPS antibodies in a serum sample reacting to the antigen on the blot strip are visualised by animal species-specific (phosphatase) conjugate and the NBT-BCIP (Nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl-phosphate) colour system. The immunoblot is scored positive if the banding pattern of the B. mallei LPS ladder within the 20–60 kDa region is clearly visible, suspicious if a weak colour reaction is detected and negative if no reaction is seen. 171 sera of glandulous horses and mules from Pakistan and Brazil and 305 sera of negative German horses were investigated and a sensitivity and specificity, both of 100% were found. For the time being, this test is the best evaluated serological test available. It has to be stressed that this test is not able to differentiate glanders from melioidosis infection and that it has not yet been evaluated for use in donkeys because of the lack of a significant number of positive control sera.

### 2.4. Other serological tests

The Rose Bengal plate agglutination test (RBT) has been described for the diagnosis of glanders in horses and other susceptible animals; and has been validated in Russia. In a study in Pakistan the RBT showed a sensitivity of 90% and a specificity of 100% (Naureen et al., 2007). The antigen is a heat-inactivated bacterial suspension coloured with Rose Bengal, which is used in a plate agglutination test.

The accuracy of other agglutination and precipitin tests is unsatisfactory for control programmes. Horses with chronic glanders and those in a debilitated condition give negative or inconclusive results.

### 3. Tests for cellular immunity

#### 3.1. The mallein test

The mallein purified protein derivative (PPD), which is available commercially, is a solution of water-soluble protein fractions of heat-treated B. mallei. See section C below for details of its preparation and availability. The test is not generally recommended because of animal welfare concerns, however it can be useful in remote endemic areas where sample transport or proper cooling of samples is not possible. It 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 diagnostic methods.

The intradermo-palpebral test is the most sensitive, reliable and specific mallein test for detecting infected perissodactyls or odd-toed ungulates, and has largely displaced other methods. 0.1 ml of concentrated mallein PPD is injected intradermally into the lower eyelid and the test is read at 24 and 48 hours. A positive reaction is characterised by marked oedematous swelling of the eyelid, and there may be a purulent discharge from the inner canthus or conjunctiva. This is usually accompanied by a rise in temperature. With a negative response, there is usually no reaction or only a little swelling of the lower lid.

### C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No vaccines are available.
Mallein PPD is available commercially. The following information outlines the requirements for the production of mallein PPD.

1. Seed management

Three strains of *Burkholderia mallei* are employed in the production of mallein PPD, namely Bogor strain (originating from Indonesia), Mukteswar strain (India) and Zagreb strain (Yugoslavia). The seed material is kept as a stock of freeze-dried cultures. The strains are subcultured on to glycerol agar at 37°C for 1–2 days. For maintaining virulence and antigenicity, the strains may be passaged in guinea-pigs.

2. Production

Dorset-Henley medium, enriched by the addition of trace elements, is used for the production of mallein PPD. The liquid medium is inoculated with a thick saline suspension of *B. mallei*, grown on glycerol agar. The production medium is incubated at 37°C for about 10 weeks. The bacteria are then killed by steaming for 3 hours in a Koch's steriliser. The fluid is then passed through a layer of cotton wool to remove coarse bacterial clumps. The resulting turbid fluid is cleared by membrane filtration, and one part trichloroacetic acid 40% is immediately added to nine parts culture filtrate. The mixture is allowed to stand overnight during which the light brownish to greyish precipitate settles.

The supernatant is decanted and discarded. The precipitate is centrifuged for 15 minutes at 2500 g and the layer of precipitate is washed three or more times in a solution of 5% NaCl, pH 3, until the pH is 2.7. The washed precipitate is dissolved by stirring with a minimum of an alkaline solvent. The fluid is dark brown and has a pH of 6.7. This mallein concentrate is centrifuged again and the supernatant diluted with an equal amount of a glucose buffer solution. The protein content of this product is estimated by the Kjeldahl method and freeze-dried after it has been dispensed into ampoules.

3. In-process control

During the period of incubation, the flasks are inspected regularly for any signs of contamination, and suspicious flasks are discarded. A typical growth of the *B. mallei* cultures comprises turbidity, sedimentation, some surface growth with a tendency towards sinking, and the formation of a conspicuous slightly orange-coloured ring along the margin of the surface of the medium.

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 applying the intradermo-palpebral test. The resulting swelling should be, at most, barely detectable and transient, without any signs of conjunctival discharge.

Preparations containing phenol as a preservative should not contain more than 0.5% (w/v) phenol. The protein content should be no less than 0.95 mg/ml and not more than 1.05 mg/ml.

Potency testing is performed in guinea-pigs and horses. The animals are sensitised by subcutaneous inoculation with a concentrated suspension of heat-killed *B. mallei* in paraffin oil adjuvant. Cattle can also be used instead of horses. The production batch is bio-assayed against a standard mallein PPD by intradermal injection in 0.1 ml doses in such a way that complete randomisation is obtained.

In guinea-pigs, the different areas of erythema are measured after 24 hours, and in horses the increase in skin thickness is measured with callipers. The results are statistically evaluated, using standard statistical methods for parallel-line assays.

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REFERENCES

ALTEMANN D. (in preparation). Pathogenesis and diagnosis of glanders in experimentally infected donkey using different infection routes and doses, Thesis Veterinary Faculty Giessen, Germany.


* * *

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

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and diagnostic biologicals for glanders.
CHAPTER 2.7.9.

OVINE EPIDIDYMITIS

(\textit{Brucella ovis})

SUMMARY

\textit{Brucella ovis} infects sheep causing a clinical or subclinical disease that is characterised by genital lesions and reduced fertility in rams, placentitis and abortions in ewes, and increased perinatal mortality in lambs. The disease has been reported in American and European countries as well as Australia, New Zealand and South Africa, but probably occurs in most sheep-raising countries.

\textbf{Identification of the agent:} Clinical lesions (epididymitis and orchi-epididymitis) in rams may be indicative of the existence of infection, but laboratory examinations are required to confirm the disease. Laboratory confirmation may be based on direct or indirect methods. Direct diagnosis is made by means of bacteriological isolation of \textit{B. ovis} from semen samples or tissues of rams, or vaginal discharges, milk and tissues of ewes, on adequate selective media. Molecular methods have been developed for complementary identification based on specific genomic sequences. Polymerase chain reaction (PCR) based methods can provide additional means of detection. However, indirect diagnosis based on serological tests is preferred for routine diagnosis.

\textbf{Serological tests:} The complement fixation test (CFT), agar gel immunodiffusion (AGID) test and indirect enzyme-linked immunosorbent assays (I-ELISA) using soluble surface antigens obtained from the \textit{B. ovis} REO 198 strain, should be used for diagnosis. The sensitivities of the AGID test and I-ELISA are similar and may be higher than that of the CFT. A combination in parallel of the AGID test and I-ELISA seems to give the best results in terms of sensitivity, but with regard to simplicity and cost, the AGID test is the most practicable test for diagnosing \textit{B. ovis} in non-specialised laboratories. However, because of the lack of standardised methods recognised at the international level for AGID and I-ELISA, the prescribed test for international trade remains the CFT.

\textbf{Requirements for vaccines:} Seed cultures for vaccine production should be obtained from internationally recognised laboratories. A single standard dose ($10^9$ colony-forming units) of the live \textit{B. melitensis} Rev.1 vaccine, administered subcutaneously or, better, conjunctivally, can be used safely and effectively in rams, for the prevention of \textit{B. ovis} infection. This vaccine strain should meet minimal quality standards: adequate concentration, absence of dissociation, adequate residual virulence and immunogenicity and free of extraneous agents (see Chapter 2.7.2 Caprine and ovine brucellosis [excluding \textit{Brucella ovis}]).

A. INTRODUCTION

1. Definition of the disease

\textit{Brucella ovis} produces a disease unique to sheep and is one of the most common causes of epididymitis in rams and a rare cause of infertility and abortion in ewes and neonatal mortality in lambs.

2. Causal pathogen

\textit{Brucella ovis} and \textit{B. canis} are the two presently known \textit{Brucella} species naturally in the rough phase. \textit{Brucella ovis} is similar to the other \textit{Brucella} spp. in its morphology, staining properties and cultural characteristics, except that it gives negative reactions to the oxidase and urease tests. The microbiological and serological properties of all \textit{Brucella} species and biovars are given in detail in the Chapter 2.4.3 Bovine brucellosis.
3. Description of the disease

*Brucella ovis* infects sheep causing genital lesions (epididymitis and orchi-epididymitis) and infertility in rams, placentitis, and infertility in ewes, and increased perinatal mortality in lambs. *Brucella ovis* is usually excreted in semen in infected rams. Passive venereal transmission via the ewe appears to be the most frequent route of infection, but ram-to-ram transmission is also very common (Blasco, 1990; 2010). Under the semi-extensive production systems (most common in European Mediterranean countries), rams are usually housed together. Direct ram-to-ram transmission during non-breeding periods is thus quite frequent and has been suggested to take place by several routes, including anal intercourse and, more frequently, through oral-genital contact (preputial licking).

Moreover, infected ewes may excrete *B. ovis* in vaginal discharges and milk and, accordingly, ewe-to-ram and lactating ewe-to-lamb transmission could also be determinant mechanisms of infection. Accordingly, ewes should be considered relevant in the epidemiology of infection, and this should be taken into account for the effective eradication of *B. ovis* in infected flocks (Blasco, 2010; Grilló et al., 1999).

The disease has been reported in American and European countries as well as Australia, New Zealand and South Africa, but probably occurs in most sheep-raising countries.

The demonstration of genital lesions (unilateral or bilateral epididymitis and orchi-epididymitis) by palpating the testicles of rams may suggest the presence of this infection in a given flock. However, clinical diagnosis lacks sensitivity because not all rams infected with *B. ovis* present palpable genital lesions (Blasco, 1990). Moreover, clinical diagnosis lacks specificity since many other bacteria may cause genital lesions in rams. The most frequently reported pathogens such lesions in rams include *Actinobacillus seminis*, *A. actinomycetemcomitans*, *Histophilus ovis*, *Haemophilus* spp., *Corynebacterium pseudotuberculosis ovis*, *B. melitensis*, *Chlamydia abortus* and *Pasteurella* spp. (Bulgin & Anderson, 1983; Garcia-Pastor et al., 2009; Livingstone & Hardy, 1964). Moreover, it must be emphasised that many palpable testicular lesions in rams are sterile, trauma-induced spermatic granulomas.

Although cattle, goats and deer have been proved susceptible to *B. ovis* in artificial transmission experiments, natural cases have been reported only in red deer reared in close contact with infected rams (Ridler et al., 2012).

4. Zoonotic risk and biosafety requirements

To date, no human cases have been reported, and *B. ovis* is considered to be non-zoonotic. However, in areas where *B. melitensis* infection co-exists with *B. ovis*, special care is required when handling samples, which should be transported to the laboratory in leak-proof containers (for further details see Chapter 2.7.2 Caprine and ovine brucellosis [excluding *B. ovis*] and Chapter 1.1.2 Transport of specimens of animal origin). All laboratory manipulations with live cultures or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis (Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

### B. DIAGNOSTIC TECHNIQUES

<table>
<thead>
<tr>
<th>Method</th>
<th>Population freedom from infection</th>
<th>Individual animal freedom from infection prior to movement(^a)</th>
<th>Contribute to eradication policies(^b)</th>
<th>Confirmation of clinical cases(^c)</th>
<th>Confirmation of suspect cases(^d)</th>
<th>Herd/flock prevalence of infection – Surveillance</th>
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<tr>
<td>Staining methods</td>
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<td>+/+</td>
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1 A combination of agent identification methods applied on the same clinical sample is recommended.
Chapter 2.7.9. – Ovine epididymitis (Brucella ovis)

<table>
<thead>
<tr>
<th>Method</th>
<th>Population freedom from infection</th>
<th>Individual animal freedom from infection prior to movement</th>
<th>Contribute to eradication policies</th>
<th>Confirmation of clinical cases</th>
<th>Confirmation of suspect cases</th>
<th>Herd/flock prevalence of infection – Surveillance</th>
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Detection of immune response

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose; n/a = not applicable.

Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

PCR = polymerase chain reaction; CFT = complement fixation test; I-ELISA = indirect enzyme-linked immunosorbent assay; AGID = agar gel immunodiffusion test.

aThis applies only to herds/flocks, countries or zones free from infection with Brucella ovis.
bTo improve the efficiency of eradication policies in infected herds/flocks it is recommended to associate tests in parallel to increase the sensitivity of the diagnosis, i.e. two serological tests at least, e.g. CFT (or AGID) and I-ELISA.
cIn low-prevalence or almost-free zones, the predictive value of positive results to serological tests may be very low. In such situation, the agent identification is usually needed for confirming clinical cases. In infected herds/flocks, a positive result to any serological test may be considered as a confirmation of a clinical case.
dIn infected herds/flocks, any reactor in any serological test should be considered as infected. In low-prevalence or almost-free zones, singleton serological reactors may be confirmed by culture (and/or PCR). In free countries or zones, suspect animals are those positive to both a screening and a confirmatory serological test (tests in series, e.g. I-ELISA and CFT respectively) and should be confirmed by culture (and/or PCR).

eFalse-positive results may occur.

1. Identification of the agent

1.1. Collection of samples

The most valuable samples for the isolation of B. ovis from live animals are semen, vaginal swabs and milk. For the collection of vaginal swabs and milk, see the instructions given in Chapter 2.4.3. Semen (genital fluids) can be collected in swabs taken from the preputial cavity of rams after electroejaculation. Alternatively, swabs can be taken directly from the vagina of brucellosis-free ewes immediately after being mated by the suspect ram. Clinically or sub-clinically infected rams may excrete B. ovis intermittently in their semen for years (Blasco, 2010). Vaginal swabs taken after abortion or premature lambing and milk samples are highly recommended samples to isolate B. ovis from infected ewes (Grilló et al., 1999).

For the isolation of B. ovis after necropsy, the preferred organs in terms of probability of isolation are the epididymides, seminal vesicles, ampullae, and inguinal lymph nodes in rams, and the uterus, iliac and supra-mammary lymph nodes in ewes. However, to obtain maximum sensitivity, a complete search that includes other organs and lymph nodes (spleen, cranial, scapular, pre-femoral and testicular lymph nodes) should be performed (Blasco, 2010). Dead lambs and placentas can also be examined. The preferred culture sites in aborted or stillborn lambs are abomasal content and lung.

Samples for culture should be refrigerated and transported to the laboratory to be cultured as soon as possible after collection. The organism remains viable for 48–72 hours at room temperature but if culture has to be delayed survival is enhanced by refrigerating or, preferably, freezing the tissue samples.

1.2. Staining methods

Semen or vaginal smears from clinically affected animals can be examined following staining by Stamp’s method (Alton et al., 1988) (see Chapter 2.4.3), and characteristic coccobacilli can be demonstrated in many infected animals. Examination of Stamp-stained smears of suspect tissues (ram genital tract, inguinal lymph nodes, placentas, and abomasal content and lung of fetuses) may also

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2 One of the listed serological tests is sufficient.
allow a rapid presumptive diagnosis. However, other bacteria with similar morphology or staining
characteristics (B. melitensis, Coxiella burnetii, and Chlamydia abortus) can also be present in such
samples, making the diagnosis difficult for inexperienced personnel. For such reason, microscopy
results should always be confirmed by culture of the microorganism.

1.3. Culture

Due to its specificity, the isolation and identification of B. ovis in sheep fluids and tissues is the best
direct method of diagnosis and, if positive, the only incontestable demonstration of B. ovis infection in a
given animal or flock. Semen, vaginal swabs, or milk samples can be smeared directly onto plates
containing adequate culture media and incubated at 37°C ± 2°C in an atmosphere of 5–10% CO₂.

Tissues should be macerated and ground in a small amount of sterile saline or phosphate buffered
saline (PBS) with a stomacher or blender, before plating. It is important to take into account that the
larger the amount of tissue homogenates and the higher number of culture plates inoculated per
diagnostic sample, the higher will be the final diagnostic sensitivity obtained.

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

Brucella ovis can be isolated in non-selective media, such as blood agar base enriched with 10%
stereile ovine or bovine blood, or in blood agar medium with added 5–10% sterile ovine blood.
However, since primary isolation requires 4–7 days of incubation, overgrowing fungi and commensal
and environmental bacteria frequently contaminate the non-selective culture plates, and result in a
reduced diagnostic sensitivity. Thus, the use of selective culture media is of paramount importance for
a proper bacteriological diagnosis of B. ovis infection. The modified Farrell’s selective medium used
widely for the isolation of the smooth Brucella (see Chapter 2.4.3), inhibits the growth of B. ovis and
should not be used (Marin et al., 1996). Various selective media have been described, but modified
Thayer–Martin’s (mTM) medium (Marin et al., 1996) has been used classically for isolating B. ovis.
Briefly, this medium can be prepared with GC medium base (38 g/litre Difco, USA) supplemented with
haemoglobin (10 g/litre) and colistin methane-sulphonate (7.5 mg/litre), vancomycin (3 mg/litre),
nitrofurantoin (10 mg/litre), nystatin (100,000 International Units [IU]/litre = 17.7 mg) and amphotericin
B (2.5 mg/litre). Working solutions are prepared as follows:

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

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

Antibiotic solutions (prepared freshly): colistin, nystatin and vancomycin are suspended in a mixture of
methanol/water (1/1); nitrofurantoin is suspended in 1 ml of a 0.1 M NaOH sterile solution. For
amphotericin B, it is recommended to prepare a stock solution of 10 mg/ml amphotericin B with 10 mg
dissolved first in 1 ml sterile dimethyl sulphoxide (C₂H₆OS, analytical grade) and then added to 9 ml of
sterile PBS (10 mM, pH 7.2 ± 0.1). Any stock solution remaining can be stored 5 days at 5°C ± 3°C. All
antibiotic solutions must be filtered through 0.22 µm filters before addition to the culture medium.
Another suitable, but less effective, antibiotic combination can be: vancomycin (3 mg/litre); colistin
(7.5 mg/litre); nystatin (12,500 IU/litre); and nitrofurantoin (10 mg/litre).

Once autoclaved, stabilise the temperature (45–50°C) of both solutions A and B with continuous
stirring. Mix both solutions (adding A to B), avoiding bubble formation. Add the antibiotic solutions while
stirring continuously and carefully, then distribute into sterile plates. Once prepared, the plates should
not be stored for long periods, and freshly prepared medium is always recommended.

However, the mTM is not translucent due to the haemoglobin incorporated as a basal component,
being thus unsuitable for the direct observation of colonial morphology. This has important practical
consequences since this is probably the most widely used procedure for the presumptive identification
of Brucella (Alton et al., 1988). Having this in consideration, a new culture medium (named CITa) has
been recently formulated using blood agar base as a basal component, and supplemented with 5% of
sterile calf serum and the following antibiotics: vancomycin (20 mg/litre), colistin methanesulfonate
(7.5 mg/litre), nitrofurantoin (10 mg/litre), nystatin (100,000 IU/litre), and amphotericin B (4 mg/litre).
This antibiotic mixture can be prepared as indicated above for the preparation of the mTM medium.
This new CITa medium inhibits most contaminant microorganisms but allows the growth of all Brucella
species. Moreover, CITa medium outperforms mTM for isolation of B. ovis, and is more sensitive than
both mTM and Farrell’s media for isolating all smooth *Brucella* species from field samples, and is therefore the selective medium of choice for general *Brucella* isolation (De Miguel *et al*., 2011).

All culture media used should be subjected to quality control with reference strains, to demonstrate that it performs properly.

### 1.4. Identification and typing

*Brucella ovis* colonies are not haemolytic. They are circular, convex, have unbroken edges, are always of the rough type when examined by oblique illumination, and test positive in the acriflavine test (Alton *et al*., 1988). For growth, *B. ovis* needs an incubating atmosphere containing 5–10% CO\(_2\). It lacks urease activity, fails to reduce nitrate to nitrite, and is catalase and oxidase negative. It does not produce H\(_2\)S and, although it does not grow in the presence of methyl violet, it usually grows in the presence of standard concentrations of basic fuchsin and thionin. The cultures are not lysed by *Brucella*-phages of the Tbilisi (Tb), Weybridge (Wb) and Izatnagar (Iz\(_1\)) groups at the routine test dilution (RTD) or 10\(^4\) RTD, while they are lysed by phage R/C (Alton *et al*., 1988). Most laboratories are not equipped enough for a complete identification of *Brucella* at species and biovar levels, and a practical schedule for presumptive identification is needed. Most *B. ovis* isolates can be correctly identified on the basis of growth characteristics, direct observation using obliquely reflected light, Gram or Stamp’s staining, catalase, oxidase, urease and acriflavine tests. However, it is recommended that the definitive identification be carried out by reference laboratories with experience in identification and typing of *Brucella*.

The polymerase chain reaction (PCR) and other recently developed molecular methods provide additional means of detection and identification of *Brucella* sp. (see Chapter 2.4.3), and are becoming routine in many diagnostic laboratories. The existence of semen samples heavily contaminated with overgrowing organisms or containing dead *B. ovis*, could also justify the use of PCR as a supplementary direct diagnostic test. In fact, several PCR procedures have been reported to have similar sensitivity to standard bacteriological culture when applied to semen samples from *B. ovis* infected rams (Xavier *et al*., 2010). However, the sensitivity and specificity of these PCR-based direct diagnostic procedures remain to be properly determined on other clinical samples and, for the moment, classical bacteriology should be considered the method of choice for the bacteriological diagnosis of *B. ovis*. By contrast, the use of the Bruce-ladder multiplex PCR (see Chapter 2.4.3) on DNA samples extracted from culture plate colonies is a rapid and highly specific procedure for the proper identification of all *Brucella* species including *B. ovis*.

### 2. Serological tests

The most efficient and widely used tests are the complement fixation test (CFT), the double agar gel immunodiffusion (AGID) test and the indirect enzyme-linked immunosorbent assay (I-ELISA). Several countries have adopted various standard diagnostic techniques for *B. ovis*, but the only test that has been prescribed up to now by the OIE and the European Union (EU) for international trade was the CFT. However, it has been demonstrated that the AGID test shows similar sensitivity to the CFT, and it is a simpler test to perform. Moreover, although international standardisation is lacking, numerous independent validation studies have shown that the I-ELISA is more sensitive than either the CFT or AGID test. AGID test and I-ELISA have been reported as more sensitive than the CFT. Conversely I-ELISA was sometimes reported as a less specific method, but this greatly depends on the protocol used (Estein *et al*., 2002; Nielsen *et al*., 2004; Praud *et al*., 2012).

The International Standard anti-*Brucella ovis* Serum (ISaBoS, International Standard 1985\(^3\)) is the one against which all other standards are compared and calibrated. This reference standard is available to national reference laboratories and should be used to establish secondary or national standards against which working standards can be prepared and used in the diagnostic laboratory for daily routine use.

HS antigens for use in serological tests should be prepared from *Brucella ovis* strain REO 198\(^4\) is CO\(_2\)- and serum-independent.

#### 2.1. Antigens

When rough *Brucella* cells are heat-extracted with saline (hot-saline method, HS), they yield water-soluble antigenic extracts, the major component of which precipitates with sera to rough *Brucella* (Diaz

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3 Obtainable from the OIE Reference Laboratory for Brucellosis in the United Kingdom.

4 Obtainable from the OIE Reference Laboratory for Brucellosis in France.
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& Bosseray, 1973; Myers et al., 1972). For this reason, the HS extract has been referred to as the 'rough-specific antigen' or, when obtained from B. ovis, as the 'B. ovis-specific antigen'. However, chemical characterisation of the HS extract from B. ovis has shown that it is enriched in rough lipopolysaccharide (R-LPS), group 3 outer membrane proteins and other outer membrane components (Riezu-Boj et al., 1986). Thus, the HS extract contains LPS determinants specific for B. ovis, but also additional antigenic epitopes, some of them being shared by rough and smooth Brucella (Santos et al., 1984). Such epitopes account for the cross-reactivity that is sometimes observed with the HS method and sera of sheep infected with B. melitensis or vaccinated with B. melitensis Rev.1 (Riezu-Boj et al., 1986). The HS extract is the most widely and currently used for the serological diagnosis of B. ovis infection. Its water solubility and high content in relevant cell surface epitopes explain its good performance in B. ovis serological tests. However, in areas where B. melitensis infection also exists or vaccination with B. melitensis Rev.1 is applied in sheep, the specificity of the diagnosis with regard to B. ovis has to be carefully interpreted taking into account the results of serological tests for smooth Brucella (Blasco, 2010).

Solid basal non-selective media described in Section B.1.3 are satisfactory for the growth of B. ovis REO 198.

2.1.1. Preparation of HS antigen

i) Exponentially grow the REO 198 B. ovis strain in one of the following ways: for 48 hours in trypticase-soy broth flasks in an orbital incubator at 37 C ± 2 C and 150 rpm; or in Roux bottles of trypticase-soy agar, or other suitable medium; or in a batch-type fermenter as described for B. abortus. Addition of 5% serum to the medium is optional as the REO 198 B. ovis strain is serum-independent.

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

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

iv) After cooling, the suspension is centrifuged (15,000 g, 5°C ± 3°C, 15 minutes) and the supernatant fluid is filtered and dialysed against purified water using 100 times the volume of the suspension, at 4°C; the water should be changed three times over a minimum of 2 days.

v) The dialysed fluid can be ultracentrifuged (100,000 g, 4°C, 6–8 hours), and the sediment is resuspended in a small amount of purified water and freeze-dried. When produced to be used in the CFT, the addition of control process serum replacement II (CPSRII) prior to freeze-drying may assist in stability and anti-complementary activity.

HS is then resuspended either in purified water (for use in the AGID test), veronal buffered saline (for use in the CFT), or carbonate/bicarbonate buffer (for use in the I-ELISA) and titrated accordingly.

If it is to be used in the AGID test, the resuspended HS may be kept at 5 C ± 3 C adding optionally 0.5% phenol as preservative. Freezing and thawing of antigen suspensions should be avoided (Diaz & Bosseray, 1973).

The CFT antigen should be standardised against the ISaBoS to give 50% fixation at a 1/100 serum dilution. It must be emphasised that each CFT antigen batch must be titrated with the CFT procedure that is to be followed for the routine test. Therefore before using a CFT antigen (commercial or in-house) in a particular CFT procedure, the laboratory should ensure that the antigen titre has been established with the same CFT procedure.

In the absence of well-established standardisation rules, the I-ELISA and AGID antigens should be titrated against a set of appropriate positive and negative sera.

2.1.2. Standardisation of the I-ELISA

The following criteria for standardisation of the I-ELISA have been used in a recent work in which the I-ELISA has been validated in comparison with the CFT (Praud et al., 2012):

i) A 1/64 pre-dilution of the ISaBoS made up in a negative serum (or in a negative pool of sera) must give a positive reaction;

ii) A 1/256 pre-dilution of the ISaBoS made up in a negative serum (or in a negative pool of sera) must give a negative reaction.

These criteria still need to be validated through an international ring-trial.
In any case, I-ELISA commercial or in-house kits must have been validated according to Chapter 1.1.5,

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### 2.2. Complement fixation test (the prescribed test for international trade)

There is no standardised method for the CFT, but the test is most conveniently carried out using the

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279 microtitration method. Some evidence shows that cold fixation is more sensitive than warm fixation (Ris et
280 al., 1984), but that it is less specific. Anticomplementary reactions, common with sheep serum, are,
281 however, more frequent with cold fixation.

Several methods have been proposed for the CFT using different concentrations of fresh sheep red blood
284 cells (SRBCs) (a 2–3% suspension is usually recommended) sensitised with an equal volume of rabbit anti-
SRBC serum diluted to contain several times (usually from two to five times) the minimum concentration
287 required to produce 100% lysis of SRBCs in the presence of a titrated solution of guinea-pig complement.
288 The latter is independently titrated (in the presence or absence of antigen according to the method) to
determine the amount of complement required to produce either 50% or 100% lysis of sensitised SRBCs in
289 a unit volume of a standardised suspension; these are defined as the 50% or 100% haemolytic unit of
290 complement (C'H50 or C'H100), respectively. It is generally recommended to titrate the complement before
each set of tests, a macromethod being preferred for an optimal determination of C'H50. Usually, 1.25–2
293 C'H100 or 5–6 C'H50 are used in the test.

Barbital (veronal) buffered saline (VBS) is the standard diluent for the CFT. This is prepared from tablets
available commercially, otherwise it may be prepared according to the formula described elsewhere (see
Chapter 2.4.3). The test sera should be inactivated for 30 minutes in a water bath at 60–63°C, and then
diluted (doubling dilutions) in VBS. The stock solution of HS antigen (2.5–20 mg/ml in VBS) is diluted in VBS
as previously determined by titration (checkerboard titration). Usually, only one serum dilution is tested
(generally 1/10).

#### 2.2.1. Test procedure

Using standard 96-well microtitre plates with round (U) bottom, the technique is usually
performed as follows:

i) Volumes of 25 µl of diluted inactivated test serum are placed in the well of the first and
second rows. The first row is an anti–complementary control for each serum. Volumes of
25 µl of VBS are added to the wells of the first row (anti–complementary controls) to
compensate for lack of antigen. Volumes of 25 µl of VBS are added to all other wells
except those of the second row. Serial doubling dilutions are then made by transferring
25 µl volumes of serum from the third row onwards; 25 µl of the resulting mixture in the
last row are discarded

ii) Volumes of 25 µl of antigen, diluted to working strength, are added to each well except
wells in the first row.

iii) Volumes of 25 µl of complement, diluted to the number of units required, are added to
each well.

iv) Control wells are set up to contain 75 µl total volume in each case; the wells contain

a) diluent only,

b) complement + diluent,

c) antigen + complement + diluent.

A control serum that gives a minimum positive reaction should be tested in each set of
tests to verify the sensitivity of test conditions.

v) The plates are incubated at 37°C ± 2°C for 30 minutes or at 5°C ± 3°C overnight, and a
volume (25 or 50 µl according to the techniques) of sensitised SRBCs is added to each
well. The plates are reincubated at 37°C ± 2°C for 30 minutes.

vi) The results are read after the plates have been centrifuged at 1000 g for 10 minutes at
5°C ± 3°C or left to stand at 5°C ± 3°C for 2–3 hours at least to allow unlysed cells to
settle. The degree of haemolysis is compared with standards corresponding to 0, 25, 50,
75 and 100% lysis. The titre of the serum under test is the highest dilution in which there is
50% or less haemolysis.
2.2.2. Standardisation of the results of the complement fixation test

There is a unit system that is based on the International Standard for anti-Brucella ovis Serum (ISaBoS or International Standard 1985 [see footnote 3]). This serum contains 1000 ICFTU per ml. If this serum is tested in a given method and gives a titre of, for example 200 (50% haemolysis), then the factor for an unknown serum tested by that method can be found from the formula: 1000/200 × titre of test serum = number of ICFTU (International CFT units) of antibody in the test serum per ml. It is recommended that any country using the CFT on a national scale should obtain agreement among the different laboratories performing the test by the same method, to allow the same level of sensitivity and specificity to be obtained against an adequate panel of sera from B. ovis culture positive and Brucella-free sheep. Results should always be expressed in ICFTU, calculated in relation to those obtained in a parallel titration with a standard serum, which itself may be calibrated against the International Standard.

2.2.3. Interpretation of the results

Sera giving a titre equivalent to 50 ICFTU/ml or more are considered to be positive.

2.3. Enzyme-linked immunosorbent assay

Several variations of this assay have been proposed. The assay described here is an indirect I-ELISA using ABTS (2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) as chromogen, but other procedures are also suitable, and several commercial kits are now available.

Tests are performed on 96-well flat-bottomed ELISA plates.

Reagent and serum dilutions are made in PBS, pH 7.2 (± 0.2), with the addition of 0.05% Tween 20 (PBST).

Antigen dilutions are made for adsorption in a carbonate/bicarbonate buffer (pH 9.6 ± 0.2). Plates are washed after antigen coating and between incubations, where appropriate, usually with PBST (see below). The antigen (HS) and conjugate are checkerboard titrated, and dilutions are selected to give the best discriminating ratio between negative and positive standard sera. Secondary antibodies (e.g. anti-ovine IgG [H+L chains]) are usually conjugated to horseradish peroxidase (HRPO), although other enzymes or conjugates (such as recombinant Protein G/HRPO) can be used. A monoclonal antibody to bovine IgG-–HRPO conjugate has also been found to be suitable for use in the I-ELISA (Vigliocco et al., 1997). If a peroxidase conjugate is used, the chromogen, usually ABTS, is diluted in a substrate buffer (composed of sodium citrate and citric acid, see below)5. The substrate, hydrogen peroxide (H$_2$O$_2$), is added to this, and the plates are incubated for 15–30 min at room temperature (22°C ± 4°C). The reaction may be stopped with 1 mM sodium azide or other reagents, and the colour change is read at 405–414 nm (for further details see Chapter 2.4.3).

The antigen used in the I-ELISA is the HS in stock solution at 1 mg/ml in coating buffer, titrated in a checkerboard titration manner, with different dilutions of antigen, conjugate and substrate, against a standard serum or against serial dilutions of a panel of sera from B. ovis culture positive and Brucella-free sheep to determine the most sensitive and specific working concentration. Other antigens have been reported in the literature, in particular R-LPS (Nielsen et al., 2004), but its extraction is cumbersome and dangerous, and it has no particular advantage compared with the HS that is also used in CFT and AGID.

A positive and a negative control are included in each plate. OD ranges to be obtained with these two controls must be established to define the criteria for validating each plate results. The positive control OD is the one to which each test serum OD is compared to establish the final result (negative or positive).

An additional positive serum (internal control) must be included in each plate to validate the repeatability of the test from plate to plate and from day to day.

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5 TMB (3,3',5,5'-tetramethylbenzidine) is also a popular chromogenic substrate for HRP detection in ELISA and is available in several formats. It is not carcinogenic.
2.3.1. Test procedure (example)

i) Microtitre plates of good quality polystyrene (this is important to obtain consistent results since there are differences in adsorption among different brands) are coated by the addition of 100 µl to each well of a predetermined antigen dilution in the adsorption buffer:

**Adsorption buffer** (0.06 M carbonate–bicarbonate buffer, pH 9.6 ± 0.2):

a) Solution A: 0.84 g NaHCO₃ in 10 ml purified water.

b) Solution B: 1.06 g Na₂CO₃ in 10 ml purified water.

Mix 4.53 ml of A with 1.82 ml of B and complete with purified water to 100 ml.

Sealed plates are incubated at 37°C ± 2°C overnight, preferably. Plates are then washed four times with the washing buffer to remove unbound antigen and dried by tapping firmly upside down on an absorbent paper.

**Washing buffer** (0.01 M PBS, pH 7.2 ± 0.2, and containing 0.05% Tween 20):

a) Stock solution:

Solution A: Na₂HPO₄: 10.96 g in 150 ml purified water

Solution B: NaH₂PO₄ (H₂O): 3.15 g in 150 ml purified water (3.5 g in 150 ml purified water if using NaH₂PO₄ 2(H₂O)

b) Mix A and B then complete to 400 ml with purified water.

c) Washing Buffer (PBST): 40 ml of Stock solution + 8.5 g NaCl and complete to 1000 ml with purified water, adding 0.05% Tween 20.

The coated and washed plates can be used immediately or dried and stored at 5°C ± 3°C (the stability in these conditions is usually adequate for at least 1 month). Most of HS batches perform properly when used at working concentrations of 2.5–15 µg/ml in adsorption buffer.

ii) Sera: Dilute test and positive and negative control serum samples (1/100 -1/200 are usually the optimal working dilutions, prepared by the addition of 10 µl of serum to 1–2 ml PBST, respectively). These working serum dilutions are usually the optimal when using either polyclonal or monoclonal anti-IgG conjugates. However, lower working dilutions (usually 1/50) are the optimal when using the protein G-HRPO conjugates (Marin et al., 1998). Add 100 µl/well volumes of samples in duplicate to the microtitre plates. The plates are covered or sealed, incubated at 37°C ± 2°C for 40–60 minutes, and washed three times with the PBST washing buffer.

iii) Conjugate: The optimal working dilution of titrated conjugate (the most widely used are the protein G or polyclonal rabbit anti-sheep IgG (H+L), both coupled to HRPO) in PBST is added (100 µl) to the wells, and the plates covered and then incubated for 40–60 minutes at 37°C ± 2°C After incubation, the plates are washed again three times with PBST.

iv) Substrate: There are several possibilities but the substrate most widely used is usually composed by a 0.1% solution (w/v) of ABTS (2-2’-azinobis 3- ethylbenzthiazoline sulfonic acid, diammonium salt) in citrate buffer containing 0.004% H₂O₂:

**Citrate buffer** (0.05 M, pH 4 ± 0.2):

a) Solution A: 22.97 g citric acid (C₆H₈O₇.H₂O) in 1000 ml purified water.

b) Solution B: 29.41 g sodium citrate (Na₃C₆H₅O₇.2H₂O) in 1000 ml purified water.

Mix 660 ml of A with 470 ml of B and complete to 2000 ml with purified water. Add then a 0.004% of good grade and fresh H₂O₂.

The substrate solution is added (100 µl/well) and the plates incubated for 15–30 minutes at room temperature with continuous shaking

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6 TMB (3,3’,5,5’-tetramethylbenzidine) is also a popular chromogenic substrate for HRP detection in ELISA and is available in several formats. It is not carcinogenic.
v) **Reading and interpreting the results:** Absorbance is read automatically in a spectrophotometer at 405–414 nm. Mean absorbance values may be expressed as percentages of the mean absorbance values of the positive control or, preferably, transformed into I-ELISA units calculated either manually or by using a computer and a curve-fitting program from a standard curve constructed with the series of positive control dilution results. Duplicate readings of each serum should be similar. In case of significant discrepancies, the particular serum should be retested. Before calculating the final results, each plate must be validated taking into account the OD values obtained for the positive and negative controls as well as the transformed OD of the internal control according to pre-established expected ranges of values.

The cut-off threshold to differentiate the positive and negative results should be properly established using the appropriate validation techniques (see Chapter 1.1.5) and avoiding, if possible, cut-off thresholds resulting in inconclusive results. The ISaBoS or the corresponding secondary or national standards should be used to verify or calibrate the particular test method in question as mentioned above.

2.4. Agar gel immunodiffusion test

The AGID test (Blasco, 1990) uses the following reagents: Good grade Noble agar or agarose, sodium chloride (NaCl), and borate buffer (prepared with boric acid [12.4 g]; potassium chloride [14.5 g]; purified water [1600 ml]; adjusted to pH 8.3 ± 0.02 with 0.2 M NaOH solution and made up to 2000 ml with purified water).

2.4.1. Agar gel preparation

Dissolve 1 g of agarose (or Noble agar) and 10 g of NaCl in 100 ml of borate buffer (by boiling while stirring continuously).

On a flat surface, cover clean glass slides with the necessary amount of molten gel to form a bed of 2.5 mm depth (3.5 ml approximately for standard microslides).

After the gel has solidified (15–20 minutes), wells are cut in it using a gel puncher.

The wells should be 3 mm in diameter and 3 mm apart, and should be arranged in a hexagonal pattern around a central well that is also 3 mm in diameter.

The test can be adapted to Petri dishes and other patterns.

2.4.2. Test procedure

Sera to be examined are placed in alternate wells separated by a control positive serum (infection proved by bacteriology), with the antigen at its optimum concentration in the central well.

The results are read after incubation for 24 and 48 hours at room temperature in a humid chamber.

A positive reaction is evidenced by a clearly defined precipitin line between the central well and the wells of the test sera that gives total or partial identity with that of the positive controls.

Precipitin lines not giving total identity may also appear and correspond usually to minor antigenic components of HS extracts (antibodies to these components can also be common in infections due to *B. melitensis* or in case of vaccination with Rev.1). These reactions should also be considered as positive. Before a definitive reading, it is important to wash the slides for 1 h in a 5% sodium citrate solution in purified water to clean unspecific precipitin lines.

The HS (2.5–20 mg/ml) diluted in purified water (optionally containing 0.5% phenol as a preservative) is the most widely used antigen in the AGID test (the preserved antigen can be stored refrigerated for at least 1 month). Dilutions of antigen are tested with a panel of 20–30 sera from rams naturally infected with *B. ovis* and with a panel of *Brucella*-free sheep. The optimum working concentration of antigen is the one giving the clearest precipitation lines with all control sera from *B. ovis*-infected rams, resulting simultaneously negative with the sera from *Brucella*-free animals.

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Chapter 2.7.9. – Ovine epididymitis (*Brucella ovis*)

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C. REQUIREMENTS FOR VACCINES

As both rams and ewes can play a role in the transmission of infection (Blasco, 2010; Grilló et al., 1999), vaccination of both rams and ewes is probably the most economical and practical means for medium-term control of B. ovis in areas with a high prevalence of infection. For long-term control, consideration should be given to the effect of vaccination on serological testing, and B. ovis-free accreditation programmes have to be implemented.

There is no specific vaccine for B. ovis however live B. melitensis strain Rev.1 (described in Chapter 2.7.2) is suitable to stimulate immunity against B. ovis infection (Blasco, 1990). A single standard dose A single standard dose (10^9 colony-forming units) of Rev.1 administered subcutaneously (in a 1 ml volume) or, better, conjunctivally (in a 25–30 µl volume), to 3–5 month-old animals confers adequate immunity against B. ovis. Conjunctival vaccination has the advantage of minimising the intense and long-lasting serological response evoked by subcutaneous vaccination, thereby improving the specificity of serological tests (Blasco, 1990), and facilitating the interpretation of serological results after vaccination. When used in both young and adult males, the safety of the Rev.1 vaccine is adequate enough and side-effects appear to be very rare (Marin et al., 1990; Muñoz et al., 2008). Therefore, in countries with extensive management and high levels of prevalence, it would be advisable to vaccinate both young and healthy adult animals (see Chapter 2.7.2). In countries affected by B. ovis but free of B. melitensis, before using the Rev.1 vaccine account should be taken of possible serological interferences and the conjunctival route should be preferred to minimise this problem. The B. abortus RB51 vaccine has not been proven successful against B. ovis in sheep (Jiménez De Bagües et al., 2005), and despite the promising results obtained with new generation subcellular vaccines (Cassataro et al., 2007; Da Costa Martins et al., 2010; Estein et al., 2009; Muñoz et al., 2006), no alternative vaccines to Rev.1 exist currently.

REFERENCES


induced a better degree of protection against *B. ovis* and a similar degree of protection against *B. melitensis* than Rev. 1 vaccination. *Vaccine*, **25**, 5958–5967.


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

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for ovine epididymitis (Brucella ovis).
CHAPTER 2.8.7.

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME

SUMMARY

Porcine reproductive and respiratory syndrome (PRRS) is characterised by reproductive failure of sows and respiratory problems of piglets and growing pigs. The disease is caused by the PRRS virus (PRRSV), a virus currently classified as a member of the order Nidovirales, family Arteriviridae, genus Arterivirus. The primary target cells of the virus is the alveolar macrophages of the pig, mainly alveolar but also present in other tissues. Two major antigenically different types of the virus exist: Type 1 (previously described as European – EU) and Type 2 (previously North American – NA). Historically Type 1 was restricted to Europe and Type 2 to North America; currently they are spread globally. The virus is primarily transmitted via direct contact infected pigs but also by contact with faeces, urine, semen, fomites and insect vectors (houseflies and mosquitoes). The possibility of aerogenic spread for short distances has also been confirmed. PRRS occurs in most major pig-producing areas throughout the world. The reproductive failure is characterised by infertility, late fetal mummification, abortions, stillbirths, and the birth of weak piglets that often die soon after birth from respiratory disease and secondary infections. Older pigs may demonstrate mild signs of respiratory disease, usually complicated by secondary infections. In 2006, a highly pathogenic PRRSV strain emerged in China (People’s Rep. of) causing high fever (40–42°C) in all age groups, abortions in sows and high mortality in sucking piglets, weaners and growers. No other species are known to be naturally infected with PRRSV.

Identification of the agent: Virological diagnosis of PRRSV virus infection is difficult; the virus can be isolated from tissues such as serum ascitic fluids, or organ samples such as lungs, tonsils, lymph nodes and spleen of affected pigs. As porcine alveolar macrophages are one of the most susceptible culture systems for virus of both antigenic types, these cells are recommended for virus isolation. Recent findings show that porcine monocyte-derived macrophages can also be used for PRRSV isolation and propagation in culture. MARC-145 (MA-104 clone) cells are suitable for isolation of PRRSV Type 2. There is variability between batches of macrophages in their susceptibility to PRRSV. Thus, it is necessary to identify a batch with high susceptibility, and maintain this stock in liquid nitrogen until required. The virus is identified and characterised by immunostaining with specific antiserum or monoclonal antibody. Additional techniques, such as immunohistochemistry and in situ hybridisation on fixed tissues and reverse-transcription polymerase chain reaction, have been developed for laboratory confirmation of PRRSV infection.

Serological tests: A wide range of serological tests is currently available for the detection of serum, oral fluid and meat juice antibodies to PRRSV. The immunoperoxidase monolayer assay and immunofluorescence assay using alveolar macrophages and the indirect immunofluorescence assay uses or MARC-145 cells can be used for the detection of PRRSV-specific antibodies that are usually infected using either the European or the American antigenic type genotype 1 or genotype 2 of the virus, respectively. Both assays can however be designed with both cells and PRRSV virus genotypes. Commercial or in-house enzyme linked immunosorbent assays (ELISA) are now most often used for PRRSV diagnosis. Several commercial ELISAs are specific for both the European and North American genotypes of the virus. An indirect ELISA, a blocking ELISA and a double ELISA that can distinguish between serological reactions to the European and the American Types 1 and 2 have been described. There are also commercial ELISAs specifically designed for detection of PRRSV seroconversion in oral fluid.

Requirements for vaccines: Vaccines can be of value as an aid in the prevention or control of reproductive and respiratory forms of PRRS. Modified live vaccines are not suitable for use in
Porcine reproductive and respiratory syndrome (PRRS) is characterised by reproductive failure of sows and respiratory disease in pigs (Benfield et al., 1999; Zimmerman et al., 2012). The disease PRRS was first recognised in 1987 in the United States of America, and within a few years it became a pandemic. PRRS is caused by the PRRSV virus (PRRSV). It was discovered in 1991 in The Netherlands (Zimmerman et al., 1992) (Wensvoort et al., 1993) and is considered as a member of the order Nidovirales, family Arteriviridae, genus Arterivirus (Faaberg et al., 2012). PRRSV is a single-stranded positive-sense RNA virus and the biology of the virus has been well characterised. Apart from domestic pigs, feral swine and wild boars, no other species are known to be naturally infected with PRRSV (PRRSV). The virus does not pose a zoonotic risk and it is not infectious for human or for cells of human origin. Soon after the discovery of the virus it became apparent that the North American (NA, Type 2 and European (EU, Type 1 PRRSV isolates represented two genotypes with antigenic differences (Larochelle & Magar, 1997c; Magar et al., 1997; Zimmerman et al., 2012). Additional investigations have demonstrated regional differences within each continent. These differences are now becoming blurred as Type 2 PRRSV has been introduced into Europe (in part through the use of a modified live vaccine based on a North American isolate) and Type 1 virus has been discovered in North America. Most PRRSV isolates from South America and much of Asia are of Type 2 and it is assumed these viruses were introduced through the movement of swine and/or semen. Most A reportedly highly virulent strain of Type 2 viruses in South-East Asia (highly pathogenic PRRSV) are characterised by a discontinuous 30 amino acid deletions in the NSP2 region of the genome. However, there is good experimental evidence that these deletions do not determine virulence (Shi et al., 2010a; Zhou et al., 2010).

There is an increasing diversity among strains of the two genotypes, which has been attributed to the high error rate inherent in PRRSV replication (Chang et al., 2002) and recombinations between strains (Murtaugh et al., 2010; Van Vuigt et al., 2004). There have also been recent descriptions of east European strains of Type 1 PRRSV with a high degree of polymorphism, providing further insights into the emergence of the relatively new pathogen of pigs. It was proposed to distinguish subtypes 1, 2 and 3 within Type 1. Moreover, mounting evidence indicates that an additional subtype (subtype 4) might exist (Stadejek et al., 2008; 2013). The effects of such diversity on diagnostics and vaccines are largely unknown, but do raise concerns and should be considered. Subtype 3 Lena and subtype 2 Bor strains have been shown to have higher virulence than subtype 1 strains (Karnyichuk et al., 2010; Stadejek et al., unpublished observations). Trus et al. (2014) showed that subtype 1 modified live vaccine partially protects against challenge with subtype 3 Lena strain. The overall level of diversity within Type 2 does not exceed the one observed for subtype 1, although nine different genetic lineages were identified (Shi et al., 2010b; Stadejek et al., 2013).

The reproductive syndrome is recognised by late-gestation abortions and early or delayed farrowings that contain dead and mummified fetuses, stillborn pigs, and weak-born pigs. An increase in repeat breeders during the acute phase of the epizootic is commonly reported. Infrequently, there are reports of early- to mid-gestation reproductive failure. Most probably the cause of PRRSV-related reproductive disorders is virus-induced damage to the placenta and endometrium (Karnyichuk & Nauwynck, 2013). In boars and unbred replacement gilts and sows, transient fever and anorexia may be observed. The respiratory syndrome is recognised by dyspnoea (thumping), fever, anorexia, and listlessness. Younger pigs are more affected than older animals with boars and sows (unbred) frequently having subclinical infection. An increase in secondary infections is common and mortality can be high. In PRRSV-infected boars and boars that have been vaccinated with live attenuated vaccine, PRRSV can be shed in semen, and changes in sperm morphology and function have been described (Christopher-Hennings et al., 1997). The virus is primarily transmitted directly via contact with infected pigs but also with faeces, urine and semen. It can also be spread indirectly, presumably via aerosol routes, leading to chronic re-infections of herds in swine dense areas, and possibly by mechanical vectors. Gross and microscopic lesions consistent with PRRSV infection have been well described (Halbur et al., 1995; Zimmerman et al., 2012). In general, the lesions are more severe in younger animals than older ones. Differences in virulence between PRRSV isolates within a genotype and between genotypes are believed to have been proved to exist based on field observations and some experimental studies (Halbur et al., 1995; Karnyichuk et al., 2010; Weesendorp et al., 2013). This variability has been reinforced with the emergence in 2006 of a PRRSV lineage in South-East Asia associated with porcine high fever disease, a syndrome causing high mortality in all ages of swine (Tian et al., 2007; Xiao et al., 2014). Although there is now an extensive body of research completed since the discovery of PRRSV, there

A. INTRODUCTION

Pregnant sows and gilts and in boars. Vaccination with modified live virus may result in shedding of viral vaccine in semen and vertical and horizontal transmission between sows and piglets and between vaccinated and non vaccinated pigs. Subsequent vaccine-virus-induced adverse signs have been reported. Modified live virus vaccines can persist in vaccinated animals, herds, and transmission to nonvaccinated animals and subsequent vaccine-virus-induced disease have been reported. Whole virus inactivated vaccines are becoming available too but their efficacy is questioned.
are still many gaps in the knowledge base about the apparent link between PRRSV and other diseases as well as understanding the PRRSV immune response.

### B. DIAGNOSTIC TECHNIQUES

#### Table 1. Test methods available for diagnosis of porcine reproductive and respiratory syndrome and their purpose

<table>
<thead>
<tr>
<th>Method</th>
<th>Population freedom from infection</th>
<th>Individual animal freedom from infection prior to movement</th>
<th>Contribution to eradication policies</th>
<th>Confirmation of clinical cases</th>
<th>Prevalence of infection – surveillance</th>
<th>Immune status in individual animals or populations post-vaccination</th>
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<td>Virus isolation</td>
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<td>RT-PCR</td>
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**Agent identification**

**Detection of immune response**

<table>
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<tr>
<th>Method</th>
<th>Population freedom from infection</th>
<th>Individual animal freedom from infection prior to movement</th>
<th>Contribution to eradication policies</th>
<th>Confirmation of clinical cases</th>
<th>Prevalence of infection – surveillance</th>
<th>Immune status in individual animals or populations post-vaccination</th>
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Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.

Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

RT-PCR = reverse transcription polymerase chain reaction; IHC = immunohistochemistry method; ISH = in-situ hybridisation, ELISA = enzyme-linked immunosorbent assay; IPMA = immunoperoxidase monolayer assay, IFA = immunofluorescence assay.

**1. Identification of the agent**

Identification of PRRSV can be accomplished by virus isolation, the detection of nucleic acids, and the detection of viral proteins. Following infection, swine develop a viraemia and lung infection that can persist for weeks in young pigs and days in adult animals making serum and bronchoalveolar lung lavage ideal samples to collect for detection of PRRSV. Isolation of PRRSV can be difficult as not all virus isolates (especially Type 1 viruses) can easily infect MARC-145 cells and CL-2621, a cell line clones derived from the MA-104 monkey kidney cell line (Kim et al., 1993; Provost et al., 2012; Zimmerman et al., 2012). Interestingly, this continuous cell culture system has been the only one reported to sustain a PRRSV infection. Recent findings show that porcine monocyte-derived macrophages can also be used for PRRSV isolation and propagation in cell culture (García-Nicolás et al., 2014). These can be differentiated in vitro from porcine peripheral blood mononucleated cells (PBMCs) without slaughtering animals, as opposed to collection of the lung for porcine alveolar macrophage (PAM) preparations.

Moreover, several genetically modified cell lines supporting PRRSV replication have been developed including immortalised PAM cell line expressing CD163, immortalised porcine monomyeloid cells, PK-15 expressing CD163 or CD163 and sialoadhesin as well as porcine, feline and baby hamster kidney cells expressing CD163 (Delrue et al., 2010; Provost et al., 2012). Other, non-recombined cell lines permissive for PRRSV infection have also been described (Feng et al., 2013; Provost et al., 2012). PAM will support replication of most, if not all PRRSV isolates.

However, the collection of PAM is not an easy task as only pigs of high health status and less than 8 weeks of age should be used as the PAM source (Wensvoort et al., 1993; Feng et al., 2013). Different batches of PAM are not always equally susceptible to PRRSV; it is thus necessary to test each batch before use. PAM can be stored in liquid nitrogen until needed as described below. Isolation of PRRSV using PAM is a technique that can be
performed in most diagnostic laboratories. This technique should be sensitive for isolation of all PRRSV strains and will be explained in detail. Samples for virus isolation should be stored at 4°C for not more than 48 hours because of the sensitivity of the virus to pH and temperature, otherwise freezing at –70°C is recommended.

One of the most commonly used diagnostic techniques is detection of PRRSV nucleic acid can be accomplished with reverse-transcription polymerase chain reaction (RT-PCR), nested set RT-PCR, and real-time RT-PCR (Drew, 1995b; Kleiboeker et al., 2005; Larochelle & Magar, 1997a; Mardassi et al., 1994; Wasilk et al., 2004; Wernike et al., 2012a, 2012b). The advantages of RT-PCR are high specificity and sensitivity as well as rapid evaluation of a current infection status. However, inactivated virus cannot be differentiated from infectious virus using this technique. RT-PCR-based tests are commonly used to detect nucleic acid in tissues and serum. It has been suggested that oral fluids testing also give reliable results for pen-based diagnosis (Kittawornrat et al., 2010). The above-mentioned assays are also useful when virus isolation is problematic, such as when testing semen (Christopher-Hennings et al., 1997) and when testing tissues partially degraded by autolysis or by heat during transport of specimens for virus isolation. Most of the in-house protocols and currently available commercial kits provide the possibility of multiplex PCR assay has been designed of differentiating isolates of Types 1 and 2 (Kleiboeker et al., 2005; Gilbert et al., 1997; Wernike, 2012a, 2012b). False-negative results related to high genetic diversity, and primer and probe mismatches are the major concern when using RT-PCR. Currently, no single RT-PCR assay is capable of detecting all PRRSV strains, especially within highly diverse east European subtypes of Type 1. The technique is also prone to contamination. Therefore, for interpretation, RT-PCR results should be carefully evaluated and continuous validation based on recently circulating PRRSV strains is strongly recommended (Wernike, 2012a). Reverse-transcription – loop-mediated isothermal amplification (RT-LAMP) is an alternative technique not requiring advanced equipment unlike the real-time RT-PCR (Zimmerman et al., 2012). All of these nucleic acid tests are more rapid than virus isolation and do not require cell culture infrastructure.

Restriction fragment length polymorphism analysis of PCR-amplified products was developed and used for the differentiation of field and vaccine PRRSV isolates (Zimmerman et al., 2012; Wesely et al., 1998), and recently molecular epidemiological studies of PRRSV strains were performed using phylogenetic analyses of specific structural gene sequences. However, high rates of recombination events observed in the field may influence the results of phylogenetic analysis based on short genome fragments. Although seldom used for diagnostic purposes, in situ hybridisation is capable of detecting and differentiating Type 1 and 2 PRRSV genotypes in formalin-fixed tissues (Larochelle & Magar, 1997a). The sensitivity and specificity of these methods for detection of PRRSV genome can be compromised by the very high genetic diversity of PRRSV, especially within Type 1. Immunohistochemistry can be used to identify viral proteins (Halbur et al., 1994; Larochelle & Magar, 1995) and when performed on formalin-fixed tissues enables the visualisation of antigen together with histological lesions (Zimmerman et al., 2012).

1.1. Harvesting of alveolar macrophages from lungs

Lungs should preferably be obtained from SPF pigs or from a herd of pigs that is proven to be free from PRRSV infection. Best results are obtained with pigs that are under 8 weeks of age. The macrophages should be harvested from the lung on the same day that the pig is slaughtered. The lungs should be washed three or four times with a total volume of approximately 200 ml sterile phosphate buffered saline (PBS). The harvested wash fluid is then centrifuged for 10 minutes at 1000 g. The resulting pellet of macrophages is resuspended in PBS and centrifuged (washed) twice more. The final pellet is resuspended in 50 ml PBS, and the number of macrophages is counted to determine the cell concentration. The macrophages can then be used fresh, or can be stored in liquid nitrogen according to standard procedures at a final concentration of approximately 6 × 10⁷ macrophages/1.5 ml. Macrophage batches should not be mixed.

1.2. Batch testing of alveolar macrophages

Before a batch of macrophages can be used it should be validated. This should be done by titrating a standard PRRSV with known titre in the new macrophages, and by performing an immunoperoxidase monolayer assay (IPMA) with known positive and negative sera on plates seeded with the new macrophages. The cells are suitable for use only if the standard PRRSV grows to its specified titre, (TCID₅₀ or 50% tissue culture infective dose). It is recommended that alveolar macrophages and fetal bovine serum (FBS) to supplement culture medium be pestivirus free.

1.3. Virus isolation on alveolar macrophages

Alveolar macrophages are seeded in the wells of flat-bottomed tissue-culture grade microtitre plates. After attachment, the macrophages are infected with the sample. Samples can be sera or ascitic fluids, or 10% suspensions of tissues, such as tonsils, lung, lymph nodes, and spleen. In general, the PRRSV gives a cytopathic effect (CPE) in macrophages after 1–2 days of culture, but sometimes viruses are...
found that give little CPE or give a CPE only after repeat passage. After a period of 1–2 days or once
CPE has been observed, the presence of PRRSV needs to be confirmed by immunostaining with a
specific antiserum or monoclonal antibody (MAb).

1.3.1. Seeding macrophages in the microtitre plates
Defrost one vial containing 6 × 10^7 macrophages/1.5 ml. Wash the cells once with 50 ml PBS
and centrifuge the cell suspension for 10 minutes at 300 g (room temperature). Collect the cells
in 40 ml RPMI (Rose-Peake Memorial Institute) 1640 medium supplemented with 5% FBS and
10% antibiotic mixture (growth medium). Dispense 100 µl of the cell suspension into each well
of a microtitre plate (with one vial of cells, four plates can be seeded at a concentration of
10^5 cells in each well of the plates).

1.3.2. Preparation of sample (serum, ascitic fluid, 10% tissue suspension) dilutions in a dummy
plate
Dispense 90 µl of growth medium into each well of a microtitre plate. Add 10 µl samples to the
wells of rows A and E (duplicate 1/10 dilution). Shake the plates and transfer 10 µl from rows A
and E to rows B and F (1/100 dilution). Shake the plates and transfer 10 µl from rows B and F to
rows C and G (1/1000 dilution). Shake the plates and transfer 10 µl from rows C and G to rows
D and H (1/10,000 dilution). Shake the plates.

1.3.3. Incubation of samples
Transfer 50 µl of the sample dilutions from the dilution plates to the corresponding wells of the
plate with macrophages (first passage). Incubate for 2–5 days and observe daily for a CPE. At
day 2, seed macrophages in new microtitre plates (see above). Transfer 25 µl of the
supernatants from the plates of the first passage to the corresponding wells of the freshly
seeded plates (second passage). Incubate for 2–5 days and observe daily for a CPE.

1.3.4. Reading and interpreting the results
Wells in which macrophages show CPE in the first passage only are considered to be false
positive because of the toxicity of the sample. Wells in which macrophages show CPE in both
passages or in the second passage only are considered to be suspect positive. All wells with
macrophage monolayers that do not show CPE need to be identified as PRRSV negative by
immunostaining with a PRRSV-positive antiserum or MAb. CPE-positive samples need to be
identified as PRRSV positive by culturing CPE-positive supernatant samples, or the original
sample dilutions, for both 24 and 48 hours in macrophages, followed by immunostaining with a
PRRSV-positive antiserum or MAb.

1.3.5. Immunostaining with a PRRSV-positive antiserum or MAb
Infect macrophages with 50 µl of supernatant or tissue sample as described in Section B.2.1,
and grow the infected cells for 24 and 48 hours. Prepare an appropriate dilution of a PRRSV-
positive serum in dilution buffer, and immunostain the macrophages as described in Section
B.2.1 or B.2.2.

2. Serological tests
A variety of assays for the detection of serum antibodies to PRRSV has been described. Serological diagnosis is,
in general, easy to perform, with good specificity and sensitivity, especially on a herd basis. Sera of individual pigs
too often cause difficulties because of nonspecific reactions, but this problem may be solved by resampling the
pig after 2–3 weeks. Serology is generally performed with a binding assay, such as the immunoperoxidases
monolayer assay (IPMA), immunofluorescence assay (IFA), or the enzyme-linked immunosorbent assay (ELISA)
– of which many varieties are described (Albina et al., 1992; Cho et al., 1997; Denac et al., 1997; Houben et al.,
1995; Diaz et al., 2012; Jusa et al., 1996; Sorensen et al., 1998; Venteo et al., 2012; Nodelijk et al., 1996;
Wensvoort et al., 1993; Yoon et al., 1992). These tests are often performed with viral antigen of one antigenic
type, which means that antibodies directed against the other, heterologous antigenic type may be detected with
less sensitivity. A blocking ELISA has been used extensively in Denmark and has been described as a double
ELISA set-up using both Types 1 and 2 virus as antigen and thus it can distinguish between serological reaction
to both types (Sorensen et al., 1998). The first live attenuated vaccine for PRRS based on genotype 2 virus has
been observed to spread to nonvaccinated animals (Botner et al., 1997; Torisson et al., 1998), and subsequent
development in herds of vaccine-virus-induced PRRS reproductive failures has been reported in Denmark (Botner
et al., 1997; Madsen et al., 1998). This is of high importance as Type 2 strains circulate in Europe following Type
2 modified live vaccine use and independent introduction (Botner, Madsen, Balka Stadejek et al., 2013). Reaction
to genotype 2 vaccine-like PRRSV can be anticipated in countries using or having used this vaccine; European
countries may therefore observe reactions and isolation of both antigenic types (Botner et al., 1997; Madsen et al., 1998). The identification of Type 1 strains of PRRSV in the USA and Canada has also been reported (Fang et al., 2004, Kleiboeher et al., 2005), but the prevalence of infections by such strains is not well documented. As both types of PRRSV are globally spread, serological tests should contain antigens from both types. Commercial ELISAs with good sensitivity and specificity are available and have been compared (Diaz et al., 2012, Venteo et al., 2012).

Antibodies to the virus can be detected by antibody-binding assays as early as 7–14 days after infection, and antibody levels reach maximal titres by 30–50 days. Some pigs may become seronegative within 3–6 months, but others remain seropositive for much longer. Antibodies to PRRSV have also been detected in meat juice, muscle transudate and oral fluid. Neutralising antibodies develop slowly and do not reach high titres. They can be detected from 3 to 4 weeks after infection and they can persist for 1 year or more or remain undetected. The use of complement to increase the sensitivity of the serum virus neutralisation test has been reported (Dea et al., 1996, Jusa et al., 1996). Extensive research into the duration of antibody titres after infection has not yet been done, and the results probably depend on the test used. Maternal antibodies have a half-life of 12–14 days, and maternal antibody titre can, in general, be detected until 4–8 weeks after birth, depending on the antibody titre of the sow at birth and the test used. In an infected environment, pigs born from seropositive females can seroconvert actively from the age of 3–6 weeks.

This chapter describes the IPMA in detail as this test can easily be performed in laboratories where virus isolation procedures using macrophages have been established, and can be used with virus of both antigenic types. This assay can also be adapted to the MARC-145 cell line for both the European and North American types (Jusa et al., 1996). An indirect immunofluorescence assay (IFA) using MARC-145 cells can also be performed for PRRSV serology and is included in the present chapter. Commercial ELISAs with good sensitivity and specificity are available and have been compared (Drew, 1995a).

2.1. Detection of antibodies with the immunoperoxidase monolayer assay

Alveolar macrophages are seeded in the wells of microtitre plates. After attachment, the macrophages are infected with PRRSV. The objective is to infect approximately 30–50% of the macrophages in a well so as to be able to distinguish nonspecific sera. After an incubation period, the macrophages are fixed and used as a cell substrate for serology. An alternative method is to use MARC 145 cells instead of macrophage cells. On each plate, 11 sera can be tested in duplicate. Test sera are diluted and incubated on the cell substrate. If antibodies are present in the test serum, they will bind to the antigen in the cytoplasm of the macrophages. In the next incubation step, the bound antibodies will be detected by an anti-species horseradish-peroxidase (HRPO) conjugate. Finally, the cell substrate is incubated with a chromogen/substrate solution. Reading of the test is done with an inverted microscope.

2.1.1. Seeding macrophages in the microtitre plates

i) Defrost one vial containing 6 x 10⁷ macrophages/1.5 ml.

ii) Wash the cells once with 50 ml of PBS and centrifuge the cell suspension for 10 minutes at 300 g (room temperature).

iii) Collect the cells in 40 ml RPMI 1640 medium supplemented with 5% FBS, 100 IU (International Units) penicillin and 100 µg streptomycin (growth medium).

iv) Dispense 100 µl of the cell suspension into each well of a microtitre plate (with one vial of cells, four plates can be seeded at a concentration of 10⁵ cells in each well of the plates).

v) Incubate the plates for 18–24 hours at 37°C in a 5% CO₂ incubator, under humid conditions. Alternatively, use HEPES buffer (N-2-hydroxyethylpiperazine, N-2-ethane-sulphonic acid) in the medium.

Preparation of chromogen solution
Stock solution of chromogen (3-amino-9-ethyl-carbazole [AEC]): (a) 4 mg AEC; (b) 1 ml N,N-dimethyl-formamide. Dissolve (a) in (b) and store the AEC stock solution at 4°C in the dark.

Preparation of chromogen/substrate solution (prepare shortly before use)
Prepare 0.05 M sodium acetate buffer, pH 5.0, as follows: Dissolve 4.1 g sodium acetate in 1 litre distilled water. Adjust the pH to 5.00 with 100% acetic acid. Add 1 ml AEC stock solution to 19 ml of 0.05 M sodium acetate buffer. Add 10 µl 30% H₂O₂ for each 20 ml of chromogen/substrate solution. Filter the solution through a 5 µm filter.
2.1.2. Infection of cells with PRRSV

i) Add to each well 50 µl of a virus suspension containing 10^5 TCID_{50}/ml, but leave two wells uninfected to act as controls.

ii) Incubate the plates for 18–24 hours at 37°C in a 5% CO₂ incubator.

2.1.3. Fixation of the cells

i) Discard the growth medium and rinse the plates once in saline.

ii) Knock the plates gently on a towel to remove excess liquid and then dry them (without lid) for 45 minutes at 37°C.

iii) Freeze the plates (without a lid) for 45 minutes at –20°C. (Plates that are not used immediately for testing must be sealed and stored at –20°C.)

iv) Incubate the cells for 10 minutes at room temperature with cold 4% paraformaldehyde (in PBS). Alternatively the cells could be fixed in ice-cold absolute ethanol for 45 minutes at 5°C or in ice-cold 80% acetone for 45 minutes.

v) Discard the paraformaldehyde and rinse the plates once in saline.

2.1.4. Preparation of serum dilutions in a dilution plate

i) Dispense 180 µl of 0.5 M NaCl with 4% horse serum and 0.5% Tween 80, pH 7.2 (dilution buffer), to the wells of rows A and E of the dummy plate(s).

ii) Dispense 120 µl of dilution buffer to all other wells.

iii) Add 20 µl of the test serum or control sera to the wells of rows A and E (= 1/10 dilution), and shake.

iv) Dilute the sera four-fold by transferring 40 µl from rows A and E to rows B and F, and so on to provide further dilutions of 1/40, 1/160 and 1/640.

2.1.5. Incubation of sera in the plate with fixed macrophages

i) Transfer 50 µl from each of the wells of the dummy plate(s) to the corresponding wells of the plate with the fixed macrophages. Seal the plate(s) and incubate for 1 hour at 37°C.

ii) Discard the serum dilutions and rinse the plate(s) three times in 0.15 M NaCl + 0.5% Tween 80.

2.1.6. Incubation with conjugate

i) Dilute the rabbit-anti-swine (or anti-mouse, if staining isolation plate with MAb) HRPO conjugate to a predetermined dilution in 0.15 M NaCl + 0.5% Tween 80. Add 50 µl of the conjugate dilution to all wells of the plate(s). Seal the plate(s) and incubate for 1 hour at 37°C. Rinse the plates three times.

2.1.7. Staining procedure

i) Dispense 50 µl of the filtered chromogen/substrate (AEC) solution to all wells of the plate(s) (see footnote 3).

ii) Incubate the AEC for at least 30 minutes at room temperature.

iii) Replace the AEC with 50 µl of 0.05 M sodium acetate, pH 5.0 (see footnote 3).

2.1.8. Reading and interpreting the results

If antibodies are present in the test serum, the cytoplasm of approximately 30–50% of the cells in a well are stained deeply red by the chromogen. A negative test serum is recognised by cytoplasm that remains unstained. A serum that reacts nonspecifically might stain all cells in a well (compared with a positive control serum). The titre of a serum is expressed as the reciprocal of the highest dilution that stains 50% or more of the wells. A serum with a titre of <10 is considered to be negative. A serum with a titre of 10 or 40 is considered to be a weak positive. Often nonspecific staining is detected in these dilutions. A serum with a titre of ≥160 is considered to be positive.
2.2. Detection of antibodies with the indirect immunofluorescence assay

Although there is no single standard accepted immunofluorescence assay in use at this time, several protocols have been developed and are used by different laboratories in North America. The IFA can be performed in microtitre plates or eight-chamber slides using the MARC-145 cell line and a MARC-145 cell-line-adapted PRRSV isolate. To prevent cross-reactivity with pestivirus, it is recommended that cells and FBS, to supplement culture medium, be pestivirus free. After an incubation period, PRRSV-infected cells are fixed and used as a cell substrate for serology. Serum samples are tested at a single screening dilution of 1/20 and samples are reported as being negative or positive at this dilution. Each porcine serum to be tested is added to wells or chambers containing PRRSV-infected cells. Antibodies to PRRSV, if present in the serum, will bind to antigens in the cytoplasm of infected cells. Following this step, an anti-porcine-IgG conjugated to fluorescein is added, which will bind to the porcine antibodies that have bound to PRRSV antigens in the infected cells. The results are read using a fluorescence microscope. Microtitre plates may also be prepared for serum titration purposes (see Section B.2.3 below).

2.2.1. Seeding and infection of MARC-145 cells in microtitre plates

i) Add 50 µl of cell culture medium (e.g. Minimal Essential Medium [MEM] containing 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU penicillin and 100 µg streptomycin) without FBS to each well of columns 2, 4, 6, 8, 10 and 12 of a 96-well plate using a multichannel pipettor.

ii) Trypsinise confluent MARC-145 cells (grown in culture flasks) to be used for seeding 96-well microtitre plates and resuspend cells in cell culture medium containing 8% FBS at a concentration of 100,000–125,000 cells/ml. The MARC-145 cells are trypsinised from culture flasks for IFA once a week using trypsin/EDTA (ethylene diamine tetra-acetic acid) and are seeded in culture flasks at a concentration of 250,000 cells/ml. After 4 days in culture flasks, new cell culture medium containing 2% FBS is added for 3 additional days.

iii) Using a multichannel pipettor, add 150 µl of the cell suspension to each well of the 96-well plate.

iv) Dilute PRRSV preparation in MEM without FBS to $10^{2.2}$ TCID$_{50}$/50 µl and distribute 50 µl in each well of columns 1, 3, 5, 7, 9 and 11.

v) Incubate the plates for approximately 48–72 hours at 37°C in a humidified 5% CO$_2$ incubator to obtain a monolayer with approximately 40–50% of the cells infected as determined by indirect immuno-fluorescence. Alternatively, microtitre plates may first be seeded with MARC-145 cell suspensions (e.g. concentration of 100,000 cells/ml in medium supplemented with 5–10% FBS) and incubated for up to 72 hours until they are confluent. Then volumes of 50 µl of PRRSV preparations (e.g. $10^5$ TCID$_{50}$/ml) are added per well and the plates are incubated for an additional 48–72 hours prior to fixation. The use of organic buffers such as HEPES in medium has been suggested to stabilise the pH when CO$_2$ incubators are not available.

2.2.2. Seeding and infection of MARC-145 cells in eight-chamber glass slides

i) Add 500 µl of a MARC-145 cell suspension (e.g. in MEM supplemented with 10% FBS) at a concentration of 100,000 cells/ml to each chamber of eight-chamber glass slides.

ii) Incubate the cells for approximately 48–72 hours at 37°C in a humidified 5% CO$_2$ incubator until they are confluent.

iii) Add to each chamber 50 µl of PRRSV suspension containing $10^5$ TCID$_{50}$/ml and further incubate cells for approximately 18 hours at 37°C in a humidified 5% CO$_2$ incubator. At this time 15–20 infected cells per field of view may be observed by indirect immunofluorescence.

2.2.3. Fixation of the cells

i) Discard the medium, rinse once with PBS and discard the PBS. For chamber slides, remove the plastic chamber walls, leaving the gasket intact.

ii) Add volumes of 150 µl cold (4°C) acetone (80% in water) to each well of the 96-well plate. Incubate the plates at 4°C for 30 minutes. For chamber slides, acetone (80–100%) at room temperature is used to fix the cells for 10–15 minutes at room temperature. Some
manufactured brands of acetone will degrade the chamber slide gasket leaving a film on
the slide. It is recommended to check the acetone before using for routine fixation.

iii) Discard the acetone and dry the plates and slides at room temperature.

iv) The plates can then be placed in a plastic bag, sealed and stored at –70°C until use.
Chamber slides can be kept similarly in slide cases.

2.2.4. Preparation of serum dilutions

i) Dilute serum samples to a 1/20 dilution in PBS (0.01 M; pH 7.2) in separate 96-well plates
(e.g., add 190 µl of PBS using a multichannel pipettor followed by 10 µl of the sera to be
tested).

ii) Include as controls reference PRRSV antibody positive and negative sera of known titre.

2.2.5. Incubation of sera with fixed MARC-145 cells

i) Stored plates are removed from the –70°C freezer and when the plates reach room
temperature rehydrate the cells with 150 µl PBS for a few minutes. Discard the PBS by
inverting the plates and blotting dry on paper towels. Cells of eight-chamber slides are not
rehydrated.

ii) Add volumes of 50 µl of each diluted serum to one well containing the fixed noninfected
cells and to one well containing the fixed infected cells. Add similar volumes for each
serum to a single chamber.

iii) Add volumes of 50 µl of the negative control serum and positive control serum dilutions in
the same manner.

iv) Incubate the plates with their lids on at 37°C for 30 minutes in a humid atmosphere. Slides
should be incubated similarly in boxes or slide trays with a cover.

v) Remove the serum samples and blot the plates dry on paper towels. A total of six washes
using 200 µl of PBS are performed. The PBS is added to each well, followed by inversion
of the plates to remove the PBS. After removing serum samples, slides are rinsed in PBS
followed by a 10-minute wash.

2.2.6. Incubation with conjugate

i) Add volumes of 50 µl of appropriately diluted (in freshly prepared PBS) rabbit or goat anti-
swine IgG (heavy and light chains) conjugated with FITC (fluorescein isothiocyanate) to
each well using a multichannel pipettor. Similar volumes are added to individual chambers.

ii) Incubate plates or slides with their lids on at 37°C for 30 minutes in a humid atmosphere.

iii) Remove the conjugate from the plates and blot the plates dry on paper towels. A total of four washes using PBS are performed as described above. Discard the conjugate from the
slides, rinse in PBS, wash for 10 minutes in PBS and rinse in distilled water. Tap the slides
on an absorbent pad to remove excessive water.

iv) The plates and the slides are read using a fluorescence microscope.

2.2.7. Reading and interpreting the results

The presence of a green cytoplasmic fluorescence in infected cells combined with the absence
of such a signal in noninfected cells is indicative of the presence of antibodies to PRRSV in the
serum at the dilution tested. The degree of intensity of fluorescence may vary according to the
amount of PRRSV-specific antibody present in the serum tested.

Absence of specific green fluorescence in both infected and noninfected cells is interpreted as
absence of antibody to PRRSV in that serum at the dilution tested. The test should be repeated
if the fluorescence is not seen with the use of the positive control sera on infected cells or if
fluorescence is seen using the negative control serum on infected cells. No fluorescence should
be seen on noninfected cells with any of the control sera. Any test serum giving suspicious
results should be retested at a 1/20 dilution and if results are still unclear, a new serum sample
from the same animal is requested for further testing.
2.3. Evaluation of sera for antibody titres by IFA

Microtitre plates and IFA may also be used for serum titration purposes. Up to 16 sera may be titred per 96-well microtitre plate.

2.3.1. Test procedure

i) Seed 96-well microtitre plates with MARC-145 cells and incubate at 37°C in a humidified 5% CO₂ incubator until they are confluent.

ii) Inoculate all wells with the PRRSV preparation except the wells of columns 1, 6 and 11, and incubate the plates at 37°C in a humidified 5% CO₂ incubator for 48–72 hours.

iii) Discard culture medium and rinse the monolayers once with PBS (0.01 M, pH 7.2). Fix the monolayers with cold acetone (80% aqueous solution) for 10 minutes at ambient temperature. Discard the acetone, air-dry the plates and keep the plates with lids at –20°C for short-term storage or –70°C for long-term storage, until use.

iv) Serially dilute sera including a PRRSV-positive control serum using a four-fold dilution in PBS, beginning at 1/16 or 1/20. Dilute a negative control serum at 1/16 or 1/20 dilution. Dispense 50 µl of each dilution (1/16, 1/64, 1/256, 1/1024 or 1/16, 1/80, 1/320, 1/1280) in wells containing viral antigen of columns 2, 3, 4, 5 or 7, 8, 9, 10. For each serum, also dispense 50 µl of dilution 1/16 or 1/20 in control wells of columns 1 and 6. Similarly dispense dilutions of positive and negative control sera in wells of columns 11 and 12.

v) Incubate the plates at 37°C for 30 minutes in a humid chamber. Discard the sera and rinse the plates three times using PBS.

vi) Add 50 µl of appropriately diluted anti-swine IgG conjugated with FITC and incubate plates at 37°C for 30 minutes in a humid chamber. Discard conjugate, rinse plates several times and tap the plates on absorbent material to remove excessive liquid.

2.3.2. Reading and interpreting the results

Following examination with a fluorescence microscope, the titre of a serum is recorded as the reciprocal of the highest serum dilution in which typical cytoplasmic fluorescence is observed. For paired serum samples, a four-fold increase in titre with a 2-week interval is indicative of active infection in an individual animal. No specific fluorescence should be observed with test sera or positive and negative control sera on noninfected control cells. No fluorescence should be seen on infected cells with negative control serum. Specific fluorescence should be observed on infected cells with positive control serum at appropriate dilutions. The IFA end-point may vary among laboratories. Test results may also vary depending on the PRRSV isolate used in the test because of antigenic diversity.

2.4. Detection of antibodies with the enzyme-linked immunosorbent assay

The ELISA is one of the most commonly used techniques for detection of antibodies specific to PRRSV, allowing fast, specific and sensitive confirmation of exposure to the virus. Several laboratories have developed ELISAs (indirect or blocking) for serological testing (Albina et al., 1992; Cho et al., 1997; Denac et al., 1997; Houben et al., 1995; Diaz et al., 2012; Sorensen et al., 1998; Venteo et al., 2012). A double-blocking ELISA format that can distinguish between serological reactions to the European Type 1 and the American antigenic Type 2 has been described (Sorensen et al., 1998). Another study reported the development of an ELISA that allows differentiation of high pathogenic PRRSV strains infections (Xiao et al., 2014). ELISA kits are available commercially to determine the serological status of swine towards PRRSV, also in the oral fluids as a diagnostic matrix (Kitlowski et al., 2010; Venteo et al., 2012). These kits use as antigens either one of the European or the North American PRRSV-types separately or combined antigens of both Types 1 and 2. Their main advantage is the rapid handling of a large number of samples. Commercial ELISAs are available that use recombinant proteins of both PRRSV types as antigens. The potential application of ELISA based on the nonstructural proteins NSP1, NSP2 and NSP7 was also suggested. The performance of NSP7 ELISA was reported to be comparable to commercial ELISA kit. Moreover, it allowed for differentiation of type-specific humoral response and resolved 98% of false-positive results of commercial assay (Brown et al., 2009).
C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Several modified-live (MLV) and inactivated (killed) PRRSV vaccines are licensed and commercially available in many countries for the control of the reproductive and/or respiratory forms of PRRS in the USA and Europe. A killed virus vaccine is licensed as an aid in the reduction of abortions and weak piglets caused by the reproductive form of PRRS. All PRRS vaccines currently licensed in the USA contain the antigenic type 2. In Europe, three MLV vaccines are licensed and available commercially (Murtaugh et al., 2011)—one is based on antigenic type 2 and two others are type 1. It is assumed the most benefit from vaccination occurs when the vaccine virus is more closely related antigenically to the field virus (Larochelle & Magar, 1997b; Scortti et al., 2006). Although vaccination of pigs does not prevent PRRSV infection, it may be helpful in herds experiencing problems with PRRS or herds at high risk of PRRSV infection. The killed vaccines are licensed to be used as an aid in the reduction of abortions and weak piglets caused by the reproductive form of PRRS. MLV vaccines are intended to be used in sows and gilts 3–6 weeks prior to breeding and in piglets from 3 weeks of age or older as an aid in the reduction of diseases caused by PRRS. MLV vaccines are not intended to be used in naïve herds, pregnant sows or gilts or boars of breeding age. Vaccine virus can persist in boars and be disseminated through semen (Christopher-Hennings et al., 1997). MLV vaccine virus may be shed and transmitted to non-vaccinated contact pigs (Torrison et al., 1996; Zimmerman et al., 2012).

Vaccines based on biotechnology are under development but not available yet on the market. Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

The isolate of PRRSV used for vaccine production must be accompanied by a history describing its origin and passage history. The master seed virus (MSV) must be safe in swine at the intended age of vaccination and provide protection against challenge. Isolates for a MLV vaccine must be shown not to revert to virulence after passage in host animals.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The MSV should be free from bacteria, fungi and mycoplasma. The MSV must be tested for and free from extraneous viruses, including transmissible gastroenteritis virus, porcine respiratory coronavirus, porcine epidemic diarrhoea virus, porcine adenovirus, porcine circovirus type 1 and 2, porcine haemagglutinating encephalitis virus, porcine parvovirus, bovine viral diarrhoea virus, reovirus, and rabies virus by the fluorescent antibody technique. The MSV must be free from extraneous virus by CPE and haemadsorption on the Vero cell line and an embryonic swine cell type.

2.2. Method of manufacture

2.2.1. Procedure

The PRRSV is propagated in a continuous cell line African green monkey kidney cell line, such as MARC-145 (clone of MA-104) or Vero cells. Viral propagation should not exceed five passages from the master seed virus (MSV) unless further passages prove to provide protection in swine.

The African green monkey kidney cell line is seeded into suitable vessels. MEM supplemented with FBS is used as the medium for production. Cell cultures are inoculated directly with PRRS working virus stock, which is generally from 1 to 4 passages from the MSV. Inoculated cultures are incubated for 1–8 days before harvesting the culture medium. During incubation, the cultures are observed daily for CPE and bacterial contamination.

Killed virus vaccines are chemically inactivated with either formalin or binary ethylenimine and mixed with a suitable adjuvant. MLV vaccines are generally mixed with a stabiliser before
bottling and lyophilisation. If formalin is used as an inactivant, the final product should be tested for residual formaldehyde concentration, which should not exceed 0.74 g/litre.

### 2.2.2. Requirements for substrates and media

The FBS must be free from pestivirus and antibodies to pestivirus and free from bovine spongiform encephalopathy risk.

### 2.2.3. In-process control

Production lots of PRRSV for MLV and for inactivated (killed) virus vaccines must be titrated in tissue culture for standardisation of the product. Low-titred lots may be concentrated or blended with higher-titred lots to achieve the correct titre.

### 2.2.4. Final product batch tests

Final container samples are tested for purity, safety and potency. MLV vials are also tested for the maximum allowable moisture content.

#### i) Sterility and purity

Samples are examined for bacterial, fungal and pestivirus contamination. To test for bacteria in a MLV vaccine, ten vessels, each containing 120 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The ten vessels are incubated at 30–35°C for 14 days and observed for bacterial growth. To test for fungi, ten vessels, each containing 40 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The vessels are incubated at 20–25°C for 14 days and observed for fungal growth. Killed vaccines require 1.0 ml from ten final container samples be inoculated into the appropriate ten vessels of media.

#### ii) Safety

Safety tests can be conducted in a combination of guinea-pigs, mice or pigs.

#### iii) Batch potency

Final container samples of an MLV vaccine are titrated (log_{10}) in microtitre plates for determination of the titre.

- **Test procedure**
  
  i) Prepare tenfold dilutions from 10^{-1} through 10^{-5} by using 0.2 ml of rehydrated test vaccine and 1.8 ml of MEM. An internal positive control PRRSV should be titrated in the appropriate range.
  
  ii) Inoculate 0.1 ml/well from each dilution into five wells of a 96-well plate containing African green monkey kidney monolayers.
  
  iii) Incubate the plate at 37°C in a CO_{2} atmosphere for 5–7 days.

  iv) Read the plates microscopically for CPE. The internal positive control PRRSV should give a titre within 0.3 log_{10} TCID_{50} from its predetermined mean.

  v) Determine the TCID_{50}/dose by the Spearman–Kärber method. The release titre must be at least 1.2 logs higher than the titre used in the immunogenicity trial. The 1.2 logs include 0.5 logs for stability throughout the shelf life of the product and 0.7 logs for potency test variability.

Killed virus vaccines may use host animal or laboratory animal vaccination/serology tests or vaccination/challenge tests to determine potency of the final product. Parallel-line assays using ELISA antigen-quantifying techniques to compare a standard with the final product are acceptable in determining the relative potency of a product. The standard should be shown to be protective in the host animal.

### 2.3. Requirements for authorisation

#### 2.3.1. Safety requirements

- i) Target and non-target animal safety
Field trial studies should be conducted to determine the safety of the vaccine. Non-vaccinated sentinel pigs should be included at each site for monitoring the shed of the attenuated virus.

ii) Reversion-to-virulence for attenuated/live vaccines

MSV must be shown not to revert to virulence after several passages in host animals, although the definition of virulence with such a virus is difficult. Attenuated PRRSV isolates are known to cause viraemia and will transmit to susceptible animals. The MSV should be shown to be avirulent in weaned piglets and pregnant animals by five serial passages (up to ten passages depending on country) of the MSV through susceptible swine using the most natural route of infection.

iii) Environmental consideration

Not applicable

2.3.2. Efficacy requirements

i) For animal production

In an immunogenicity trial, the MSV at the highest passage level intended for production must protect susceptible swine against a virulent, unrelated challenge strain. For the respiratory form, 3-week-old piglets are vaccinated with the highest passage level of MSV. The piglets are challenged with a virulent isolate of PRRSV 2–16 weeks later to determine protection from respiratory clinical signs of PRRS. To determine protection from the losses caused by the reproductive form of PRRS, vaccinated animals are challenged at approximately 85 days’ gestation. A prevented fraction, the proportion of potential PRRS disease occurrence reduced due to vaccination, is calculated to determine if there is acceptable protection, based on the proposed label claims, in the vaccinated compared with the controls.

Duration of immunity studies are conducted before the vaccine receives final approval. For the respiratory form of PRRS, duration should be shown up to the market age in pigs. Duration of immunity for the reproductive form should be shown through weaning of the piglets.

ii) For control and eradication

Not applicable

2.3.3. Stability

All vaccines are initially given a shelf life of 24 months before expiry. Real-time stability studies are then conducted to confirm the appropriateness of the expiry date.

Multiple batches of MLV vaccines should be re-titrated periodically throughout the shelf-life to determine vaccine variability. The release value should be adjusted if the titres are insufficient or highly variable.

Killed vaccines using in-vivo potency tests should be retested at expiry to demonstrate stability. Parallel-line assays using ELISA antigen-quantifying techniques should demonstrate the stability of the standard.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

None Under development but not available yet on the market.

3.2. Special requirements for biotechnological vaccines, if any

None Not applicable yet.
REFERENCES


Chapter 2.8.7. – Porcine reproductive and respiratory syndrome


interlaboratory ring trial to evaluate real-time reverse transcription polymerase chain reaction detection methods.

* * *


* * *

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

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for porcine reproductive and respiratory syndrome.
**CHAPTER 2.8.8.**

**SWINE-INFLUENZA A VIRUS OF SWINE**

**SUMMARY**

Swine Influenza A viruses of swine (IAV-S) are cause a highly contagious viral infection of pigs. Swine influenza virus (SIV), IAV-S infections cause respiratory disease characterised by coughing, sneezing, nasal discharge, elevated rectal temperatures, lethargy, breathing difficulty, and depressed appetite. In some instances, IAV-S-SIV infections are associated with reproductive disorders such as abortion. Clinical signs and nasal shedding of virus can occur within 24 hours of infection. Morbidity rates can reach 100% with IAV-S-SIV infections, while mortality rates are generally low. Secondary bacterial infections can exacerbate the clinical signs following infection with IAV-S-SIV. Transmission is through contact with IAV-S-SIV-containing secretions such as nasal discharges and aerosols created by coughing or sneezing.

Identification of the agent: Samples for virus identification should be collected within 24–72 hours after development of clinical signs. The animal of choice is an untreated, acutely ill pig with an elevated rectal temperature. Virus can readily be detected in lung tissue and nasal swabs. Oral fluids collected from cotton ropes hung in a pig pen are becoming more popular as a diagnostic specimen due to the ease of sample collection. Virus isolation can be conducted in embryonated chicken fowl eggs and on continuous cell lines. Isolated viruses can be subtyped using the haemagglutination inhibition (HI) and the neuraminidase inhibition tests, or by reverse transcription-polymerase chain reaction assays. Immunohistochemistry can be conducted on formalin-fixed tissue and a fluorescent antibody test can be conducted on fresh tissue. Enzyme-linked immunosorbent assays (ELISA) are commercially available for detection of type A influenza viruses.

Serological tests: Historically, the primary serological test for detection of IAV-S-SIV antibodies is the HI test conducted on paired sera. The HI test is subtype specific. The sera are generally collected 10–21 days apart. A four-fold or greater increase in titre between the first and second sample is suggestive of a recent IAV-S-SIV infection. Additional serological tests that have been described are the agar gel immunodiffusion test, indirect fluorescent antibody test, virus neutralisation, and ELISA. Due to the increasing amount of antigenic diversity in influenza A viruses of swine and the need to use multiple H types in HI assays, there is a general trend towards increasing use of commercially available ELISAs that are not subtype specific.

Requirements for vaccines: Inactivated, adjuvanted IAV-S-SIV vaccines are commercially available. Vaccines may be in the form of a single IAV-S-SIV subtype or may contain multiple IAV-S-SIV subtypes. Vaccines should reflect the current antigenic profile of field viruses, containing subtypes and strains that are changed as needed to assure protection. The finished vaccine must be shown to be pure, safe, potent, and efficacious.

**A. INTRODUCTION**

Swine Influenza A of swine (IAV-S) is a highly contagious viral infection of pigs that can have significant economic impact on an affected herd (Olsen et al., 2005). IAV-S swine influenza virus (SIV) is a type A orthomyxovirus with a segmented RNA genome. The type A swine influenza viruses are further subdivided based on their haemagglutinin and neuraminidase proteins. Subtypes of IAV-S-SIV that are most frequently identified in pigs include classical and avian H1N1, human (hu) H1N1 and H1N2, reassortant (r) H3N2, and rH1N2 (Choi et al., 2004; Gramer, 2007; Marozin et al., 2002; Olsen et al., 2002; Schrader & Süss, 2004). Other subtypes that have been identified in pigs include H1N7, rhH3N1, H2N3, avian (av) H4N6, avH3N3, and avH9N2 (Brown et al., 1997; Karasin et al., 2000a; 2004; Ma et al., 2007; Olsen et al., 2005; Peiris et al., 2001). The H1N1, H1N2 and H3N2...
viruses found in Europe are antigenically and genetically different from those found in America (Brown, 2013; Brown et al., 1998; Castrucci et al., 1993; Done & Brown, 1997; Karasin et al., 2000a; 2000b; 2002; 2004; Noble et al., 1993; Olsen, 2002; Sheerar et al., 1989; Vincent et al., 2009; Webby et al., 2000; 2004; Zhou et al., 1999). Pigs have receptors in their respiratory tract that will bind influenza A viruses of swine, human, and avian species. Consequently, pigs have been called ‘mixing vessels’ for the development of new influenza viruses when influenza A viruses of swine, avian, and/or humans undergo genetic reassortment in pigs. IAV-S infections are described as causing respiratory disease characterised by coughing, sneezing, nasal discharge, elevated rectal temperatures, lethargy, breathing difficulty and depressed appetite. Other agents that may cause respiratory disease in pigs include porcine reproductive and respiratory syndrome virus, Aujeszky’s disease (pseudorabies) virus, porcine respiratory coronavirus, Actinobacillus pleuropneumoniae, Mycoplasma hypopneumoniae and other bacterial agents. However, most of these have other signs that do not mimic IAV-S infections. swine influenza, Actinobacillus pleuropneumoniae, in the acute form of the infection, has clinical signs most similar to IAV-S infections: swine influenza, such as dyspnoea, tachypnoea, abdominal breathing, coughing, fever, depression and anorexia. Clinical signs and nasal shedding of SIV influenza A virus can occur within 24 hours of infection, and shedding typically ceases by day 7–10 after infection. Two forms of the disease occur in swine, epidemic or endemic. In the epidemic form, the virus quickly moves through all phases of a swine unit with rapid recovery, provided there are not complicating factors such as secondary bacterial infections. In the endemic form, clinical signs may be less obvious and not all pigs may demonstrate traditional clinical signs of infection. Morbidity rates can reach 100% with IAV-S-SIV infections, while mortality rates are generally low. The primary economic impact is related to retarded weight gain resulting in an increase in the number of days to reach market weight. Transmission is through contact with IAV-S-SIV-containing secretions such as nasal discharges and aerosols created by coughing or sneezing. Human infections with IAV-S-SIV can occur and a limited number of deaths have been reported (Lindstrom et al., 2012; Myers et al., 2007; Olsen et al., 2002). Precautions should be taken to prevent human infection as described in Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities. Conversely, human influenza A viruses can occasionally be transmitted from animal caretakers to people. Similarly, influenza viruses can also be transmitted occasionally from poultry to pigs as well as from pigs to poultry. In the spring of 2009 a newly identified H1N1 virus was detected in people in the Western Hemisphere. This novel virus was composed entirely of swine genes of which were of swine, avian, and human lineages. The matrix and neuraminidase were from European H1N1 IAV-S swine influenza viruses of avian lineage and the remaining genes were from North American IAV-S swine influenza viruses of swine, avian, and human lineage. The virus spread rapidly throughout the world through human-to-human transmission. Swine cases in both Northern and Southern Hemispheres occurred simultaneously and subsequently the virus has become endemic in many swine populations worldwide. In addition to continued independent circulation in humans, have occurred since the virus was first recognised in humans (http://www.oie.int/eng/en_index.htm). Current information on recent research can be found at a number of websites, including, but not limited to http://www.defra.gov.uk/vla/science/sci_si.htm and http://www.ars.usda.gov/2009H1N1/.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of IAV-S and their purpose

<table>
<thead>
<tr>
<th>Method</th>
<th>Population freedom from infection</th>
<th>Individual animal freedom from infection prior to movement</th>
<th>Contribution to eradication policies</th>
<th>Confirmation of clinical cases</th>
<th>Prevalence of infection surveillance</th>
<th>Immune status in individual animals or populations post-vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agent identification 1</td>
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<td>+++</td>
<td>+++</td>
<td>=</td>
</tr>
<tr>
<td>Virus isolation</td>
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<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>=</td>
</tr>
<tr>
<td>Real-time RT-PCR</td>
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<td>+++</td>
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<td>Conventional PCR</td>
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</tr>
</tbody>
</table>

1 A combination of agent identification methods applied on the same clinical sample may be needed in some situations.
### Chapter 2.8.8. – Influenza A virus of swine

<table>
<thead>
<tr>
<th>Method</th>
<th>Population freedom from infection</th>
<th>Individual animal freedom from infection prior to movement</th>
<th>Contribution to eradication policies</th>
<th>Confirmation of clinical cases</th>
<th>Prevalence of infection – surveillance</th>
<th>Immune status in individual animals or populations post-vaccination</th>
</tr>
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<tbody>
<tr>
<td>HI</td>
<td>±</td>
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<td>ELISA</td>
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</tbody>
</table>

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose; n/a = not applicable.

Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

RT-PCR = reverse-transcription polymerase chain reaction; HI = haemagglutination inhibition; ELISA = enzyme-linked immunosorbent assay. Note that antigen ELISA assays are designed for use in clinically ill animals. Their reliability in clinically healthy animals is questionable.

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1. **Identification of the agent**

Because IAV-S-SIV is a potential human pathogen, all work with potentially infectious tissues, swabs, diagnostic specimens, embryonated eggs, and cell cultures should be done in a class II biological safety cabinet. Additional safety precautions (personal protective equipment) may be considered when working with infected pigs such as the use of respirators during laboratory work and eye protection.

#### 1.1. Culture

1.1.1. **Sample processing**

Lung tissue can be processed for virus isolation in a variety of ways, for example with a mortar and pestle, stomacher, homogeniser, or mincing with a scalpel blade or scissors. Processing of the tissue is done in cell culture medium with antibiotic supplement (e.g. 10 × working strength), at a final concentration of 10–20% weight to volume. Nasal swabs should be collected in cell culture medium or phosphate buffered saline (PBS), supplemented with antibiotics and bovine serum albumin (5 mg/ml). Fetal bovine serum should not be included. Oral fluids may require adjustments to sample processing method used for nasal swabs due to the viscous nature of the specimen and increased propensity for bacterial contamination. Samples should ideally be shipped to a diagnostic laboratory overnight on wet ice, not frozen (see http://offlu.net for guidance on sample collection and sample shipment). Upon receipt at the laboratory, the nasal swabs are vigorously agitated by hand or on a vortex mixer. The nasal swab and lung materials are centrifuged at 1500–1900 g for 15–30 minutes at 4°C. The supernatant is collected and maintained at 4°C until inoculated. If supernatant is to be held for longer than 24 hours before inoculation, it should be stored at –70°C or colder. Lung supernatant is inoculated without further dilution. Nasal swab supernatant can also be inoculated without dilution or diluted 1/3 in cell culture medium. Antibiotics are added to the cell culture medium used for processing and/or the supernatant can be filtered to reduce bacterial contamination, but this may decrease virus titre. For filtration, low protein adsorption membrane, such as PVDF membrane, is recommended to minimise virus loss. As an alternative, the virus preparation may be treated with antibiotics such as gentamicin (100 µg/ml) or penicillin (10,000 units/ml) streptomycin (10,000 units/ml) and 2% fungizone (250 mg/ml) for 30–60 minutes at 4°C prior to inoculating the embryos or cell culture.

1.1.2. **Cell culture virus isolation**

i) Virus isolation can be conducted in cell lines and primary cells susceptible to SIV-influenza A virus infection. Madin–Darby canine kidney (MDCK) is the preferred cell line, but primary swine kidney, swine testicle, swine lung, or swine tracheal cells can be used.

ii) Wash confluent cell monolayers (48–72 hours post-seeding) three times with cell culture medium containing a final concentration of 1 µg/ml of TPCK3-treated trypsin; however, the concentration will depend on the type of trypsin and the cells used (0.3–10 µg/ml may be

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2 One of the listed serological tests is sufficient provided antigens in the HI are representative of viruses known to circulate in the region.

3 TPCK: tosylphenylalanylchloromethane
used). The cell culture medium can be supplemented with antibiotics, but is not supplemented with fetal bovine serum.

iii) Inoculate cell cultures with an appropriate amount of tissue suspension, oral fluids, or swab supernatant. Note: The volume of inoculum will vary with the size of the cell culture container. In general, 100–200 µl are inoculated in each well of a 24-well culture plate, 1 ml in each Leighton tube, and 1–2 ml into a 25 cm² flask.

iv) Incubate inoculated cell cultures for 1–2 hours at 37°C with occasional rocking. When using cell culture containers that are open to the environment, such as culture plates, incubation should be done in a humidified incubator with 5% CO₂.

v) Remove the inoculum and wash the cell monolayer three times with the cell culture medium containing trypsin.

vi) Add an appropriate volume of the cell culture maintenance medium (as noted in ii above) to all containers and incubate at 37°C for 5–7 days with periodic examination for cytopathic effect (CPE). If CPE is not observed at the end of the incubation period, the cell culture container can be frozen at −70°C or colder, thawed, and blind passaged as described above (step iii). If CPE is observed, an aliquot of the cell culture medium can be tested for haemagglutinating viruses or by reverse transcription-polymerase chain reaction (RT-PCR) for conserved influenza virus genes such as nucleoprotein or matrix, and can be collected and used as inoculum for confirmation by the fluorescent antibody technique (see Section B.1.5 below). Cover-slips (Leighton tube, 24-well cell culture plate) or chamber slides with MDCK (or other appropriate cell) monolayer can be inoculated for this purpose. The isolation procedure is as described above (step iii). In some instances, it may be necessary to make tenfold dilutions of the cell culture virus in order to have appropriate CPE on the cover-slip. Influenza subtypes can be determined by the haemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests, or by RT-PCR with primers validated for sensitive and specific amplification of individual HA and NA genes (Chiapponi et al., 2012; Hoffman et al., 2001; Nagarajan et al., 2010; Phipps et al., 2004). However, validation using endemically circulating strains in the region should be done to ensure fitness for purpose of tests since endemic strains of IAV-S may vary genetically between regions.

1.1.3. Egg inoculation (Senne, 1998)

i) Use 10- to 11-day-old embryonated chicken fowl eggs (Senne, 1998).

ii) Inoculate 0.1–0.3 ml of inoculum into the allantoic cavity and amniotic sac; many laboratories only inoculate via the allantoic route with similar sensitivity. Generally, 3–4 eggs are inoculated per sample.

iii) Incubate eggs at 35–37°C for 3–4 days and candle daily. Eggs with embryos that have died within 24 hours of inoculation are discarded (assumed to be trauma-induced deaths associated with the inoculation process).

iv) Refrigerate eggs with embryos that have died later than 24 hours after inoculation. Harvest amniotic and allantoic fluids from eggs with dead embryos and from eggs with viable embryos at the end of the incubation period. All egg materials should be considered to be potentially infectious and should be treated accordingly to prevent IAV-S-SIV exposure to the laboratory worker.

v) Centrifuge fluids at 1500–1900 g for 10–20 minutes at 4°C. Transfer the supernatant to another tube for testing.

vi) Fluids are evaluated for the presence of IAV-S-SIV with the haemagglutination (HA) test (see below).

vii) Repass (up to 1–2 passages) fluids negative for haemagglutinating activity (negative for IAV-S-SIV) in eggs or on cell lines as described above. Isolation may be improved by making tenfold dilutions of the fluid in cell culture medium. Antibiotics may be added to the cell culture fluid.

1.1.4. Haemagglutination test

i) Prepare a 0.5% erythrocyte suspension from male turkey or chicken blood. Dispense whole blood into a tube and add PBS. For example, 10–20 ml whole blood in a 50 ml centrifuge tube to which PBS is added to fill the tube. Gently invert the tube several times to wash the erythrocytes. Centrifuge at 800 g for 10 minutes in a refrigerated centrifuge. Aspirate PBS anduffy coat (white blood cell layer) from the tube. Refill the tube with fresh
Chapter 2.8.8. – Influenza A virus of swine

PBS and resuspend erythrocytes thoroughly. Repeat the washing and centrifugation cycle two additional times. Once washing is complete, add sufficient erythrocytes to PBS to make a 0.5% solution. Certain virus strains agglutinate turkey rather than chicken erythrocytes to greater or lesser degrees. Therefore, it may be necessary to choose the species of erythrocytes based on the strains circulating in a given area. Washed erythrocytes and 0.5% suspensions of erythrocytes can be stored at 4°C for up to 1 week. Discard if haemolysis is observed.

ii) Dispense 50 µl PBS in a row of 8–12 wells on a 96-well V- or U-bottom microtitre plate for each unknown virus. U-bottom plates are generally preferred over V-bottom plates. One additional row of wells should be included for a positive control.

iii) Add 50 µl of undiluted isolate to the first well of each corresponding row.

iv) Serially dilute the isolate with a micropipette set to deliver 50 µl. The resulting dilutions will range from 1/2 (well 1) to 1/2048 (well 11). Well 12 contains PBS only and serves as a cell control.

v) Add 50 µl of 0.5% erythrocyte suspension to each well and agitate the plate to mix thoroughly. Note: keep erythrocytes thoroughly suspended during the dispensing process.

vi) Cover the plate with sealing tape and incubate at room temperature (24°C) or 4°C until a distinct button has formed (30–60 minutes) in the control well.

vii) Wells with complete haem agglutination (positive HA, IAV-S SIV present) will have erythrocytes spread throughout the well in a ‘mat’ type appearance. Wells with a distinct button of erythrocytes at the bottom of the well are negative for haemagglutinating activity (negative for IAV-S SIV). Incomplete HA activity is demonstrated by partial buttons characterised by fuzzy margins or ‘donut-like’ appearance. When interpretation between negative and incomplete inhibition is doubtful, tilt the microtitre plate to about a 45-degree angle for 20–30 seconds and look for streaming, which produces a tear-drop appearance and translucency around the cells in wells with negative hemagglutination. Wells with partial inhibition will not produce a tear drop.

1.2. Typing influenza A viruses of swine (IAV-S) SIV isolates

1.2.1. Haemagglutination inhibition test

i) Dilute reference HA antigens (H1, H3, etc.) to a concentration of 8 HA units (HAU) per 50 µl (4 HAU/25 µl) in 0.01 M PBS, pH 7.2–7.4. Reference antigens should represent what is actively circulating in the region where the pigs are located. For guidance, the OIE Reference Laboratory in the region should be consulted regarding reference antigens.

ii) Standardise unknown influenza A viruses to contain 8 HAU in 50 µl.

iii) Conduct a back titration (HA test) for all unknown isolates and the H subtype antigens to assure that the correct HAUs are present. The back titration is performed as described in the HA procedures except that six well dilutions are used instead of eleven.

iv) Treat each reference serum (specific for an individual HA subtype, and representative of actively circulating viruses in the region) with RDE (receptor-destroying enzyme); add 50 µl serum to 200 µl RDE (1/10 dilution in calcium saline solution equalling 100 units per ml). Incubate overnight (12–18 hours) in a 37°C water bath. Add 150 µl 2.5% sodium citrate solution and heat inactivate at 56°C for 30 minutes. Combine 200 µl treated sample and 25 µl PBS. Note: RDE treatment is recommended as it will reduce nonspecific reactions and will enhance the identification of H1N2 and H3N2 isolates.

v) Remove natural serum agglutinins from the sera by treating diluted serum with 0.1 ml packed, washed erythrocytes per 1 ml diluted serum. Incubate for 30 minutes at room temperature with occasional mixing to keep the erythrocytes suspended. Centrifuge the treated serum at 800 g for 10 minutes and then retain the serum.

vi) Dispense 25 µl of standardised antigen (unknown isolate or positive control antigen) into three wells of a 96-well V- or U-bottom microtitre plate. Add 50 µl of PBS to several wells to serve as an erythrocyte cell control. Note: 25 µl of PBS can be used in place of the 25 µl of standardised antigen.

vii) Add 25 µl of the appropriate reference serum to the first well of the H subtype being evaluated. Serially dilute the antiserum in 25 µl volumes in the antigen wells with a pipette set to deliver 25 µl. Repeat this procedure for each H subtype being evaluated. Note: If 25 µl of PBS was used in place of the 25 µl of standardised antigen in step vi, add 25 µl of standardised antigen to each well containing the reference serum.
vii) Cover plate(s) and incubate at room temperature for 10–30 minutes.

ix) Add 50 µl 0.5% erythrocyte suspension to each well and shake/agitate the plate(s) to mix thoroughly. Keep the erythrocytes thoroughly suspended during the dispensing process.

x) Cover the plate(s) with sealing tape and incubate at room temperature (24°C) or 4°C until a distinct button has formed in the positive control wells (usually 30–60 minutes). Observe the plates after about 20 minutes’ incubation for evidence of haemagglutination as some isolates may begin to elute (detach from erythrocytes) in 30 minutes.

xi) Read test results as described above for the HA test. A sample is considered positive for a specific H subtype if haemagglutination is inhibited. The test is considered valid if the positive reference antigen and its homologous antiserum demonstrate the expected HI titre and the back titration of each antigen (unknown and positive control) is 4 or 8 HAUs. If these conditions are not met, the test should be repeated.

xii) If erythrocytes in the cell control wells do not settle into a well-defined button, check the following as possible causes: incorrect formulation of PBS, excessive evaporation from the plates, erythrocytes too old, or incorrect concentration of erythrocytes.

1.2.2. Neuraminidase inhibition test

Reliable subtype identification based on the NI test is beyond the scope of many laboratories. Reference laboratories should be consulted for N typing of isolates.

1.3. Fluorescent antibody test

1.3.1. Test procedure

i) This technique can be used for tissue sections, cover-slips/slides, or 96-well plates of infected cell monolayers (Vincent et al., 1997). Positive and negative controls should be included with all staining procedures.

ii) Note this technique is highly dependent on use of reference reagents representative of circulating viruses in the region and on skilled readers who can differentiate between positive results and background staining (specificity). This method of virus detection is of lower sensitivity compared with other available assays such as PCR.

iii) Inoculated cells are incubated for an appropriate length of time to allow 10–25% of the cells to become productively infected with virus. Rinse the cover-slip or slide once in PBS, place in 100% acetone for 5–10 minutes and air-dry. Acetone should be used in a vented hood.

iv) Prepare frozen tissue sections on glass slides. Fix the glass slides in acetone for 5–10 minutes and air-dry.

v) Apply conjugate (fluorescein-labelled IAV-S swine influenza antibody) and incubate in a humid chamber at 37°C for 30 minutes. Preferably the conjugate contains Evans blue for counter staining.

vi) Rinse in PBS, pH 7.2, soak for 5–10 minutes in fresh PBS, rinse in distilled water, and air-dry.

vii) Place cover-slips on glass slides, cell side down, with mounting fluid. Remove the rubber gasket from chamber slides and add mounting fluid followed by a glass cover-slip. Mounting fluid followed by a glass cover-slip is also placed over tissue sections on the slide. If 96-well plates are used, mounting medium and cover-slips are not required.

viii) Observe stained slides in a darkened room with the use of an ultraviolet microscope. Cells infected IAV-S-SIV are identified by the presence of bright apple-green fluorescence. It is recommended that the person examining the slides receive training in reading fluorescein-labelled slides as they can be difficult to interpret. Known positive and negative slides should be included when testing unknowns to verify the test procedure worked and to use as a basis for differentiating between positive (IAV-S) staining and negative (background) staining. It is also important to use an antibody that recognises all possible viruses circulating in the area (e.g. a pan-anti-influenza A nucleoprotein antibody).
1.4. Immunohistochemistry (Vincent et al., 1997)

1.4.1. Test procedure

i) Slice formalin-fixed, paraffin-embedded lung in 4-µm thick sections and place on poly-L-lysine-coated slides (alternatively, commercially available charged slides can be used and are deemed superior to lysine-coated slides by some). Positive and negative control tissues should be included with all tests.

ii) Heat slides at 60°C for 15 minutes, deparaffinise, and rehydrate through immersions in decreasing concentrations of ethanol and then in distilled water.

iii) Treat samples with 3% hydrogen peroxide for 10 minutes and rinse twice in distilled water.

iv) Digest samples with 0.05% protease for 2 minutes and rinse twice for 2 minutes in 0.1 M Tris/PBS buffer, pH 7.2, at room temperature.

v) Apply primary mouse anti-IAV-S SIV monoclonal antibody (directed against the viral nucleoprotein) to each slide and incubate at room temperature for 1 hour or overnight at 4°C. Rinse slides with Tris/PBS buffer.

vi) Apply secondary antibody (biotinylated goat anti-mouse antibody) for 10 minutes at room temperature. Rinse with Tris/PBS buffer.

vii) Apply tertiary antibody (peroxidase-conjugated streptavidin) for 10 minutes at room temperature. Rinse with Tris/PBS buffer.

viii) Apply diaminobenzidine tetrahydrochloride solution for 5 minutes at room temperature. Rinse twice in distilled water.

ix) Counterstain slides in Gill’s haematoxylin for 10–30 seconds, wash in water for 2 minutes, dehydrate, clear, and add cover-slips.

x) IAV-S SIV-infected tissues are identified by the presence of brown staining in bronchiolar epithelium and pneumocytes.

1.5. Antigen-capture enzyme-linked immunosorbent assays

Type A antigen-capture enzyme-linked immunosorbent assays (ELISAs) are commercially available for detection of human and animal influenza viruses. These types of assays have been used for detection of IAV-S SIV in lung tissue and nasal swabs (Swenson et al., 2001). The assays are generally available through human health and animal health care companies. These assays tend to be of lower sensitivity compared with other assays such as PCR.

1.6. Polymerase chain reaction

RT-PCR tests have been developed for the diagnosis of swine influenza and for hemagglutinin and neuraminidase typing (Landolt et al., 2004). The highly conserved matrix protein is the best target for screening for infection with IAV-S by RT-PCR. Following the identification of the novel (pandemic) H1N1 in 2009, molecular assays based on an avian influenza matrix real-time PCR (Spackman et al., 2002) were adapted for use in swine (Brookes et al., 2009; Slomka et al., 2010). Modifications to the assay vary by country and a swine influenza reference laboratory should be consulted (http://offlu.net) for the most suitable matrix PCR assay.

The IAV-S real-time RT-PCR procedure described in this chapter targets the matrix (M) gene of Influenza A viruses. The matrix primer/probe set is a quasi-multiplex real-time RT-PCR that uses a single forward primer, probe and two reverse primers. The two reverse primers can generically detect the Eurasian, North American and pandemic 2009 H1N1 matrix lineages.

The real-time RT-PCR uses a one-step procedure. Specific primers are designed to amplify the target region (see Table 2). Non-extendable fluorogenic hydrolysis probes measure the target PCR product formation during each cycle of the PCR reaction. The probes are labelled at the 5’ end with a reporter dye, and non-fluorescing quencher at the 3’ end. Once the probe hybridises to the target sequence, the 5’ nuclease activity of Taq polymerase will hydrolyse the probe, and separate the quencher from the reporter dye. This results in the fluorescence of the separated reporter dye, which is detected spectrophotometrically and recorded. The amount of fluorescence recorded and the cycle number of detection is proportional to the amount of target template in the samples.
For this procedure, it is critical to have separate preparation areas and equipment for nucleic acid extraction, RNA transfer, and master mix preparation. A “clean” area is needed to prepare reagents used for PCR that is free of amplified c-DNA or sample RNA.

### Table 2. IAV-S matrix hydrolysis probe and primer sequences

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Description</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Matrix</strong></td>
<td>M+25° 5’ Primer</td>
<td>5’-AgA TgA gTC TTC TAA CCg Agg TCg-3’</td>
</tr>
<tr>
<td>(any influenza virus)</td>
<td>M+64° Probe</td>
<td>5’-FAM-TCA gqC CCC CTC AAA gCC gA-BHQ-1 -3’</td>
</tr>
<tr>
<td><strong>A virus</strong></td>
<td>M-124° 3’ Primer</td>
<td>5’-TgC AAA AAC ATC TTC CAg TCT CTq-3’</td>
</tr>
<tr>
<td></td>
<td>M-124° SIV 3’ Primer**</td>
<td>5’-TgC AAA gAC ACT TTC CAg TCT CTq-3’</td>
</tr>
</tbody>
</table>

*Refers to the nucleotide position where the 5’ end of the probe or primer anneals to the genome.
**Primer detects the 2009 H1N1 pandemic matrix.

i) Extract nucleic acid from sample. A positive and negative extraction control (PEC and NEC, respectively) will need to be used to confirm that the extraction was successful.

ii) Prepare RT-PCR master mix in a “clean” PCR room (Table 2).

iii) Aliquot 17 µl of reaction mix to each well in a 96-well plate. Transfer 8 µl of RNA template to each reaction in a designated RNA transfer room. When using a 96-well plate, use a support base to protect the bottom of the plate from scratches, finger prints, or picking up particles that could interfere with the optical system and alter the background fluorescence.

a) The following controls will need to be included in the PCR run to verify that the PCR and RNA extraction were successful: positive extraction control (PEC), negative extraction control (NEC), positive amplification (template) control (PAC), and negative amplification (template) control (NAC). PACs are diluted by each diagnostic lab, and must have a C<sub>t</sub> value in the range of 21–29 for the run to be valid.

iv) Place samples in thermocycler and run at appropriate parameters.

v) Analyse results. The PCR run will be valid if:

a) The PAC C<sub>t</sub> value is 21–29
b) The PEC is positive
c) Both NEC and NAC are negative
d) All samples and controls that are positive have “sigmoidal curve”
e) If the above conditions are not met, the test will need to be repeated.

### Table 2. Example Real-time RT-PCR Master Mix for a one step kit

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Concentration</th>
<th>Volume per reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>–</td>
<td>0.83</td>
</tr>
<tr>
<td>2× RT-PCR buffer</td>
<td>1×</td>
<td>12.5</td>
</tr>
<tr>
<td>M+25 5’ primer (20 µM)</td>
<td>200 nM</td>
<td>0.25</td>
</tr>
<tr>
<td>M-124 3’ primer (20 µM)</td>
<td>200 nM</td>
<td>0.25</td>
</tr>
<tr>
<td>M-124 SIV 3’ primer (20 µM)</td>
<td>200 nM</td>
<td>0.25</td>
</tr>
<tr>
<td>25× RT-PCR enzyme mix</td>
<td>1×</td>
<td>1</td>
</tr>
<tr>
<td>M+64 probe (6 µM)</td>
<td>60 nM</td>
<td>0.25</td>
</tr>
<tr>
<td>Detection enhancer (15×)</td>
<td>1×</td>
<td>1.67</td>
</tr>
<tr>
<td>Template</td>
<td>–</td>
<td>8</td>
</tr>
<tr>
<td><strong>Total Reaction Volume</strong></td>
<td>–</td>
<td>25</td>
</tr>
</tbody>
</table>
Table 3. Example thermocycler parameters

<table>
<thead>
<tr>
<th>Stage</th>
<th>Cycles</th>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>10 minutes</td>
<td>45°C</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>10 minutes</td>
<td>95°C</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>denaturation</td>
<td>1 second</td>
<td>94°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>annealing*</td>
<td>30 seconds</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>extension</td>
<td>15 seconds</td>
<td>72°C</td>
</tr>
</tbody>
</table>

*Collection of fluorescence

Viral isolates can be subtyped using conventional methods or by real-time PCR assays that can differentiate the genetically distinct novel H1 viruses from other known strains (Chiapponi et al., 2012). Increasingly, differential real-time PCRs are being used in many regions, conventional H1N1 based on differentiable matrix real-time or N1 real-time assays have also been developed for use in North America. Matrix PCR diagnostic specimens can also be subtyped through use of subtyping PCRs. Samples with high matrix CT's may not be detectable by subtyping PCRs and it may be necessary to attempt virus isolation prior to identifying the subtype. Screening and subtyping PCR reagents are commercially available; however, laboratories need to ensure they will detect currently circulating influenza viruses in their area. In many instances it is necessary to conduct partial or complete gene sequencing of one or more of the IAV-S-SIV genes (i.e. matrix, neuraminidase, haemagglutinin) to ascertain the subtype of detected virus. Furthermore, virus genotyping based on gene sequencing several or all gene segments is increasingly being used to determine and monitor virus diversity. Tests should be validated for the region in which they are to be applied given the worldwide variability in IAV-S. Population-wide validation data for these tests are not currently available.

2. Serological tests

The primary serological test for detection of IAV-S-SIV antibodies is the HI test and it is subtype specific. Reference antigens should reflect what is circulating in the region and as broadly cross reactive as possible with the specific subtype. It should be conducted on paired sera collected 10–21 days apart. A four-fold or greater increase in titre between the first and second sample is suggestive of a recent IAV-S-SIV infection. Additional serological tests that have been described but not commonly used are the virus neutralisation, agar gel immunodiffusion test, and indirect fluorescent antibody test. ELISA technology for detection of IAV-S antibodies has been described in the literature and commercial kits have been marketed (Barbé et al., 2009; Ciacci-Zanella et al., 2010; Lee et al., 1993).

2.1. Haemagglutination inhibition test

2.1.1. Test procedure

i) Dilute reference HA antigens (H1, H3, etc.) to a concentration of 4–8 HAU/25 µl in 0.01 M PBS, pH 7.2.

ii) H1N1 test: Heat inactivated sera for 30 minutes at 56°C. Dilute 1/10 in PBS. Add 0.1 ml packed, washed erythrocytes to 1 ml of heat-inactivated, diluted serum and mix. Incubate at room temperature for 30 minutes with periodic shaking every 10–15 minutes. Centrifuge at 800 g for 10 minutes at 4°C. Note: Sera can be treated with RDE and erythrocytes as described below in step iii as an alternative to heat inactivation and treating with packed erythrocytes. While the use of RDE is encouraged, there may be regional variability in its use for treatment of sera depending on serum specificity for some antigens used in the HI assay.

iii) H1N2 and H3N2 test: Add 50 µl serum to 200 µl RDE (1/10 dilution in calcium saline solution equalling 100 units per ml). Incubate overnight (12–18 hours) in a 37°C water bath. Add 150 µl 2.5% sodium citrate solution and heat inactivate at 56°C for 30 minutes. Combine 200 µl treated sample and 25 µl PBS. Add 50 µl of 50% erythrocytes. Shake and incubate for 30 minutes at room temperature or overnight at 4°C. Centrifuge at 800 g for 10 minutes at 4°C.

iv) Dispense 50 µl treated serum into two wells of a V- or U-bottom 96-well plate. Dispense 25 µl of treated serum into two wells to be used as a serum control. Positive and negative control sera are treated in the same way as the unknown sera.
v) Dispense 25 µl PBS in the serum control wells and all empty wells except two wells identified as the cell control wells. Add 50 µl PBS in the cell control wells.

vi) Make serial twofold dilutions of the serum in 25 µl volumes in the plate and then add 25 µl of appropriate antigen to all test wells except the serum control wells and the cell control wells.

vii) Incubate covered plates at room temperature (24°C) or 4°C for 30–60 minutes.

viii) Add 50 µl of 0.5% erythrocyte suspension to each well, shake, and incubate at room temperature (24°C) or 4°C for 20–30 minutes until a distinct button forms at the bottom of the cell control wells. Keep erythrocytes thoroughly suspended during the dispensing process.

ix) Conduct a HA test using the HI test antigens prior to and simultaneously to conducting the HI test to verify that antigen concentrations are appropriate.

x) For the test to be valid, there should be no haemagglutination in the serum control well, no inhibition of haemagglutination with the negative serum, the positive serum should have its anticipated HI titre and the HA back titration should indicate 4–8 HAU per 25 µl.

2.2. Enzyme-linked immunosorbent assay (Barbé et al., 2009; Ciacci-Zanella et al., 2010; Lee et al., 1993)

ELISA technology for detection of IAV-S antibodies has been described in the literature and ELISAs are available as commercially produced kits.

C. REQUIREMENT FOR VACCINES

1. Background

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

1.1. Rationale and intended use of the product

IAV-S infections Swine influenza can cause significant economic impact for producers because of reduced feed intake during illness resulting in decreased weight gain, increased days to market, and decreased feed efficiency. Where vaccination is practiced, vaccine is used to reduce the economic impact of disease by reducing the severity and duration of clinical signs. In addition, vaccines can reduce the level of viral shedding and the duration of viral shedding. Decreasing the amount of virus shed and duration of shedding can be important in reducing virus transmission while minimising the risk of exposure for pigs and people swine caretakers.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Strains used in vaccine production should be antigenically relevant to IAV-S strains circulating in the field. Haemagglutination inhibition and neutralisation tests demonstrating cross-reactivity between antisera from animals pigs vaccinated with the candidate vaccine strain and current field isolates can be used for the selection.

Identity of the seed should be well documented, including the source and passage history of the organism virus. All defining characteristics such as haemagglutinin and neuraminidase subtype should be established. Haemagglutination inhibition and neuraminidase inhibition by subtype-specific antisera or real-time RT-PCR and sequencing can be used to establish the H and N subtypes. Also, aliquots of the master seed virus (MSV) can be neutralised with specific antiserum, e.g. antiserum produced against H1N1 or H3N2 IAV-S, then inoculated into the allantoic sac of 10-day old embryonated chicken eggs or on susceptible cell lines such as the MDCK cell line. Allantoic fluid or cell culture supernatant is harvested 72–96 hours post-
inoculation and tested for HA activity. Identity is demonstrated by the lack of HA activity in the
neutralised seed, and the presence of HA activity in the non-neutralised seed. Significant
antigenic differences present in a given strain that set it apart from other members of its
subtype, and that purportedly have a beneficial impact on its use as a vaccine, should be
confirmed.

Factors that may contribute to instability during production, such as replication on an unusual cell
line, should be investigated. If production is approved for five passages from the master seed,
then sequencing of the genes for H and N at the maximum passage may be warranted to confirm
the stability of the viral seed.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The purity of the seed and cells to be used for vaccine production must be demonstrated. The
MSV should be free from adventitious agents, bacteria, or Mycoplasma, using tests known to be
sensitive for detection of these microorganisms. The test aliquot should be representative of a
titre adequate for vaccine production, but not such a high titre that hyperimmune antisera are
unable to neutralise seed virus during purity testing. Seed virus is neutralised with monospecific
antisera or monoclonal antibody against IAV-S SIV and the virus/antibody mixture is cultured
on several types of cell line monolayers. Cultures are subpassaged at 7-day intervals for a total
of at least 14 days, then tested for cytopathogenic and haemadsorbing agents.

2.2. Method of manufacture

2.2.1. Procedure

Once the vaccine is shown to be efficacious, and the proposed conditions for production are
acceptable to regulatory authorities, approval may be granted to manufacture vaccine. IAV-S
SIV can be grown in eggs or in cell culture. Selection of a culture method is dependent on the
degree of virus adaptation, growth in medium, rate of mutation, and viral yield in the specific
culture system. IAV-S SIV vaccine products should be limited to five passages from the MSV to
avoid genetic/antigenic variation. Generally, large-scale monolayer or suspension cell systems
are operated under strict temperature-controlled, aseptic conditions and defined production
methods, to assure lot-to-lot consistency. When the virus has reached its maximum titre, as
determined by HA, CPE, fluorescent antibody assay, or other approved technique, the virus is
clarified, filtered, and inactivated. Several inactivating agents have been used successfully,
including formalin or binary ethylenimine. Typically, adjuvant is added to enhance the immune
response.

2.2.2. Requirements for substrates and media

Cells are examined for adventitious viruses that may have infected the cells or seed during
previous passages. Potential contaminants include bovine viral diarrhoea virus, reovirus, rabies
virus, Aujeszky's disease (pseudorabies) virus, transmissible gastroenteritis virus, porcine
respiratory coronavirus, porcine parvovirus, porcine adenovirus, haemagglutinating
encephalomyelitis virus, porcine rotavirus, porcine circovirus, and porcine reproductive and
respiratory syndrome virus. Cell lines on which the seed is tested include: an African green
monkey kidney (Vero) cell line (rabies and reoviruses), a porcine cell line, a cell line of the
species used to propagate the seed, if not of porcine origin, and cell lines for any other
species through which the seed has been passaged. Additionally, a cell line highly permissive
for bovine viral diarrhoea virus, types 1 and 2, is recommended. Bovine viral diarrhoea virus is a
potential contaminant introduced through the use of fetal bovine serum in cell culture systems.

2.2.3. In-process controls

Cell cultures should be checked macroscopically for abnormalities or signs of contamination
and discarded if unsatisfactory. A lot is ready to harvest when viral CPE has reached 80–90%.
Virus concentration can be assessed using antigenic mass or infectivity assays.

2.2.4. Final product batch tests

Vaccine candidates should be shown to be pure, safe, potent, and efficacious.

i) Sterility and purity

During production, tests for bacteria, Mycoplasma, and fungal contamination should be
conducted on both inactivated and live vaccine harvest lots and confirmed on the
completed product (see Chapter 1.1.7 Tests for sterility and freedom from contamination of biological materials).

### ii) Safety

An inactivation kinetics study should be conducted using the approved inactivating agent on a viral lot with a titre greater than the maximum production titre and grown using the approved production method. This study should demonstrate that the inactivation method is adequate to assure complete inactivation of virus. Samples taken at regular timed intervals during inactivation, and then inoculated on to a susceptible cell line or into the allantoic sac of embryonated eggs, should indicate a linear and complete loss of titre by the end of the inactivation process. This is represented as less than one infectious particle per $10^4$ litres of fluids following inactivation.

### iii) Batch potency

During production, antigen content is measured to establish that minimum bulk titres have been achieved. Antigen content is generally measured before inactivation and prior to further processing. Relative potency ELISA, HA, and HI are among the assays that can be used to determine antigen content in final product. It is necessary to confirm the sensitivity, specificity, reproducibility, and ruggedness of such assays.

The potency assay established at the time of the minimum antigen protection study should be used to evaluate new lots for release. The assay needs to be specific and reproducible. It must reliably detect vaccines that are not sufficiently potent. If laboratory animal serology is used instead of swine serology, it should first be demonstrated that vaccination of the laboratory animal induces a specific, sensitive, dose-dependent response as measured in the potency assay and is correlated to protection in swine.

### 2.3. Requirements for authorisation

#### 2.3.1. Safety requirements

### i) Target and non-target animal safety

Final container samples of completed product from inactivated vaccines should be tested in young mice for safety. Generally, healthy pigs of weaning age or older and pregnant sows at any stage of gestation may be safely vaccinated with inactivated IAV-S SIV vaccines. Final product may be evaluated in the host animal using two animals of the minimum age recommended for use, according to the instructions given on the label; the animals are observed for 21 days. Field safety studies conducted on vaccinates, in at least three divergent geographical areas, with at least 300 animals per area, are also recommended.

### ii) Reversion-to-virulence for attenuated/live vaccines

Reversion-to-virulence for live viral vaccines is often demonstrated by back passage through susceptible species. Virus is isolated from the vaccinated animal and the isolated virus is then used to inoculate additional animals. Sequential passage through animals should show that animals remain clinically healthy with no demonstration of typical vesicular stomatitis lesions IAV-S signs.

### iii) Environmental consideration

Inactivated IAV-S SIV vaccines present no special danger to the user, although accidental inoculation may result in an adverse reaction caused by the adjuvant and secondary components of the vaccine. Modified live virus vaccines may pose a hazard to the user depending on the level of inactivation of the virus.

Preservatives should be avoided if possible, and where not possible, should be limited to the lowest concentration possible. The most common preservative is thimerosal, at a final concentration not to exceed 0.01% (1/10,000). Antibiotics may be used as preservatives in IAV-S SIV vaccines but are limited as to kinds and amounts. Also restricted are residual antibiotics from cell culture media that may be present in the final product. For example, the total amount of preservative and residual gentamicin is not to exceed 30 mcg per ml of vaccine.
Vaccine bottles, syringes, and needles may pose an environmental hazard for vaccines using adjuvants or preservatives and for modified live virus vaccines. Instructions for disposal should be included within the vaccine packaging information and based on current environmental regulations in the country of use.

### 2.3.2. Efficacy requirements

#### i) For animal production

A vaccination/challenge study in swine, using homologous and heterologous challenge strains, will indicate the degree of protection afforded by the vaccine. Swine used in vaccination/challenge studies should be free of antibodies against IAV-S\_SIV at the start of the experiments. Vaccination/challenge studies should be conducted using virus produced by the intended production method, at the maximum viral passage permitted, and using swine of the minimum recommended age listed on the label. Initially, lots are formulated to contain varying amounts of viral antigen. The test lot containing the least amount of antigen that demonstrates protection becomes the standard against which future production lots are measured. The most valuable criterion for blind trial evaluations of treatment groups is a statistically significant reduction of virus (titres and duration of shedding) in the respiratory tract of vaccinated pigs. Differences in clinical observations and lung lesions are also among the criteria used in evaluation of a successful trial. If in\_vivo or in\_vitro test methods are to be used to determine the potency of each production lot of vaccine, those assays should be conducted concurrent with the minimum antigen studies in order to establish the release criteria. Combination vaccines containing more than one strain of IAV-S\_SIV are available in some countries. The efficacy of the different components of these vaccines must each be established independently and then as a combination in case interference between different antigens exists.

The duration of immunity and recommended frequency of vaccination of a vaccine should be determined before a product is approved. Initially, such information is acquired directly using host animal vaccination/challenge studies. The period of demonstrated protection, as measured by the ability of vaccinates to withstand challenge in a valid test, can be incorporated into claims found on the vaccine label. Once a suitable potency assay has been identified, should antigenic drift require replacement of strains within the vaccine, strains of the same subtype can be evaluated in either the host animal or a correlated laboratory animal model. However, circulating strains may show significant antigenic differences from the vaccine strain, but the vaccine strain may still provide protection. Also, the vaccine may not protect against a new strain that appears to be antigenically similar to the vaccine. Other factors that play a role include the adjuvant and the antigenic dose. Consequently, it would appear that the efficacy of a vaccine will always have to be evaluated in swine.

If the vaccine is to be used in swine destined for market and intended for human consumption, a withdrawal time consistent with the adjuvant used (generally 21 days) should be established by such means as histopathological examination submitted to the appropriate food safety regulatory authorities.

#### ii) For control and eradication

The same principles apply as for animal production usage. In addition, it should be noted that antibody responses in vaccinated animals may not be differentiated from animals exposed to field virus. Therefore, vaccinated animals will need to be clearly identified if serological methods will be used in conjunction with compatible clinical signs to assess field virus exposure.

### 2.3.3. Stability

Vaccines should be stored with minimal exposure to light at 4°C±2°C, or as approved by the designated regulatory authorities. The shelf life should be determined by use of the approved potency test over the proposed period of viability.

### REFERENCES

Chapter 2.8.8. – Influenza A virus of swine

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Chapter 2.8.8. – Influenza A virus of swine


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

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for swine influenza.
2.9.6. **NIPAH AND HENDRA AND NIPAH VIRUS DISEASES**

### SUMMARY

Hendra virus (HeV) and Nipah virus (NiV) emerged in the last decade of the twentieth century as the causes of outbreaks of respiratory and neurological disease that infected a number of animal species. In 1994, HeV caused severe respiratory disease and the death of 13 horses and a horse trainer at a stable in Brisbane, Australia. NiV appeared in the human population between September 1998 and April 1999 as the cause of fatal encephalitis, after spreading unrecognised as primarily a respiratory or encephalitic infection in Malaysian pigs. NiV appeared in the human population there and was the cause of fatal encephalitis. Over one million pigs were culled to stop spread of the disease. HeV has caused the death of four of seven infected people while it has been reported that there have been **585,400** cases of NiV in humans, with approximately **300,200** deaths, in Malaysia, Singapore, Bangladesh and India. Fruit bats (flying foxes) in the genus Pteropus are natural hosts of both viruses.

HeV infection of horses is can be characterised progressively by high fevers, facial swelling, severe respiratory difficulty and, terminally, copious frothy nasal discharge. Ataxia and myoclonus may also be seen. Some horses display neurological signs while others have presented with colic-like signs. The most common post-mortem observations are dilated pulmonary lymphatic vessels, severe pulmonary oedema and congestion. The underlying lesion is generalised degeneration of small blood vessels in a range of organs. Syncytiotrophoblastic cells containing viral antigen are common in capillaries and arterioles. HeV infection of horses is not uniformly fatal and some horses manifesting clinical signs survive infection. Hendra virus does not appear to be highly contagious among horses, and close contact seems to be necessary for it to spread. Infected horses on pastures have rarely transmitted the virus. However, transmission appears to occur more readily in closed environments such as stables.

NiV infection of pigs is highly contagious, but it was not initially identified as a new disease because morbidity and mortality were not marked and clinical signs were not significantly different from other known pig diseases. Observations made during the outbreak investigation and during experimental infections confirmed that NiV infection of pigs is characterised by fever with respiratory involvement. In animals showing disease, nervous signs have been frequently reported, but many infections are subclinical. Some infected animals display an unusual barking cough. Abortion is reported in sows. Immunohistological lesions are found in either or both the respiratory system (tracheitis and bronchial and interstitial pneumonia) and the brain (meningitis) of infected animals. Syncytiotrophoblastic cells containing viral antigen are seen in small blood vessels, lymphatic vessels and the respiratory epithelium.

Both viruses affect companion animals. Experimentally, HeV causes pulmonary disease in cats similar to that observed in horses while dogs may appear clinically well. Natural infection of dogs with NiV causes a distemper-like syndrome with a high mortality rate; there is serological evidence that some dogs survive infection. Experimentally NiV causes a similar disease to HeV in cats. Syncytiotrophoblastic cells containing viral antigen were demonstrated in both HeV and NiV infections in cats and in NiV infection in dogs.

Infection of humans is from animal contact, usually from an amplifier host rather than directly from the natural, reservoir host: NiV from swine and HeV from horses. However investigations of outbreaks of human NiV in Bangladesh have indicated human infection from Pteropid bats without an intermediary host. Human-to-human transmission has not been seen with HeV or with NiV in
Chapter 2.9.6. – Nipah and Hendra virus diseases

Malaysia and Singapore, but limited human-to-human transmission is suspected in recent outbreaks of NIV in Bangladesh.

HeV and NIV are closely related members of the family Paramyxoviridae. Differences between them and other family members have led to their classification in a new genus, Henipavirus, in the subfamily Paramyxovirinae, family Paramyxoviridae. HeV and NIV are dangerous human pathogens such as are designated in laboratory risk management analyses as requiring biosafety level 4 (BSL4) containment. It is important that samples from suspect animals be transported to authorised laboratories only under biologically secure conditions according to international regulations.

Identification of the agent: Both HeV and NIV may be propagated in a range of cultured cells. Virus isolation from unfixed field samples should be attempted, but only in situations where operator safety can be assured. Identification procedures following virus isolation include immunostaining of infected cells, neutralisation with specific antisera and molecular characterisation. Real-time reverse-transcriptase polymerase chain reaction (RT-PCR) is now available as a diagnostic test.

Viral antigen is present in vascular endothelium, and in the case of NIV in pigs, the respiratory epithelium. A wide range of formalin-fixed tissues can be examined to detect HeV and NIV antigens. Submissions for immunohistochemistry should include samples of brain at various levels including meninges, lung, spleen and kidney. In pregnant animals or in cases of abortion, uterus, placenta and fetal tissues should be included as appropriate. Specimens for virus isolation and molecular detection of virus should be fresh tissues from the same organs, and/or urine, es-throat or nasal swabs.

Serological tests: Virus neutralisation tests (VNT) and enzyme-linked immunosorbent assay (ELISA) are available. VNT is currently accepted as the reference procedure. The ability of antisera to HeV and NIV to cross-neutralise to a limited degree means that a single VNT using either virus does not provide definitive identification of antibody specificity. Neutralising antibodies to HeV and NIV can be differentiated by the greater capacity to neutralise the homologous compared with the heterologous virus. This may not be a major impediment in outbreak situations where the causative agent is known, but serum samples from suspect cases or from areas of the world other than Australia and Malaysia should be subjected to VNT analyses with both HeV and NIV. The serological relationship between HeV and NIV ensures that ELISAs using HeV or NIV antigen can be used to detect antibodies to both viruses.

Requirements for vaccines and diagnostic biologicals: There is a vaccine available for HeV, registered for use in horses in Australia. There is no vaccine currently available for either HeV or NIV.

A. INTRODUCTION

Hendra virus (HeV) and Nipah virus (NIV) are classified in the family Paramyxoviridae subfamily Paramyxovirinae, genus Henipavirus. They have morphological and physicochemical properties typical of paramyxoviruses. The viruses are pleomorphic in shape and enveloped, with herringboned nucleocapsids. Virions are 40–600 nm in diameter. Glycoprotein and fusion protein spikes project through a lipid envelope. HeV and NIV have a non-segmented, single-stranded, negative-sense RNA genome (18.2 kb) consisting of six genes which code for six major structural proteins, namely: N (nucleocapsid protein), P (phosphoprotein), M (matrix protein), F (fusion protein), G (glycoprotein) and L (large protein).

HeV and NIV occur naturally as viruses of fruit bats commonly known as 'flying foxes'. These are members of the genus Pteropus, family Pteropodidae. Antibodies to HeV are found in all approximately 50% of the four Australian pteropus species with seroprevalence varying over time and location (Young et al., 1996). Serological surveys of antibodies to NIV show seroprevalances up to 20% in Malaysian pteropid bats (Epstein et al., 2006; Johara et al., 2005). In one bat colony, seroprevalence steadily increased from 45% to 69% over a 2-year period supporting a model of endemic infection in the populations. Antibodies to NIV or putative closely related viruses have subsequently been detected in pteropid bats in Bangladesh (Hsu et al., 2004), Cambodia (Olson et al., 2002; Reynes et al., 2005), Indonesia (Sendow et al., 2006), Madagascar (Iehle et al., 2007) and Thailand (Wacharapluesadee et al., 2005). HeV has been isolated from Australian flying foxes (Halpin et al., 2000), and NIV from flying foxes from Malaysia and Cambodia (Chua et al., 2002; Reynes et al., 2005). NIV RNA has been detected by polymerase chain reaction (PCR) in pteropid bat urine, saliva and blood in Thailand (Wacharapluesadee & Hemachudha, 2007; Wacharapluesadee et al., 2005). In Ghana, 39% of Eidolon helvum, a non-pteropus fruit bat, had NIV reactive
HeV disease emerged in Brisbane, Australia, in September 1994 in an outbreak of acute respiratory disease that killed 13 horses and a horse trainer (Murray et al., 1995). The virus was initially called equine morbillivirus, but subsequent genetic analyses indicated that it did not resemble morbilliviruses sufficiently closely enough to merit inclusion in that genus. There have been other instances of fatal HeV infection of horses in northern Queensland and further instances of infection of people. Two horses developed an acute disease and died almost 1 month before the Brisbane outbreak, but HeV was determined to be the cause of death only after the horse owner, who probably acquired HeV during necropsy of the horses, died 13 months later with HeV-mediated encephalitis (Rogers et al., 1996). Since then there have been more than 40 outbreaks involving more than 75 horses but with only two outbreaks involving more than three horses. The seven human cases have resulted in four deaths (57%). All infected people have had very close contact with infected body fluids from horses through performing invasive procedures and/or have not worn fully protective PPE. A third horse died in January 1999 with no associated human disease (Field et al., 2000). Two further equine cases occurred in 2004, one confirmed and the other unconfirmed, the latter identified by an associated human infection (Hanna et al., 2006). In 2006 Australia reported two further cases in horses, one in Southern Queensland and one in northern New South Wales. Detailed reports of the three most recent human cases have not been published, but all three people were infected by contact with horses. One of the two cases reported in 2008, as a well as a case the occurred in August 2009, were fatal.

In Malaysia, retrospective studies of archival histological specimens indicate that NiV has caused low mortality in pigs since 1996, but remained unknown until 1999 when it emerged as the causative agent of an outbreak of encephalitis in humans that had commenced in 1998 (Chua et al., 2000; Nor et al., 2000). Unlike respiratory disease caused by HeV in horses, which was frequently fatal but characterised by poor transmissibility (Williamson et al., 1998), respiratory disease caused by NiV in pigs was often subclinical but highly contagious (Hooper et al., 2001), properties that led to rapid virus dispersal through the Malaysian pig population and forced authorities to choose culling as the primary means to control spread (Nor et al., 2000). Over one million pigs were destroyed; 106 of 267 infected humans, mostly pig farmers in Malaysia and abattoir workers in Singapore who had direct contact with live pigs, died of encephalitis (Chua et al., 2000; Paton et al., 1999). Dogs and horses were also infected on infected pig farms during that outbreak (Hooper et al., 2001) but the infections were not epidemiologically significant.

New foci of human NiV disease have subsequently been identified on an annual basis in Bangladesh, with a few outbreaks in West Bengal, in neighbouring India. In outbreaks in 2001 and 2003 an animal source of the human infections was not identified (Hu et al., 2004), but pteropid bats, Pteropus giganteus, were present and had antibodies capable of neutralising Nipah virus. Clustering of cases and time–sequence studies indicated that there is human-to-human transmission but at low levels (Hu et al., 2004). In another outbreak in 2004 in which 27 of 36 infected humans died, epidemiological evidence indicated person-to-person transmission and serological studies identified seropositive fruit bats at the location (Anonymous, 2004). Drinking fresh date palm sap contaminated by fruit bat saliva, urine or excreta has been identified as one possible route of transmission from the wildlife reservoir to humans (Luby et al., 2006). As a result of these ongoing outbreaks it is estimated that across Malaysia, Singapore, Bangladesh and India there have now been up to 585 deaths in NIV in humans, with approximately 334,200 deaths.

NIV and HeV are classified taxonomically as paramyxoviruses in the subfamily Paramyxovirinae, and have been grouped in a separate and new genus, the henipaviruses (Eaton et al., 2006).

Diagnosis of disease caused by henipaviruses is by virus isolation, detection of viral RNA in clinical or post-mortem specimens or demonstration of viral antigen in tissue samples taken at necropsy (Daniels et al., 2001). Detection of specific antibody can also be useful particularly in pigs where NIV infection may go unnoticed. Identification of HeV antibody in horses is less useful because of the high case fatality rate of infection in that species. Human infections of both HeV and NIV have been diagnosed retrospectively by serology. Demonstration of specific antibody to HeV or NIV in either animals or humans is of diagnostic significance because of the rarity of infection and the serious zoonotic implication of transmission of infection.

The henipavirus genus is expanding, with new viruses recently identified. Cedar virus was isolated from the urine of pteropid bats in Australia, but it remains to be seen if it has the capacity to spill over to other species, and if so, cause disease (Marsh et al., 2012). A number of other henipa-like viruses have been detected by PCR and sequencing, but have not yet been isolated by traditional virus isolation techniques (Wu et al., 2014).
Chapter 2.9.6. – Nipah and Hendra virus diseases

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of henipaviruses and their purpose

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Agent identification

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Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.

Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

RT-PCR = reverse-transcription polymerase chain reaction; IHC = Immunohistochemistry; IFA = Indirect fluorescent antibody; ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation test.

1. Laboratory biosafety

HeV and NiV are classified as risk group 4 agents for human infection, as they are dangerous human pathogens with a high case fatality rate and for which there is no human vaccination or effective antiviral treatment (WHO, 2004). All laboratory manipulations with live cultures (including serological tests using live virus) or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities). This would generally be at Biosafety Level (BSL) 4 as defined by WHO (2004). Primary virus isolation from suspect samples may of necessity be conducted under BSL3 conditions but as soon as suspected growth is detected the culture should be safely inactivated or transferred to BSL4. Virus propagation should always be at BSL4. See further guidance below under virus isolation.

2. Identification of the agent

2.1. Virus isolation and characterisation

Virus isolation greatly facilitates identification procedures and definitive diagnosis should be undertaken where operator safety can be guaranteed. Isolation is especially relevant in any new case or outbreak, particularly in countries or geographical areas where infection by HeV or NiV has not been previously documented. However, molecular detection techniques which do not require handling of live virus, can identify the presence of viral genome in samples. Implication of wildlife species as natural hosts of the viruses requires positive serology, PCR or virus isolation from wild-caught animals (Daniels et al., 2007).

1 A combination of agent identification methods applied on the same clinical sample specimen is recommended.
2 One of the listed serological tests is sufficient.
1.1.3. Methods of identification

The serological cross reactivity between HeV and NiV means that polyclonal antibodies (MAbs) are currently not available to give a rapid diagnostic result. At present the Henipavirus genus consists of HeV and NiV and there are no known antigenically related viruses. The serological cross reactivity between HeV and NiV means that polyclonal antiserum to either virus or mono-specific antisera to individual proteins of either virus, will fail to differentiate between HeV and NiV. Monoclonal antibodies (MAbs) are currently being generated and tested to fulfill this function both in primary identification of the virus.

1.1.3. Methods of identification

i) Immunostaining of fixed cells

The speed with which HeV and NiV replicate and the high levels of viral antigen generated in infected cells make immunofluorescence a useful method to rapidly identify the presence of henipaviruses using either anti-NiV or anti-HeV antiserum. At present the Henipavirus genus consists of HeV and NiV and there are no known antigenically related viruses. The serological cross reactivity between HeV and NiV means that polyclonal antiserum to either virus or mono-specific antisera to individual proteins of either virus, will fail to differentiate between HeV and NiV. Monoclonal antibodies (MAbs) are currently being generated and tested to fulfill this function both in primary identification of the virus.
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upon isolation and for use in immunohistochemical examination of tissues from suspect cases.

Test procedure

Under BSL4 laboratory conditions, monolayers of Vero or RK-13 cells grown on glass cover-slips or in chamber slides are infected with the isolated virus, and the monolayers are examined for the presence of syncytia after incubation for 24–48 hours at 37°C. It is recommended that a range of virus dilutions (undiluted, 1/10, 1/100) be tested because syncytia are more readily observed after infection at low multiplicity. Once visible syncytia are detected, infected cells are fixed by immersion in a vessel filled completely with acetone. The vessel is sealed and surface sterilised prior to removal to a less secure laboratory environment, for example BSL2, where the slides are air-dried. Viral antigen is detected using anti-HeV or anti-NiV antiserum and standard immunofluorescent procedures. A characteristic feature of henipavirus-induced syncytia is the presence of large polygonal structures containing viral antigen. These are observed most readily with monospecific and MAbs to the nucleocapsid protein N and phosphoprotein P.

Immunoelectron microscopy

The high titres generated by HeV and NiV in cells in vitro permits their visualisation in the culture medium by negative-contrast electron microscopy without a centrifugal concentration step. Detection of virus–antibody interactions by immunoelectron microscopy provides valuable information on virus structure and antigenic reactivity, even during primary isolation of the virus. Other ultrastructural techniques, such as grid cell culture (Hanna et al., 2006), in which cells are grown, infected and visualised on electron microscope grids, and identification of replicating viruses and inclusion bodies in thin sections of fixed, embedded cell cultures and infected tissues complement the diagnostic effort. The details of these techniques and their application to the detection and analysis of HeV and NiV have been described (Hyatt et al., 2001).

1.2. Virus neutralisation: differentiation of HeV and NiV

Neutralisation tests rely on quantification methods and three procedures are available to titre HeV and NiV. In the traditional plaque and microtitre assay procedures, the titre is calculated as plaque forming units (PFU) or the tissue culture infectious dose capable of causing CPE in 50% of replicate wells (TCID50), respectively.

In an alternative procedure, the viruses are titrated on Vero cell monolayers in 96-well plates and after 18–24 hours, foci of infection are detected immunologically in acetone-fixed cells using anti-viral antiserum (Crameri et al., 2002). The virus titre is expressed as focus-forming units (FFU)/ml.

Neutralisation assays using these three methods are described below. A virus isolate that reacts with anti-HeV and/or anti-NiV antisera in an immunofluorescence assay is considered to be serologically identical to either HeV or NiV if it displays the same sensitivity to neutralisation by anti-HeV and anti-NiV antisera as HeV or NiV. Anti-HeV antiserum neutralises HeV at an approximately four-fold greater dilution than that which neutralises NiV to the same extent. Conversely, anti-NiV antiserum neutralises NiV approximately four times more efficiently than HeV (Chua et al., 2000). Virus quantification procedures should be conducted at BSL4. A new version of the differential neutralisation test has been recently described, which avoids the use of infectious virus by the use of ephrin-B2-bound biospheres (Bossart et al., 2007). Although the test has yet to be formally validated, it appears to have the potential to be a screening tool for use in countries without BSL4 facilities.

1.2.1. Microtitre neutralisation Plaque reduction

This procedure is dependent on the availability of anti-serum, specific for HeV and NiV, as well as stock viruses. Stock HeV and NiV and the unidentified henipavirus are diluted in media and replicates of each virus containing approximately 100 PFU in 50–100 µl are mixed with an equal volume of either Eagle’s minimal essential media (EMEM) or a range of dilutions of anti-HeV or anti-NiV antiserum in EMEM. The virus–antiserum mixtures are incubated at 37°C for 45 minutes, adsorbed to monolayers of Vero cells at 37°C for 45 minutes and the number of plaques determined by traditional plaque assay procedures after incubation at 37°C for 3 days.
1.3. Molecular methods – detection of nucleic acid

iii) Immune plaque assay

Vero cells (2 × 10^4 in 200 µl medium/well) are added to flat-bottom microtitre plates grown overnight at 37°C. Stock HeV and NiV and the unidentified Henipavirus are diluted and replicates containing about 60 FFU/50 µl are mixed with an equal volume of either EMEM or a range of dilutions of anti-HeV and anti-NiV antisera diluted in EMEM. Virus-antiserum mixtures are incubated for 45 minutes at 37°C and adsorbed to Vero cell monolayers for 45 minutes at 37°C with 5% CO₂. Virus-antiserum mixtures are removed, 200 µl EMEM is added to each well and incubation is continued at 37°C. After 18-24 hours the culture medium is discarded and plates are immersed in cold, absolute acetone for 10 minutes and then placed in plastic bags, which are filled with acetone, heat sealed and surface sterilised with 4% (v/v) lysol during removal from the BSL4 laboratory. Glutaraldehyde can also be used for sterilisation at concentrations as low as 0.1% for 24 hours. It is recommended that each laboratory determine the concentration of gluteraldehyde required for sterilisation within the time frame required. Acetone-fixed plates are air-dried, the wells are blocked with phosphate buffered saline (PBS) containing 0.05% Tween 20 and 2% skim milk powder, and incubated for 30 minutes at 37°C with antiserum to either HeV or NiV or a monospecific antiserum to a virus protein. Anti-viral antibody binding to syncytia can be detected using alkaline phosphatase-conjugated species-specific antibody and the substrate 5-bromo-4-chloro-3-indolyl phosphate and p-nitro blue tetrazolium substrate (NBT/BCIP; Promega, Catalog number S3771). When purple plaques appear against a clear background (10-30 minutes), the substrate is removed and the plates are rinsed with distilled water and air-dried. Plaques are counted using a magnifying glass.

1.3. Nucleic acid based recognition methods

The complete genomes of both HeV and NiV have been sequenced (Wang et al., 2001), and as more isolates come to hand their sequences have been deposited on Genbank. PCR-based methods are commonly used to detect virus. They have the biosafety advantage of not propagating live infectious virus and they have been validated in a number of laboratories. They are also highly sensitive and specific.

1.3.1. Real-time reverse transcription polymerase chain reaction

A particularly sensitive and useful approach to the detection of henipavirus genome in specimens is real-time RT-PCR (see Table 2). This method has the biosafety advantage of not propagating live infectious virus. Test methods and primers used depend on the technology platform and associated chemistry being used in individual laboratories (Mungall et al., 2006; Wacharapluesadee & Hemachudha, 2007). The HeV M gene (Smith et al., 2001) and N gene (Feldman et al., 2009) TaqMan assays are the primary tests for Hendra virus disease diagnosis. The virus-specific reagents used in one such assay (Mungall et al., 2006) based on TaqMan chemistry are as follows:

Table 2. Real-time RT PCR (Taqman) assays for the detection of HEV and NIV

<table>
<thead>
<tr>
<th>Assay</th>
<th>Oligo</th>
<th>Name</th>
<th>Primer sequence (5’–3’)</th>
<th>Probe label (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeV TOM M</td>
<td>Forward</td>
<td>HeV M 5755F</td>
<td>CTT-CGA-CAA-AGA-CGG-AAA-CAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>HeV M 5823R</td>
<td>CCA-GCT-CGTT-GCA-AAA-TT</td>
<td></td>
</tr>
<tr>
<td>Hendra</td>
<td></td>
<td>HeV M 5778P</td>
<td>TGG-CAT-CTT-TGC-TCC-ATC-TGG-CCA-TGG-CCA-TGG</td>
<td>FAM-TAMRA</td>
</tr>
</tbody>
</table>

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1.3.2. Conventional RT-PCR and Sanger sequencing

Two semi-nested conventional PCR assays, targeting the M gene and the P gene, can also be used for the detection of HeV. These two assays are used as supplementary tests to confirm the results from the TaqMan assays when unusual/atypical results arise from the TaqMan assays. They are also used for characterisation of detected HeVs when followed by Sanger (di-deoxy) sequencing using the same primers (see Table 3).

Table 3. Primers used for conventional PCR and sequencing of HEV

<table>
<thead>
<tr>
<th>Target</th>
<th>Assay</th>
<th>Type</th>
<th>Name</th>
<th>Primer Sequence (5'-3')</th>
<th>PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M gene</td>
<td>Primary PCR</td>
<td>Forward</td>
<td>HeV M 5481F</td>
<td>GCC-CGC-TTC-ATC-ATC-TCT-T</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
<td>HeV M 5781R1</td>
<td>CCA-CTT-TGG-TTC-GTG-CTT-TG</td>
</tr>
<tr>
<td></td>
<td>Semi-nested PCR</td>
<td>Forward</td>
<td>HeV M 5481F</td>
<td>GCC-CGC-TTC-ATC-ATC-TCT-T</td>
<td>211 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
<td>HeV M 5691R2</td>
<td>TGG-CAT-CTT-TCA-TGC-ATC-TCG-G</td>
</tr>
<tr>
<td></td>
<td>P gene</td>
<td>Primary PCR</td>
<td>Forward</td>
<td>HeV P 4464F1</td>
<td>CAG-GAG-GTG-GCC-AAT-ACA-GT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
<td>HeV P 4798R</td>
<td>GAC-TTG-GCA-CAA-CCC-AGA-AT</td>
</tr>
<tr>
<td></td>
<td>Semi-nested PCR</td>
<td>Forward</td>
<td>HeV P 4594F2</td>
<td>TCA-ACC-ATT-CAT-AAC-CCG-TCA-G</td>
<td>205 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse</td>
<td>HeV P 4798R</td>
<td>GAC-TTG-GCA-CAA-CCC-AGA-AT</td>
</tr>
</tbody>
</table>

i) PCR conditions

a) Primary RT-PCR

1× 48°C for 30 minutes, 94°C for 2 minutes
40× 95°C for 30 seconds, 53°C for 30 seconds, 68°C for 45 seconds
1× 68°C for 7 minutes

b) Semi-nested PCR

1× 95°C for 5 minutes
30× 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 45 seconds
1.4. Henipavirus antigen detection in fixed tissue — immunohistochemistry

Immunohistochemistry is also a useful test in HeV and NiV detection. Performed on formalin-fixed tissues or formalin-fixed cells, it is safe and has allowed retrospective investigations on archival material. As virus replication and the primary pathology occur in the vascular endothelium (Hooper et al., 2001), there is a wide range of tissues in which HeV and NiV antigen can be detected (Daniels et al., 2001). It is thought that HeV antigens may be cleared from lung tissue early in the course of infection, and so the sample submitted should include a range of tissues, not just lung. HeV antigen has been detected in the kidney of a horse 21 days post-infection (Williamson et al., 1998) and so this organ should always be submitted. Ideally a submission for immunohistochemistry would include samples of the brain at various levels, lung, mediastinal lymph nodes, spleen and kidney. In pregnant animals the uterus, placenta and fetal tissues should be included.

A range of antisera to HeV and NiV may be used in immunohistochemical investigations of HeV- and NiV-infected tissues, but rabbit antisera to plaque-purified HeV and NiV have been found to be particularly useful. Some MAbs are also available. The Nipah Virus Pathology Working Group has described a detection system (Wong et al., 2002). A biotin-streptavidin peroxidase-linked detection system has also been used successfully (Hooper et al., 2001). The following detection system is an anti-rabbit/anti-mouse dextran polymer-linked reagent conjugated with alkaline phosphatase.

Immunohistochemistry is a powerful tool that allows the visualisation of viral antigen within cell and tissue structures. Nucleoprotein viral antigen in usually located within particulate structures of variable size and form within the cytoplasm. Because of the morphological aspect to the interpretation, colour signal can be effectively evaluated for its specificity. The test is done on formalin-fixed tissues, allowing the procedure to be done safely under non-microbiologically-contained conditions.

Henipavirus antigen replicates in a range of cell types, including endothelium, vascular smooth muscle, lung parenchyma, kidney glomeruli, neuron cell bodies, lymphoid tissues and connective tissues (Hooper et al., 2001; Marsh et al., 2011; Middleton et al., 2002; Munhall et al., 2006). Antigen is particular dense in syncytia and in macrophages within lesions. Therefore, suitable tissues for diagnosis of henipavirus infection include lung, brain, lymph nodes, spleen and kidney. In the absence of these tissues, it is worthwhile examining any tissue type, as antigen can be found in occasional blood vessels throughout the vascular bed. Unless full protective clothing can be worn and suitable disinfection protocols be implemented, it is safer to remove only small pieces of tissue through ‘keyhole’ sampling. Lung tissue and sub-mandibular lymph nodes are good tissues to remove in this manner.

Rabbit polyclonal antisera raised against recombinant henipavirus nucleoprotein are highly reliable for use as primary antibodies for diagnostic immunohistochemistry. Detection of phosphoprotein antigens is also suitable for diagnostic purposes, although phosphoprotein tends to be less expressed than...
nucleoprotein. There are various secondary detection systems on the market that can be used. The following is an example of an immunohistochemical procedure using an immunoperoxidase system and AEC chromagen. Other methods can be used, with slight variation of the method for different enzymes and chromagens.

1.4.1. Test procedure

i) **Dewax slides containing formalin-processed the fixed tissues according to routine histological procedures into paraffin-embedded test material and wax blocks and cut sections onto glass slides.** Cut positive control sections and negative control tissue sections, if appropriate.

ii) **Dewax the slides** by immersion in three times in consecutive xylene baths for 3–4 minutes each. Hydrate sections through two changes of 98–100% ethanol, one change of 70% ethanol and running tap water to remove residual alcohol.

iii) **Antigen retrieval can be done through heating in a citrate buffer (pH 9) for 20 minutes at 97°C, or by proteinase K digestion for 5 minutes.**

iv) **At this point and between each successive step till after step vii, wash the slides in TRIS buffer (pH 7.6) multiple times.**

v) **Block endogenous compound at this stage.** This will depend on the detection system used, for example, if an immunoperoxidase system is used then endogenous peroxidase needs to be blocked with 3% aqueous H$_2$O$_2$ for 10 minutes.

vi) **Add the primary antibody at a pre-characterised dilution for 45 minutes.**

vii) **Add the secondary antibody conjugate.** Many different systems are available: the simplest and most robust consist of a single step. Consult the manufacturer’s product guidelines for the correct use.

viii) **Add the chromagen** (for example, 3-amino-9-ethylcarbazole (AEC), or 3,3’ diaminobenzidine (DAB) for 10 minutes. Refer to the product guidelines for the correct use.

ix) **Wash in distilled water to stop colour development.**

x) **Counterstain in haematoxylin for 30 seconds to 3 minutes (depending on type).**

xi) **Rinse in tap water. Add Scott’s solution (0.04 M sodium bicarbonate, 0.3 M magnesium sulphate), for 1 minute and wash well in running tap water.**

xii) **Mount with a cover-slip using aqueous mounting medium.**

xiii) **Viral antigen can be visualised by the brown/ red stain, the colour depending on the chromagen used.**

iv) **Rinse slides in distilled water, immerse in 0.01 M CaCl$_2$ (adjusted to pH 7.8 with 0.1 M sodium hydroxide) containing 0.1% (w/v) trypsin (Difco Trypsin 250) for 20 minutes at 37°C and wash in distilled water.**

v) **Lay slides flat in a humid chamber and rinse with PBS for 5 minutes. Add 200 µl 3% aqueous H$_2$O$_2$ to each slide for 20 minutes at room temperature to block endogenous peroxidase. Rinse slides in PBS for 5 minutes.**

vi) **Add 200 µl of an appropriate dilution of rabbit anti-Nipah or anti-Hendra antibody in PBS containing 0.1% (w/v) skim milk powder to test tissue slides and positive and negative control slides.** To a duplicate set of test and positive and negative control slides add rabbit antibody to an unrelated pathogen. Cover the slides and incubate at 37°C for 1 hour.

vii) **Rinse slides in PBS for 5 minutes and apply 2–3 drops of Envision™ solution (anti-rabbit IgG conjugated to peroxidase-labelled dextran polymer [DAKO Corporation, 6392 Via Real, Carpinteria, CA 93013]). Incubate at 37°C for 20 minutes.**

viii) **Prepare the substrate by dissolving 2 mg 3-amino-9-ethylcarbazole (AEC) in 200 µl dimethyl formamide (Merck) and add to 10 ml 0.02 M acetate buffer, pH 5.0. Add 5 µl H$_2$O$_2$ (30% w/v) and mix.** Check the positive control slide for sufficient staining, usually 2–5 minutes, and stop the reaction by rinsing in distilled water. The substrate solution should be made fresh prior to use.

ix) **Counterstain the slides in haematoxylin for 1–3 minutes, rinse in tap water, add Scott’s solution (0.04 M sodium bicarbonate, 0.3 M magnesium sulphate), and wash well in...**
running tap water. Rinse the slides in distilled water and mount with a cover-slip using aqueous mounting medium.

All the above test methods should be considered as a guide only; each test parameter will need to be optimised for each testing laboratory, as they will vary according to specific laboratory conditions.

2. Serological tests

In laboratories doing serological testing, particularly in outbreak situations, several strategies have been adopted to reduce the risk of exposure of laboratory personnel to HeV and NiV. Sera may be gamma-irradiated (6 kiloGreys) or diluted 1/5 in PBS containing 0.5% Tween 20 and 0.5% Triton-X100 and heat-inactivated at 56°C for 30 minutes. The process used will be based on a risk assessment. Specimens for surveillance testing and testing for animal movement certification may be considered a lesser biosafety risk than those for disease investigation. In some circumstances heat inactivation may be adopted as a sufficient precaution. However there is value in having a standardised approach for all samples in managing a test, rather than be maintaining multiple test methods.

2.1. Virus neutralisation tests

Henipaviruses can be quantified by plaque, microtitre or immune plaque assays and these assays can be modified to detect anti-virus antibody (see above). The virus neutralisation test (VNT) (Kaku et al., 2009; Tamin et al., 2009) is accepted as the reference standard. In the most commonly used microtitre assay, which is performed under BSL4 conditions, sera are incubated with virus in the wells of 96-well microtitre plates prior to the addition of Vero cells. Sera are screened starting at a 1/2 dilution although this may lead to problems with serum-induced cytotoxicity. Where sample quality is poor or sample volumes are small, as may be the case with flying fox or microbat sera, an initial dilution of 1/5 may be used. Cultures are read at 3 days, and those sera that completely block development of CPE are designated as positive. If cytotoxicity is a problem the immune plaque assay described above has merit because the virus/serum mixtures are removed from the Vero cell monolayers after the adsorption period, thereby limiting their CPE toxic effect.

2.2. Enzyme-linked immunosorbent assay

Henipavirus antigens derived from tissue culture for use in the enzyme-linked immunosorbent assay (ELISA) are irradiated with 6 kiloGreys prior to use, a treatment that has negligible effect on antigen titre. In the indirect ELISA developed in response to the initial outbreak at Hendra in 1994, antigen was derived from HeV-infected cells subjected to several cycles of freezing and thawing and treatment with 0.1% (w/v) sodium dodecyl sulphate (P. Selleck, unpublished data). More recently, the use of a recombinant expressed soluble form of the Hendra G protein (Bossart et al., 2005) has been applied for improvements in Hendra immunossays (McNabb et al., 2014). In the national swine surveillance programme in Malaysia in 1999 (Daniels et al., 2000) a similar programme in Malaysia in 1999, an indirect ELISA format was used in which antigen was derived from non-ionic detergent treatment of NiV-infected cells. Subsequently, to control for high levels of nonspecific binding activity in some porcine antisera, a modified ELISA was developed based on the relative reactivity of sera with NiV antigen and a control antigen derived from uninfected Vero cells. At the Centers for Disease Control (CDC), Atlanta, USA, the approach has been to not only have an indirect ELISA for detection of IgG but also to use a capture ELISA for detection of IgM. For NiV, an ELISA using a recombinant nucleocapsid antigen has also been described (Yu et al., 2006), which is also configured to detect either IgG or IgM.

The specificity of the indirect NiV ELISA (98.4%) (Ong et al., 2000) means that in surveillance programmes the test will yield false positives. This may not be a significant problem in the face of a NiV outbreak where a high proportion of pigs are infected and the purpose of the surveillance is to detect infected farms. However, this level of test specificity creates a problem in the absence of an outbreak or if the number of samples to be tested is limited. If a positive ELISA result was indicative of a bona fide infection, failure to respond may lead to virus spread and human fatalities. In contrast, initiating control measures in response to a false positive ELISA result would be wasteful of resources (Daniels et al., 2001). The current approach is to test all ELISA reactive sera by VNT, with sera reacting in the VNT considered to be positive. Confirmatory VNT should be done under BSL4 conditions and this may entail sending the samples to an internationally recognised laboratory.

The following procedure for the NiV ELISA has been developed at Australian Animal Health Laboratory (AAHL) for porcine sera and standardised after collaborative studies in the Veterinary Research Institute, Ipoh, Malaysia.
2.2.1. Test procedure

i) Preparation of NiV antigens

a) Grow Vero cells until confluent in roller bottles in EMEM containing 10% (v/v) fetal calf serum (FCS). To infect with virus, pour off all but 5 ml of the medium from each roller bottle and, in a BSL4 laboratory, add low passage, plaque-purified NiV to a multiplicity of infection of 0.1 TCID50/cell.

b) Rotate roller bottles for 30 minutes at 33°C to adsorb virus, add 60 ml EMEM containing 10% FCS to each bottle and roll for a further 48 hours at 33°C. The multiplicity of infection, incubation time and temperature are chosen so that although the majority of cells become infected and are incorporated into syncytia within 48 hours, few cells detach into the culture medium. The culture medium of cells infected under these conditions is an excellent source of virus for further purification.

c) Wash monolayers of virus-infected cells once with cold 0.01 M PBS and, using a large scraper, scrape cells from each roller bottle into 5–10 ml ice-cold PBS.

d) Pool scraped cells into 50 ml tubes kept in ice and pellet the cells at 300 \( g \) for 5 minutes at 4°C. Pour off PBS and resuspend cells in ice cold TNM (10 mM Tris, 10 mM NaCl, 1.5 mM MgCl\(_2\) - pH 7.2), approximately 0.5 ml TNM per roller bottle.

e) Add NP40 (non-ionic detergent, Nonidet P40) to 1% (by addition of 1/10 volume of 10% [v/v] NP-40 in water) and lysis cells using 5–10 strokes of a Dounce homogeniser. This also releases from the cytoskeleton viral antigen that would otherwise be removed by centrifugation (step i.f).

f) Pellet the nuclei at 600 \( g \) for 10 minutes at 4°C. The nuclei will not lyse under these conditions and should form a tight white pellet.

g) Gently remove the supernatant cytoplasmic extract into a clean tube and add ethylene diamine tetra-acetic acid to 1.5 mM. Make up to 10 ml with TNE, aliquot in small amounts, freeze at –80°C and gamma-irradiate with 6 kiloGreys. Store aliquots at –80°C.

ii) Preparation of control, uninfected Vero cell antigen

a) Grow Vero cells in roller bottles in EMEM containing 10% FCS. When confluent, wash monolayers once with cold PBS and scrape the cells from each roller bottle into 5–10 ml ice cold PBS. Proceed as described for virus-infected cells in steps i.d–i.g above.

Detailed methodology for production and/or supply of irradiated NiV and uninfected Vero cell antigens are available from the Australian Animal Health Laboratory.

i) Preparation of test sera

a) In a biological class II safety cabinet with appropriate personal protective equipment or a class III cabinet, dilute test serum 1/5 in PBS containing 0.5% (v/v) Triton X-100 and 0.5% (v/v) Tween 20 in the wells of a 96-well microtitre plate. Seal the microtitre plate. Laboratory personnel should wear gowns and gloves and spray both their hands and the sealed microtitre plate with suitable disinfectant (e.g. 1% Virkon) before removing the microtitre plate from the biosafety cabinet to heat at 56°C for 30 minutes.

b) Mix 22.5 \( \mu l \) heat-inactivated serum with an equal volume of uninfected Vero cell antigen diluted 1/100 in PBS. Mix thoroughly and incubate at 18–22°C for 30 minutes.

c) Add 405 \( \mu l \) blocking solution (PBS containing 5% chicken serum and 5% skim milk powder) to give a final serum dilution of 1/100 and incubate at 18–22°C for 30 minutes. Aliquots of 100 \( \mu l \) are added to two wells containing NiV antigen and two wells containing uninfected Vero cell control antigen as described in step vi.

ii) ELISA procedure

a) Dilute Vero cell control and NiV antigens in PBS to ensure that control and virus antigen wells are coated with a similar concentration of protein. Antigen is usually diluted 1/1000 to 1/4000, but a specific dilution factor must be determined for each batch of antigen. Add 50 \( \mu l \) virus and cell control antigen to the wells of a Nunc
Maxisorp 96-well microtitre plate as follows: virus antigen in columns 1, 3, 5, 7, 9 and 11 and cell control antigen in columns 2, 4, 6, 8 and 12 (Fig. 1). Incubate at 37°C for 1 hour with shaking. Plates can be also incubated at 4°C overnight.

b) Wash ELISA plates three times with PBS containing 0.05% Tween 20 (PBST) (250 μl/well) and block with PBS containing 5% chicken serum and 5% skim milk powder (100 μl/well) for 30 minutes at 37°C on a shaker.

c) Wash plates three times with PBST and add 100 μl of inactivated, absorbed sera from step iii to each well as indicated in the format below. Add 100 μl PBST containing 5% chicken serum and 5% skim milk powder to conjugate and substrate control wells. Incubate the plates without shaking for 1 hour at 37°C and wash three times with PBST.

d) Dilute protein A/G-horseradish peroxidase conjugate (Protein-A/G-Conjugate Supplied by Pierce through Progen Biosciences Thermo Scientific Product No. 32490,) in PBST containing 1% (w/v) skim milk powder. The dilution factor is approximately 1/50,000. Mix well and add 100 μl protein A-conjugate to all wells except the substrate control wells. Add 100 μl PBST containing 1% skim milk powder to the substrate control wells. Incubate the plates for 1 hour at 37°C without shaking and wash four times with PBST.

e) Prepare the substrate (3,3’,5,5’-tetramethylbenzidine; TMB; Sigma, catalogue number T 3405) by dissolving one tablet (1 mg) in 10 ml of 0.05 M phosphate citrate buffer, pH 5.0, and add 2 μl of fresh 30% (v/v) H₂O₂. Add 100 μl of the TMB substrate to each well. Incubate for 10 minutes at 18–22°C and stop the test by adding 100 μl 1 M sulphuric acid to each well.

f) Read plates after blanking on a substrate control well. The optical density (OD) at 450 nm on NiV antigen and control Vero cell antigen are used to calculate an OD ratio for each serum (OD on NiV antigen/OD on Vero control antigen).

**Interpretation of results**

a) An OD ratio >2.0 with an OD on NiV antigen >0.20 is considered positive.

b) An OD ratio >2.0 with an OD on NiV antigen <0.20 is considered negative.

c) Sera displaying an OD ratio between 2.0 and 2.2 should be considered doubtful.

**Fig. 1.** ELISA plate format and result sheet.

<table>
<thead>
<tr>
<th>A</th>
<th>Test serum #1</th>
<th>Test serum #1</th>
<th>Test serum #1</th>
<th>Test serum #1</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>Test serum #2</td>
<td>Test serum #2</td>
<td>Test serum #2</td>
<td>Test serum #2</td>
</tr>
<tr>
<td>C</td>
<td>Test serum #3</td>
<td>Test serum #3</td>
<td>Test serum #3</td>
<td>Test serum #3</td>
</tr>
<tr>
<td>D</td>
<td>Test serum #4</td>
<td>Test serum #4</td>
<td>Test serum #4</td>
<td>Test serum #4</td>
</tr>
<tr>
<td>E</td>
<td>Test serum #5</td>
<td>Test serum #5</td>
<td>Test serum #5</td>
<td>Test serum #5</td>
</tr>
<tr>
<td>F</td>
<td>Test serum #6</td>
<td>Test serum #6</td>
<td>Test serum #6</td>
<td>Test serum #6</td>
</tr>
<tr>
<td>G</td>
<td>Test serum #7</td>
<td>Test serum #7</td>
<td>Test serum #7</td>
<td>Test serum #7</td>
</tr>
<tr>
<td>H</td>
<td>Test serum #8</td>
<td>Test serum #8</td>
<td>Test serum #8</td>
<td>Test serum #8</td>
</tr>
</tbody>
</table>

**Interpretation of results**

Samples with NiV antigen O.D. value less than 0.20 are negative. Samples with NiV antigen OD value greater than 0.2 are assessed by OD ratio value accordingly as:

a) an OD ratio >2.0 (with an OD on NiV antigen > 0.20) are considered positive

b) an OD ratio between 2.0 and 2.2 should be considered doubtful
Doubtful and positive sera should be tested by VNT.

2.3. Bead-based assays

Bead-based assays can be used. The methods below are examples of such assays.

Two multiplexed bead-based serological assays have been developed using LumineX technology and incorporate identification of antibodies to both HeV or NiV in a single test (Bossart et al., 2007; McNabb et al., 2014). Both assays measure antibodies to recombinant expressed soluble glycoprotein (sG) of HeV and NiV. One assay measures antibodies that bind directly to sG (binding assay) and the other assay measures the ability of antibodies to block the henipavirus receptor EphrinB2 binding to sG (blocking assay). The recombinant HeV or NiV sG proteins are first coupled to individually identifiable magnetic beads. The coupled beads are then mixed with test sera. For the binding assay, bound sera are then detected using a biotinylated protein A/G secondary conjugate and Streptavidin-phycoerythrin (S-PE). For the blocking assay, sera must compete with biotinylated ephrinb2 for binding to the sG and S-PE is again used to quantify the reaction. The beads are then interrogated by lasers in a LumineX machine and the results recorded as the median fluorescent intensity (MFI) of 100 beads. The assays use completely recombinant reagents and can be performed at PC2, whereas the traditional ELISA requires PC4 containment to produce antigen. Similar to the approach taken with ELISA, any suspect positive sera are then tested by VNT at BSL4 for confirmation.

2.3.1. Bead-coupling procedure

i) Bead activation

a) Bring the bead activation buffer (0.1 M NaH₂PO₄, pH 6.2) to room temperature prior to use.

   NOTE: Be careful to protect the beads from light as they photobleach (cover tubes with foil where possible).

b) Select the MagPlex carboxylated beads (Luminex corp., supplied as 1.25 × 10⁷ beads/ml) for the protein coupling reaction (usually HeV: Bead#29 & NiV: Bead#30). Vortex the beads for 30 seconds at medium speed, then sonicate the beads by bath sonication for ~30–60 seconds. It is important that the beads are completely resuspended as single monodisperse particles.

c) Transfer 300 µl of MagPlex carboxylated beads #28 & #30 (3.75 × 10⁶ beads) into 2 ml sarstedt tubes. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet.

d) Wash beads by adding 300µl of PBS-T to the tubes and vortexing. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet. Repeat.

e) Add 600 µl of bead activation buffer to the tubes and vortex. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet. Repeat.

f) Add 240 µl of bead activation buffer to the tubes, cover with foil and shake for 3 minutes.

g) Prepare EDC (Pierce) and S-NHS (Pierce) in bead activation buffer immediately prior to use to a concentration of 50 mg/ml (20 µl buffer/mg powder). Add 30 µl of the freshly made 50 mg/ml EDC into the tubes, closely followed by 30 µl of the freshly made 50 mg/ml S-NHS into the tubes. NOTE: Discard unused portion and make fresh each time.

h) Cover the tubes with aluminum foil and shake the beads at room temperature for 20 minutes.

i) While beads are incubating, prepare sG proteins. Use 90 µg each of HeV sG & NiV sG and use PBS (do not use PBS-T, as it blocks carboxy groups) to bring proteins up to a final volume of 300 µl.

j) After incubation, the beads are now activated and ready for coupling. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the
ii) Protein coupling

a) Wash beads by adding 300 µl of PBS to the tubes and vortexing (do not use PBS-T as it blocks carboxy groups). Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet.

b) Add all of the 300 µl of prepared protein, above, to the activated beads.

c) Cover the tubes with aluminium foil and shake the beads moderately at room temperature for 2 hours.

d) The protein is now coupled to the beads. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet.

e) Wash the beads twice with 300 µl of PBS-T as described above. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet.

f) Resuspend the coupled beads in 1.8 ml bead storage buffer (10 ml PBS, 1% BSA, 0.05% sodium azide and 1 protease inhibitor tablet (Roche) and store at 4°C.

NOTES: Check reactivity of sG with panel of henipavirus sera before use. Use 1 µl of coupled beads per well for henipavirus binding and blocking serological assays (this procedure couples enough beads to test around 1800 sera). Coupled beads are able to be stored at 4°C for at least 1 year and maintain reactivity.

2.3.2. Henipavirus luminex binding assay procedure

i) Test procedure

a) Select previously coupled HeV and NiV sG beads. Vortex the beads for 30 seconds at maximum speed, then sonicate the beads by bath sonication for ~30–60 seconds.

b) Dilute beads in blocker (2% skim milk in PBS-T) at an appropriate concentration for the number of sera to be tested (1 µl of each bead set/well).

c) Add 100 µl of diluted beads to appropriate wells of a 96-well NUNC TC flat bottom plate.

d) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

e) Place plate on magnetic holder and allow separation to occur for 30–60 seconds. With the plate still in the magnetic holder, flick contents into the sink and gently blot on paper towel, remove plate from magnetic holder.

f) Wash twice with PBST or alternatively, use automated magnetic plate washer.

g) Add 100 µl of control and test sera diluted 1/100 in PBS-T to the wells (bat sera dilute 1/50).

NOTE: All sera should be heat-inactivated for 35 minutes at 56°C before testing.

h) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

i) Place plate on magnetic holder and allow separation to occur for 30–60 seconds. With the plate still in the magnetic holder, flick contents into the sink and gently blot on paper towel, remove plate from magnetic holder.

j) Wash twice with PBST or alternatively, use automated magnetic plate washer.

k) Dilute biotinylated protein A (Pierce) 1/500 (2 µg/ml) and biotinylated protein G (Pierce) 1/250 (2 µg/ml) in the same tube in PBS-T and add 100 µl to the wells.

l) Cover plate in foil and shake at RT for 30 min on a plate shaker.
m) Place plate on magnetic holder and allow separation to occur for 30–60 seconds.

With the plate still in the magnetic holder, flick contents into the sink and gently blot
on paper towel, remove plate from magnetic holder.

p) Wash twice with PBST or alternatively, use automated magnetic plate washer.

o) Add 100 µl of Streptavidin R-PE (QIAGEN) diluted 1/1000 (1 ug/ml) in PBS-T to the
wells.

Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

Read plate using an appropriate Luminex machine and software.

i) Interpretation of results

The results can be interpreted from the raw MFI values or can be transformed into a
percentage relative to the MFI for the positive control (%P) using the following formula:

\[(\text{MFI test serum/MFI positive control}) \times 100\]

A sample giving an MFI >1000 or %P >5 should be first retested in the Binding assay and
Blocking assay. If the sample is still positive it should be tested further by VNT for
confirmation.

2.3.3. Henipavirus luminex blocking assay procedure

i) Test procedure

a) Select previously coupled HeV and NiV sG beads. Vortex the beads for 30 seconds
at max speed, then sonicate the beads by bath sonication for ~30–60 seconds.

b) Dilute beads in blocker (2% skim milk in PBS-T) at an appropriate concentration for
the number of sera to be tested (1 µl of each bead set/well).

c) Add 100 µl of diluted beads to appropriate wells of a 96 well NUNC TC flat-bottom
plate.

d) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

e) Place plate on magnetic holder and allow separation to occur for 30–60 seconds.

With the plate still in the magnetic holder, flick contents into the sink and gently blot
on paper towel, remove plate from magnetic holder.

f) Wash twice with PBST. Or, alternatively, use automated magnetic plate washer.

g) Add 100 µl of control and test sera diluted 1/50 in PBS-T to the wells (bat sera dilute
1/25).

NOTE: All sera should be heat-inactivated for 35 minutes at 56°C before testing.

h) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

i) Place plate on magnetic holder and allow separation to occur for 30–60 seconds.

With the plate still in the magnetic holder, flick contents into the sink and gently blot
on paper towel, remove plate from magnetic holder.

j) Wash twice with PBST or alternatively, use automated magnetic plate washer.

k) Dilute biotinylated ephrinB2 (RnD Systems) 1/1000 (50 ng/ml) in PBS-T and add
100 µl to the wells.

l) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

m) Place plate on magnetic holder and allow separation to occur for 30–60 seconds.

With the plate still in the magnetic holder, flick contents into the sink and gently blot
on paper towel, remove plate from magnetic holder.

n) Wash twice with PBST or alternatively, use automated magnetic plate washer.

o) Add 100 µl of streptavidin R-PE (QIAGEN) diluted 1/1000 (1 ug/ml) in PBS-T to the
wells.

p) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

Read plate using an appropriate Luminex machine and software.
i) Interpretation of results

For the blocking assay, the raw MFI readings are converted into percentage inhibition (%I) using the following formula: 

\[ \text{%I} = \left( 1 - \frac{\text{MFI test serum}}{\text{MFI NSC}} \right) \times 100 \]

A sample giving a %I > 15 should be first retested in the binding assay and blocking assay. If the sample is still positive it should be tested further by VNT for confirmation.

2.4. DIVA

Now that a vaccine exists for Hendra virus for use in horses, the ability to differentiate vaccinated horses from unvaccinated naturally infected horses may seem desirable to some parties. Traditionally DIVAs have been applied on the premise that vaccinated animals will only have antibodies to the viral protein(s) used in the vaccine (in the case of HeV, that is the G protein) whereas naturally infected animals will have antibodies to all viral proteins, both structural and non-structural. However, caution must be exerted when interpreting such serological results. Experimentally it has been shown in ferrets that not all individuals mount an immune response to all of the viral proteins of Hendra virus that are detectable using conventional assay systems (Middleton D., unpublished data). In particular, serological profiles of unvaccinated infected animals may be indistinguishable from those that are vaccinated.

C. REQUIREMENTS FOR VACCINES

Veterinary vaccines for henipaviruses

1. Background

1.1. Rationale and intended use of the product

There is no commercially produced vaccine against Hendra and/or Nipah virus available. The original outbreak of NIV in Malaysia and Singapore was linked to transmission of the virus from pigs to humans, and all of the human infections with Hendra virus in Australia have been linked to contact with sick horses. Development of veterinary vaccines against henipaviruses is important both to protect susceptible domestic animal species (i.e. porcine, equine, feline, and canine) and to reduce transmission from domestic animals to humans. The original outbreak of NIV in Malaysia and Singapore was linked to transmission of the virus from pigs to humans, and all of the human infections with Hendra virus in Australia have been linked to contact with sick horses. This was the rationale for development of the vaccine for HeV which is currently available for use in horses in Australia.

A vaccine that protects against both NIV and HeV, and could be used in a number of species (i.e. equine, swine, feline, canine) would be desirable. In addition, a vaccine for wildlife may be advantageous to assist in outbreak control. Henipaviruses are considered to be bioterrorism and agroterrorism threats, which increase the need for development and production of safe and effective vaccines for domestic animals.

2. Outline of production and minimum requirements for conventional vaccines

The desired profile for henipavirus vaccines includes a manufacturing process that is safe under low containment conditions, yielding a large number of doses at a reasonable cost. The vaccine should be highly efficacious with a quick onset of immunity following a single dose. The vaccine should ideally cross-protect against both NIV and HeV and be safe in a wide range of species, and across all ages. Vaccination should prevent virus transmission to susceptible animals and people, and should prevent virus entry into the brain. The vaccine should allow detection of vaccinated animals which become infected (differentiation of infected from vaccinated animals [DIVA]) and should have duration of immunity of at least 1 year.

Vaccine production techniques which require growing large quantities of henipaviruses have not been considered to date because of the requirement for biosafety level 4 containment, and subsequent extensive safety testing. This precludes the development of conventional killed, split, split-subunit or live attenuated vaccines.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages
The use of biotechnology presents opportunities for production of safe and effective henipavirus vaccines. Live vectored vaccines expressing the fusion (F) and/or attachment proteins (G) and subunit vaccines containing recombinant F and/or G proteins have been produced and shown to be safe and effective under experimental conditions.

The vaccine currently under development for commercialisation is a recombinant canarypox virus expressing Nipah virus F and/or G proteins. The ALVAC canarypox vector has been successfully used to produce licensed, commercially available live vectored vaccines in equine, feline, and canine species. This vector will infect mammalian cells and produce viral encoded protein, but does not replicate in mammalian hosts or cells. These properties provide an acceptable safety and efficacy profile and provide a vaccine that is DIVA compatible.

### 3.2. Special requirements for biotechnological vaccines, if any

Live recombinant vectored vaccines must meet all requirements for safety, efficacy, potency, and purity required of all vaccines. In addition they must be shown to stably express the recombinant proteins upon passage in vitro and growth in cell culture to produce vaccine virus. A risk assessment should be conducted before biotechnology-derived vaccines are released into the environment. The risk assessment should include information on the design, construction, and testing of the biotechnology-derived vaccine. Detailed information should be provided about the documented genetic characteristics and history of the organisms used to construct the final recombinant biological agent and its survivability in the environment.

### 3.3. Experimental vaccines based on biotechnology

The data on experimental vaccines published by mid-2009 indicate that experimental henipavirus vaccines can prevent clinical disease, elicit systemic and mucosal immunity, and prevent viral replication in target tissues in several mammalian animal species. In addition there are indications that the HeV vaccine formulations can cross protect against NiV.

#### 3.3.1. Canarypox vectored NiV vaccines

The ALVAC canarypox virus-based recombinant vaccine vector was used to construct two experimental NiV vaccines, carrying the gene for NiV glycoprotein (ALVAC-G) or the fusion protein (ALVAC-F). The efficacy of both the ALVAC-G and ALVAC-F were tested in 10-week old pigs, either as monovalent vaccine or in combination (ALVAC-G/F) in a pilot protection study (Weingartl et al., 2006).

The vaccination regimen was two doses administered intramuscularly 14 days apart, each of them containing $10^8$ PFU. Both non-vaccinated controls and vaccinated pigs were challenged intra-nasally with $10^5.4$ PFU of NiV 2 weeks later.

The combined ALVAC-F/G vaccine induced the highest levels of neutralising antibodies, and stimulated both type 1 and type 2 cytokine responses. Virus was not isolated from the tissues of any of the vaccinated pigs post-challenge, and no virus shedding was detected in vaccinated animals, in contrast to challenge control pigs. Histopathological findings indicated that there was no enhancement of lesions in the challenged vaccinates. Based on the data generated in this one study, the combined ALVAC-F/G vaccine appears in particular to be a very promising vaccine candidate. The canarypox vaccine vector has been licensed for commercially available vaccines for dogs, cats, and horses. The canarypox (ALVAC) vaccine vectors induce antibody and cytotoxic T cell responses in a range of mammalian species, and the replication of canarypox viral vectors is abortive in mammalian cells, eliminating some of the safety concerns.

#### 3.3.2. Vaccinia vectored NiV vaccine

The NiVAC vaccine virus-based recombinant vaccine vector was used to construct experimental NiV vaccines where the vaccinia virus expresses either NiV-G or F-glycoprotein (Guillaume et al., 2004). The recombinant vaccines were used for subcutaneous immunisation of hamsters, either individually or in combination, using $10^7$ PFU/animal in two doses (1 month apart). Both of the NiV glycoproteins G and F vaccinia virus recombinants induced an immune response in hamsters that protected against a lethal intraperitoneal challenge with $10^6$ PFU of NiV/animal. This team also demonstrated that passive transfer of antibody induced by the glycoproteins protected the animals against NiV, and also against HeV (Guillaume et al., 2009).
Although NYVAC vaccinia-based vector is highly attenuated, it still has the potential to infect people, creating safety concerns for use of the vaccine in domestic animals (or humans). However, vaccinia vectors were successfully used in wildlife rabies vaccination campaigns in Europe with the advantage for lending themselves to oral immunisation.

2. Soluble G henipavirus vaccine

Studies using NiV in cats (Mungall et al., 2006) and monkeys (Bossart et al., 2012) and HeV in ferrets (Pallister et al., 2011) provided strong evidence that a HeV soluble G (HeVsG) glycoprotein subunit-based vaccine could prevent not only disease but often infection in animals exposed to otherwise lethal doses of NiV or HeV. The horse vaccine has been formulated using a proprietary adjuvant (Zoetis). The henipavirus surface-expressed G glycoprotein has the critical role of initiating infection by binding to receptors on host cells, and antibodies directed against this protein can neutralize virus. The vaccine, Equivac HeV (Zoetis) was released under a Minor Use Permit for use in Australia in November 2012, and is only available for administration by accredited veterinarians.

For primary immunisation two doses of vaccine should be administered 3 weeks apart in horses four months of age or above. For continued effect, a booster dose every 6 months is currently recommended by the manufacturer.

3. Experimental vaccines

In preliminary evaluation of experimental subunit vaccine formulations containing either soluble forms of HeV sG or NiV sG glycoprotein in a NiV challenge study in cats, good crossreactivity was demonstrated with HeV sG possibly providing better protection (Mungall et al., 2006). In a subsequent study, a subunit formulation containing CpG as an adjuvant and HeV sG with its cytoplasmic tail and transmembrane domains replaced by an immunoglobulin kappa leader sequence coupled with an S-peptide tag to facilitate purification, was evaluated as a potential NiV vaccine. Intramuscularly vaccinated cats developed varying levels of NiV-specific Ig systemically and importantly, all vaccinated cats possessed antigen-specific IgA on the mucosa. Upon oronasal challenge with NiV (10^4.7 TCID50), all vaccinated animals were protected from disease although virus was detected on day 21 post-challenge in one animal. However shedding was detected at 6 and 8 days based on virus genome detection at about the same levels in both vaccinated and control challenged animals (McEachern et al., 2008). In addition NiV genome was detected in the brain of several vaccinated challenged animals compared to challenge control animals raising some concerns about significance of this phenomenon in the light of the observed late encephalitis in humans (Paterson et al., 1998; Tan et al., 2002). Beside the potential efficacy concerns, cost of production may prohibit development of this vaccine for veterinary application.

Current experimental vaccines for protection from NiV infection have focused on the use of NiV glycoprotein (G) and/or fusionprotein (F) as immunogens in various platforms, including DNA vaccines, subunit vaccines, non-replicating vectors, as well as replicating vectors. Efficacy of most candidates required a prime/boost(s) approach, which would not favour their use in an emergency situation for rapid dissemination during an outbreak. A live-attenuated vaccine vector based on recombinant vesicular stomatitis viruses (rVSV) expressing NiV glycoproteins (G or F) or nucleoprotein (N) has been evaluated. Vaccination of Syrian hamsters with a single dose of the rVSV vaccine vectors resulted in strong humoral immune responses with neutralizing activities found only in those animals vaccinated with rVSV expressing NiV G or F proteins suggesting that these may be prime candidates for emergency vaccines to be utilised in NiV outbreak management. A similar construct consisting of a replication-defective vesicular stomatitis virus (VSV)-based vaccine vectors expressing either the NiV fusion (F) or attachment (G) glycoproteins protected hamsters from over 1000 times LD50 NiV challenge when vaccinated with a single dose.

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Chapter 2.9.6. – Nipah and Hendra virus diseases


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Chapter 2.9.6. – Nipah and Hendra virus diseases


Chapter 2.9.6. – Nipah and Hendra virus diseases


**NB:** There is an OIE Reference Laboratory for Hendra and Nipah virus diseases (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)).

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Hendra and Nipah virus diseases.
CHAPTER 2.9.12.

ZOONOSES TRANSMISSIBLE
FROM NON-HUMAN PRIMATES

SUMMARY

For standards for testing non-human primates, please consult the following document:


http://lan.sagepub.com/content/33/suppl_1/3.full.pdf

The Terrestrial Animal Health Code (chapter 6.11) requires tests for certain diseases in non-human primates imported for research, educational or breeding purposes. This chapter indicates where to find further information on such tests. It is important to recognise that primate species represent a significant risk of pathogen transmission to humans in contact, including the collection of samples for laboratory testing, and the handling of those samples in the laboratory. Veterinary laboratories should seek advice from medical authorities on the appropriate health protocols that should be followed by staff handling such materials. All laboratory manipulations with live cultures or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis (Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

In addition to the specific tests required by the OIE Terrestrial Code as detailed below, additional information on the health monitoring of non-human primate colonies, including a list of potential zoonotic diseases and the types of tests used for diagnosis, is provided by FELASA (1998).

1. Tuberculosis

The test procedures and preparation of reagents are described in chapter 2.4.7 Bovine Tuberculosis. The delayed hypersensitivity skin test in non-human primates is usually carried out by intradermal injection of 0.1 ml "mammalian old tuberculin" into the edge of the upper eyelid using a sterile 25–27 gauge needle. Purified protein derivatives (PPD) as described in Chapter 2.4.7 Bovine tuberculosis may also be used, but are generally considered less sensitive for non-human primates. The animal must be suitably restrained or drug-immobilised. For smaller species such as marmosets, tamarins or small prosimians the test should be carried out in the abdominal skin. A repeat test by this route may be used in other cases where the palpebral reaction is difficult to interpret. False positive and false negative reactions can occur with the tuberculin skin test and clarification can be obtained by use of additional tests including radiography for tuberculous lesions; detection of the organism in samples of gastric or bronchial lavage, faeces or tissue biopsies by culture or polymerase chain reaction (PCR) assay; the detection of cellular immunity by the gamma-interferon assay; or antibody detection by enzyme-linked immunosorbent assay (ELISA).

1 Mammalian old tuberculin is available from the Colorado Serum Company, 4950 York St, P.O. Box 16428, Denver, Colorado 80216-0428, United States of America.
2. Enteric bacteria (*Salmonella, Shigella, Yersinia*)

These organisms can be detected by standard bacteriological culture methods on samples of fresh faeces or rectal swabs. Culture techniques for *Salmonella* are described in chapter 2.9.9 *Salmonellosis*. Methods for *Shigella* are described by WHO (2003).

Enteric species of *Yersinia* include *Y. enterocolitica* and *Y. pseudotuberculosis*. Culture and enrichment are more effective if carried out at lower temperatures (4°C rather than 25°C). Details of culture methods including suitable enrichment media are described by Laukanen *et al.* (2010) and Arrausi-Subiza *et al.* (2014). The latter also describe real-time polymerase chain reaction (PCR) methods for the identification of culture isolates. A general overview of *Y. enterocolitica* and *Y. pseudotuberculosis* is given by Fredriksson-Ahomaa *et al.* (2007), including biochemical methods for the identification of culture isolates.

3. Hepatitis B

Hepatitis B virus (HBV) is classified in the family *Hepadnaviridae*. It occurs as seven distinct genotypes, designated A to G, and has a double-stranded DNA genome of approximately 3200 base pairs organized into four partially overlapping open reading frames, which encode the envelope, core, polymerase and X proteins. The surface glycoproteins of the envelope are collectively designated as hepatitis B surface antigen. Infection is widespread in the human population, despite the availability of effective vaccines, and a significant proportion of infected people progress to serious or fatal liver diseases.

Non-human primates should be tested for evidence of infection by serological methods for antibodies to hepatitis-B core antigen and surface antigen. The test methods are described by Krajden *et al.* (2005).

4. Endo- and ectoparasites

Non-human primates should be screened during quarantine for the presence of parasites by standard parasitological techniques, according to the parasite under investigation. Methods for these tests may be found in Standard parasitological textbooks (Cogswell, 2007; Smith *et al.*, 2007) or, for specific parasites, the relevant chapter in this Terrestrial Manual, such as 2.9.4 *Cryptosporidiosis*, 2.9.10 *Toxoplasmosis*.

5. Other zoonotic pathogens

As well as those infections and infestations referred to above, there is a long list of zoonotic agents that may be carried by different species of non-human primate. Further details including the likely host species, and a suitable regimen for health monitoring in primate colonies, are given in FELASA (1998, currently under review). The following table is derived from that paper.

<table>
<thead>
<tr>
<th>Table 1. Microorganisms and parasites of current concern in non-human primates (from FELASA [1998])</th>
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<tbody>
<tr>
<td><strong>(1) Viruses</strong></td>
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<tr>
<td>B virus, <em>Herpesvirus simiae</em>, Cercopithecine herpesvirus 1</td>
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<tr>
<td><em>Herpesvirus cercopithecus</em>, (SA 8), Cercopithecine herpesvirus 2</td>
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<td><em>Herpesvirus papio</em> 2 (HVP/2), Cercopithecine herpesvirus 12</td>
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<tr>
<td><em>Herpes T</em>, <em>Herpesvirus platyrhinae</em>, Saimiriine herpesvirus 1</td>
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<td><em>Herpesvirus saimiri</em>, Saimiriine herpèsvirus 2</td>
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<tr>
<td>Hepatitis A virus</td>
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<td>Hepatitis B virus</td>
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<td>SV 40</td>
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<tr>
<td>Simian haemorrhagic, fever virus</td>
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<tr>
<td><strong>(2) Bacteria</strong></td>
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<tr>
<td><em>Campylobacter jejuni</em></td>
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<td><em>Campylobacter fetus</em></td>
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<tr>
<td><strong>Leptospira interrogans (various serovars)</strong></td>
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<td><strong>Mycobacterium africanum</strong></td>
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<tr>
<td><strong>Mycobacterium bovis</strong></td>
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<tr>
<td><strong>Mycobacterium tuberculosis</strong></td>
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</table>

**Parasites**

| **Entamoeba histolytica** | **Plasmodia malariae, vivax** |
| **Toxoplasma gondii** | **Strongyloides stercoralis** |
| **Giardia spp.** | **Trichuris** |
| **Plasmodia species** | **Prosthenorchis elegans** |
| **Plasmodia cynomolgi** | **Pneumonyssus simicola** |
| **Plasmodia brasiliensis** | **Ectoparasites:** |
| | • Mites |
| | • Lice |

**Dermatomycosis**

| **Trichophyton** |

**REFERENCES**


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