GLOSSARY OF TERMS

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

- **Absorbance/optical density**

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

- **Accuracy**

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

- **Assay**

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

- **Batch**

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

- **Biohazard (CWA[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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[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)**

The process composed of biohazard identification, biorisk assessment, biorisk management and biorisk communication.

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

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 Managment Advisor (CWA 15793:2011)**

Individual who has expertise in the biohazards encountered in the organisation 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)**

Process of identifying, selecting and implementing measures that can be applied to reduce the level of biorisk.

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

Part of an organisation'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:

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

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

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

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

- **Cross-reaction**

See 'False-positive reaction'.

- **Cut-off/threshold**

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

- **Dilutions**

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

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

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

- **Dilutions used in virus neutralisation tests**

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

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

- **Efficacy**

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

- **Equivalency testing**

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

- **False-negative reaction**

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

- **False-positive reaction**

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

- **Final product (lot)**

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

- **Harmonisation**

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

- **Incidence**

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

- **In-house checks**

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

- **In-process control**

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

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

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

- **Laboratory biosafety**

See *Biosafety*.

112 • **Laboratory biosecurity**

113 See *Biosecurity*.

114 • **Master cell (line, seed, stock)**

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

118 • **Master seed (agent, strain)**

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

122 • **Performance characteristic**

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

125 • <u>**Phylogeography**</u>

126 <u>Phylogeography is the study of the genetic and geographic structure of populations and species.</u>

127 • **Potency**

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

131 • **Precision**

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

134 • **Predictive value (negative)**

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

138 • **Predictive value (positive)**

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

141 • **Prevalence**

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

144 • **Primary cells**

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

146 • **Production seed**

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

149 • **Proficiency testing**

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

153 • **Purity**

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

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

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

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

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

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

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

166 • **Reference animal**

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

169 • **Reference Laboratory**

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

172 • **Repeatability**

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

175 • **Reproducibility**

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

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

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

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

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

186 • **Room temperature**

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

190 • **Safety**

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

193 • **Sample**

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

196 • **Sensitivity (analytical)**

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

199 • **Sensitivity (diagnostic)**

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

202 • **Sensitivity (relative)**

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

205 • **Specific pathogen free (SPF)**

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

208 • **Specificity (analytical)**

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

211 • **Specificity (diagnostic)**

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

214 • **Specificity (relative)**

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

217 • **Specimen**

218 Material submitted for testing.

219 • **Standard Reagents**

220 • **International Standard Reagents**

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

223 • **National Standard Reagents**

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

226 • **Working Standards (reagents)**

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

230 • **Sterility**

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

232 • **Thermotolerant**

233 The term used to describe the ability of a vaccine and/or the parent virus/strain to retain a level of infectivity after
234 exposure to heat, that is, the delayed heat degradation of the virus. For example, for the thermotolerant I-2
235 Newcastle disease vaccine, it is defined by the length of time the vaccine will retain an infectivity titre sufficient to
236 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 I-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.

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* *

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.

41 These roles can be provided by governments (public sector laboratories), by industry (private sector laboratories),
42 by universities (university laboratories) or by external organisations. Combinations of such providers in a complex
43 matrix of services create challenges in the management and expectations of service delivery.

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

49 2.   Accountability and oversight

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

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

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

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

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

77 3.   Executive management

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

88 The director should be supported by a senior management team whose members will lead specific aspects of the
89 work of the laboratory. The size of this team, and the scope of their individual responsibilities, will depend on the
90 size of the laboratory, but it will typically involve leaders of different scientific disciplines (e.g. pathology,
91 bacteriology, virology) as well as business leaders with expertise in human resources (HR), finance, procurement,
92 engineering, information technology (IT) and communication. At least one of the senior team should be
93 designated deputy director; the deputy will work closely with and in support of the director and fulfil the director's
94 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 WHO[1] 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

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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.

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2   ISO: International Organization for Standardization

191 All countries should support OIE designated Reference Centres through submission of specimens, isolates of
192 infectious agents and other information of potential regional or international significance. It is only through receipt
193 of such submissions that the Reference Centres can fulfil their OIE mandated role on behalf of the international
194 community. Involvement with the designated Reference Centres is necessary for international public good.

195 In turn, national veterinary laboratories with special expertise in particular areas may seek recognition from
196 international bodies such as the OIE, FAO[3] or WHO as reference laboratories or collaborating centres. This is
197 encouraged, as it facilitates the harmonisation of laboratory procedures worldwide, and strongly supports the work
198 of the OIE and other international organisations. Funding for reference laboratory status needs to be allocated
199 from national sources, and this should be part of the national planning with the Veterinary Services.

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

208 ## 2. Quality assurance

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

214 The quality standards require that diagnostic tests used in the laboratory should be validated as fit for purpose.
215 The international standard for validation of diagnostic tests is established by the OIE, and is set out in chapter
216 1.1.5. Validation is not a once-for-all-time procedure but requires continual monitoring and refinement as the test
217 is used. Laboratories should strive at all times to use tests that have reached at least stage 3 on the OIE
218 validation pathway (chapter 1.1.5) and to continue refining the validation data as explained in the text.

219 ## 3. Research

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

224 # C. SUPPORT SERVICES

225 ## 1. Internal governance: policies and procedures

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

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

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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 laboratoryand 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.

343 **REFERENCES**

344 EDWARDS S. & JEGGO M.H. (2012). Governance and management of veterinary laboratories. *Rev. sci. tech. Off.*
345 *Int. Epiz.,* **31** (2), 493–503.

346 INTERNATIONAL ORGANIZATION FOR STANDARDIZATION (ISO) (2004). ISO 14001:2004. Environmental management
347 systems. Requirements with guidance for use. ISO, Geneva, Switzerland. www.iso.org.

348 INTERNATIONAL ORGANIZATION FOR STANDARDIZATION (ISO) (2005). ISO/IEC 17025:2005. General requirements for
349 the competence of testing and calibration laboratories. ISO, Geneva, Switzerland. www.iso.org.

350 WORLD HEALTH ORGANIZATION (WHO) (2006). Laboratory Biosafety Manual, Third Edition. WHO, Geneva,
351 Switzerland. www.who.int.

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C H A P T E R   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.

44   *Quality assurance is an integral part of the production of pure, safe and efficacious vaccines. This*
45   *chapter outlines critical check points, with more details provided in chapters 1.1.8*
46   Recommendations for manufacturing sites for veterinary vaccines *and 1.1.9* Quality control of
47   vaccines*. It is a step-wise and iterative process. Compliance with the full standards described in*
48   *these chapters can be achieved through risk analysis and step-wise process improvement.*

49   **NOMENCLATURE**

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

64   **VACCINE TYPES OR FORMS**

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

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

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

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

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

92 Each competent authority with power to regulate organisms and products derived from recombinant techniques
93 should ensure that the public health and the environment are protected from any potentially harmful effect.
94 Veterinary vaccines derived through rDNA technology may be divided into three broad categories. The division is
95 based on the products' biological properties and on the safety concerns they present.

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

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

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

VACCINE PRODUCTION

1. Quality assurance

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

2. Production facilities

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

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

134 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

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

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

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

144 The producer should establish a detailed record-keeping system capable of tracking the performance of
145 successive steps in the preparation of each biological product. Records kept should indicate the date that each
146 essential step was taken, the name of the person who carried out the task, the identity and quantity of ingredients
147 added or removed at each step, and any loss or gain in quantity in the course of the preparation. Records should
148 be maintained of all tests conducted on each batch/serial. All records relevant to a batch/serial of product should
149 be retained for at least 2 years after the expiry date on the label, or in line with the requirements of the competent
150 control authority.

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

4. Production

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

156 The specifications and source of all product ingredients should be defined in the Outline of Production, SOPs, or
157 other appropriate documents. The Outline of Production must be approved by the competent authority. All
158 ingredients of animal origin that are not subject to a validated sterilisation procedure should also be tested to
159 ensure freedom from extraneous bacteria, fungi, mycoplasma, and viruses as specified in Chapter 1.1.7. *Tests for*
160 *sterility and freedom from contamination of biological materials*. Their country of origin should be known.
161 Measures should be implemented by the manufacturer to avoid the risk of transmissible spongiform
162 encephalopathy (TSE) agent contamination by ingredients of animal origin.

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

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

5. Process validation

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

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

182 The manufacturer should test each of these batches/serials for purity, safety, and potency as provided in the
183 Outline of Production or other documentation of the manufacturing process. Applicable standard requirements
184 and test procedures, for example those described in CFR Title 9 Part 113, in the Annex to EU Directive
185 2001/82/EC (as amended), in the European Pharmacopoeia, or as described in this *Terrestrial Manual* may be
186 used. Satisfactory test results should be demonstrated for all three batches/serials prior to approving the
187 production of the product in the facilities and its release for marketing. Each subsequent batch/serial should be
188 tested in the same manner with satisfactory results prior to release for marketing.

6. Stability tests

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

192 All vaccines are sensitive to heat to some extent, but some are more sensitive than others. There is increasing
193 interest in the development of vaccines that can tolerate adverse storage conditions. In this *Terrestrial Manual*,
194 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 (http://www.vichsec.org/guidelines/biologicals/bio-safety/target-animal-safety.html). An overdose 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 (http://www.vichsec.org/guidelines/biologicals/bio-safety/target-animal-safety.html).

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/WC500004590.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

454 indications that raise questions regarding the purity, safety, potency, or efficacy of a product, or if it appears that
455 there may be a problem regarding the preparation, testing or distribution of a product, the manufacturer must
456 immediately notify the regulatory authorities concerning the circumstances and the action taken.

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

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

2. Enforcement

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

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

485 **INSPECTION OF PRODUCTION FACILITIES**

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

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

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

497 **FURTHER READING**

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

499 CODE OF FEDERAL REGULATIONS (OF THE UNITED STATES OF AMERICA) (CFR) (2000). Title 9, Parts 1–199. US
500 Government Printing Office, Washington DC, USA. http://www.gpo.gov/fdsys/pkg/CFR-2006-title9-vol1/pdf/CFR-
501 2006-title9-vol1-chapI.pdf or ELECTRONIC CODE OF FEDERAL REGULATIONS, accessed at http://www.ecfr.gov/cgi-
502 bin/text-idx?SID=a96ece744f88b16cc39202d9cbc5bdae&tpl=/ecfrbrowse/Title09/9tab_02.tpl

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

505 ESPESETH D.A. (1993). Licensing Veterinary Biologics in the United States. The First Steps Towards an
506 International Harmonization of Veterinary Biologicals; and Free circulation of vaccines within the EEC. *Dev. Biol.*
507 *Stand.,* **79**, 17–25.

508 ESPESETH D.A. & GOODMAN J.B. (1993). Chapter 13. *In:* Licensing and Regulation in the USA. Vaccines for
509 Veterinary Application. Butterworth Heinemann, London, UK, 321–342.

510 EUROPEAN COMMISSION (2006). The Rules Governing Medicinal Products in the European Union. Eudralex.
511 Volumes 1–9. European Commission Enterprise and Industry DG; Directorate F – Consumer goods. Latest
512 versions only available at http://pharmacos.eudra.org/F2/eudralex/index.htm.

513 GAY C.G. & ROTH H.J. (1994). Confirming the safety characteristics of recombinant vectors used in veterinary
514 medicine: a regulatory perspective. Recombinant vectors in vaccine development. *Dev. Biol. Stand.*, **82**, 93–105.

515 ROTH H.J. & GAY C.G. (1996). Specific safety requirements for products derived from biotechnology. *In:* Veterinary
516 Vaccinology, Pastoret P.-P., Blancou J., Vannier P. & Verschueren C., eds. Elseviers Science Publishers B.V.
517 Amsterdam, The Netherlands.

518 PASTORET P.P., BLANCOU J., VANNIER P. & VERSCHUEREN C., EDS (1997). Veterinary Vaccinology. Elsevier Science,
519 Amsterdam, The Netherlands.

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

521 USDA-APHIS[1]-VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1999). Categories of Inspection for
522 Licensed Veterinary Biologics Establishments. Veterinary Services Memorandum No. 800.91. Center for
523 Veterinary Biologics, 510 S. 17th Street, Suite 104, Ames, Iowa 50010, USA.

524 USDA-APHIS-VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1999). Veterinary Biological Product
525 Samples. Veterinary Services Memorandum No. 800.59. Center for Veterinary Biologics, 510 S. 17th Street, Suite
526 104, Ames, Iowa 50010, USA.

527 USDA-APHIS- VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1995). Guidelines for Submission of
528 Materials in Support of Licensure. Veterinary Biologics Memorandum No. 800.84. Center for Veterinary Biologics,
529 510 S. 17th Street, Suite 104, Ames, Iowa 50010, USA.

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

533 USDA-APHIS-VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1995). Veterinary Biologics General
534 Licensing Considerations No. 800.201, Back Passage Studies. Center for Veterinary Biologics, 510 S. 17th Street,
535 Suite 104, Ames, Iowa 50010, USA.

536 USDA-APHIS-VETERINARY SERVICES (1964–1994). Standard Assay Methods, Series 100–900. National Veterinary
537 Services Laboratories, Ames, Iowa 50010, USA.

538 USDA-APHIS- VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1984). Basic License Requirements for
539 Applicants. Veterinary Biologics Memorandum No. 800.50. Center for Veterinary Biologics, 510 S. 17th Street,
540 Suite 104, Ames, Iowa 50010, USA

541 USDA-APHIS-VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1988). Guidelines for the Preparation and
542 Review of Labeling Materials. Veterinary Services Memorandum No. 800.54. Center for Veterinary Biologics, 510
543 S. 17th Street, Suite 104, Ames, Iowa 50010, USA.

544 <div align="center">*</div>
545 <div align="center">* *</div>

1 United States Department of Agriculture (USDA), Animal and Plant Health Inspection Services (APHIS). USDA-APHIS-
Center for Veterinary Biologics Home Page: http://www.aphis.usda.gov/vs/cvb/index.html

546 APPENDIX 1.1.6.1.

547 # RISK ANALYSIS FOR BIOLOGICALS
548 # FOR VETERINARY USE

549 ## GENERAL CONSIDERATIONS

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

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

562 *
563 * *

564 APPENDIX 1.1.6.2.

565 # RISK ANALYSIS FOR VETERINARY VACCINES

566 ## INTRODUCTION

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

574 ## PRINCIPLES

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

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

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

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

587 ## MANUFACTURING PRACTICES

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

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

600 A commonly agreed system of facility inspection carried out by qualified and specialised inspectors must be in
601 place to assure confidence.

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INFORMATION TO BE SUBMITTED WHEN APPLYING FOR ~~REGISTRATION~~ <u>MARKETING AUTHORISATION</u> IN THE IMPORTING COUNTRY

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

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

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

618

CATEGORISATION OF VETERINARY VACCINES

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

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

625

VACCINOVIGILANCE

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

630

RISK COMMUNICATION

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

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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 1.1.10.

VACCINE BANKS

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 supress 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.

90   Stockpiles of antigens, ready-to-use vaccines, or service contracts will be referred to as 'banks' in this chapter.

91   **B. TYPES OF BANKS**

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

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

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

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

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

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

144   The vaccine banks often hold physical stocks of antigens or vaccines. It is also possible to establish virtual
145   vaccine banks based on contractual relationships (service contracts) between the bank holder and manufacturers

146 operating any of the above solutions, with clear obligations, price limits, maximum delays for delivery and severe
147 contractual penalties in case of failure.

C. SELECTION OF VACCINES FOR A BANK

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

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

D. QUANTITIES OF VACCINE REQUIRED IN A BANK

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

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

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

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

E. ACQUISITION OF ANTIGENS OR VACCINES FOR A BANK

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

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

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

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

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

F. REGULATORY STANDARDS – SAFETY, EFFICACY AND QUALITY

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

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

250 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 also consider the possibility of independent testing, or of greater reliance on overseeing/auditing of the manufacturer's test procedures.

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

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

307 ## H. DEPLOYMENT PLANNING

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

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

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

323 ## REFERENCES

324 EUROPEAN MEDICINES AGENCY (2004). Position paper on requirements for vaccines against foot-and-mouth
325 disease, EMEA/CVMP/775/02-FINAL 01/12/2004, available at (last accessed on 7 October 2013)
326 http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500004592.pdf

327 FORMAN A.J. & GARLAND A.J.M. (2002). Foot and mouth disease: the future of vaccine banks. *Rev. sci. tech. Off.*
328 *Int. Epiz.*, **21**, 601–612.

329 OFFICE INTERNATIONAL DES EPIZOOTIES (OIE) (2014). OIE Vaccine Banks. *The Bulletin*, No. 3, pp. 9–11. OIE, Paris,
330 France.

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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~~ is 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.*

46   ***Serological tests:*** *A number of tests can be used, particularly the indirect immunofluorescence*
47   (*IFA*) *test, the enzyme-linked immunosorbent assay* (*ELISA*), *and the complement fixation test*
48   (*CFT*). *The presence of specific IgG antibodies provides evidence of a recent* C. burnetii *infection or*
49   *a past exposure. ELISAs are preferred for practical reasons and for their higher sensitivity.*

50   ~~***Requirements for diagnostic biologicals and vaccines:***~~ *Serological antigens are based on the*
51   *two major antigenic forms of* C. burnetii: *phase I, obtained from spleens after inoculation of*
52   *laboratory animals, and phase II, obtained by repeated passages in embryonated eggs or in cell*
53   *cultures. Currently available commercial tests allow the detection of phase II or of both phases II*
54   *and I anti-*C. burnetii *antibodies.*

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

A. INTRODUCTION

59

1. Definition of the disease and transmission routes

60

61 Q fever (or Coxiellosis) is widely distributed throughout the world with the exception of New Zealand. The causal
62 agent, *Coxiella burnetii,* ~~Although Q fever~~ is present in virtually all animal kingdoms, including arthropods, but the
63 disease affects mostly humans, cattle, sheep and goats (~~Arricau-Bouvery & Rodolakis, 2005;~~ EFSA, 2010; Lang,
64 1990~~; Maurin & Raoult, 1999~~). Domestic ruminants are considered the main reservoirs of *C. burnetii*, but cats, dogs,
65 rabbits, birds, etc., have also been reported to be implicated in human disease/infection. There is clear
66 epidemiological and experimental evidence that the infection is principally transmitted by inhalation of desiccated
67 aerosol particles, and through exposure in the vicinity of infected animals, their reproductive tissues or other animal
68 products, like wool (ECDC, 2010). Ingestion has been often suggested, particularly through the consumption of dairy
69 products derived from contaminated raw milk, but no good evidence has shown significant transmission to humans
70 by food. Indeed, there are epidemiological indications of sero-conversion but no association with clinical Q fever in
71 humans. Q fever also seems very rarely transmissible from person to person, although exposure during childbirth,
72 through sexual transmission or blood transfusion is possible. In animals, vertical transmission and sexual
73 transmission could occur but their importance is not known. Finally, arthropods, principally ticks, may be involved in
74 Q fever transmission. The risk of transmission seems to be linked to wildlife animals. It could be associated with bites
75 as well as with contaminated dust from dried excrement.

2. Description of the causal pathogen

76

77 The aetiological agent, *Coxiella burnetii*, is a Gram-negative obligate intracellular bacterium, adapted to thrive within
78 the phagolysosome of the phagocyte. It has been historically classified in the *Rickettsiaceae* family. However,
79 phylogenetic investigations, based mainly on 16s rRNA sequence analysis, have shown that the *Coxiella* genus is
80 distant from the *Rickettsia* genus of the *alpha* subdivision of *Proteobacteria* (Drancourt & Raoult, 2005). *Coxiella*
81 *burnetii* has been placed in the *Coxiellaceae* family in the order *Legionellales* of the *gamma* subdivision of
82 *Proteobacteria*. The complete genome sequencing of *C. burnetii* has been achieved and confirms its systematic
83 position (Seshadri *et al.,* 2003). In general, the genomes of *C. burnetii* isolates from a wide range of biologically and
84 geographically diverse sources are highly conserved, but notable polymorphism occurs such as rearrangement of
85 syntenic blocks (Beare *et al.*, 2009). This genomic plasticity might contribute to different phenotypes and is of great
86 interest for genotyping methods (Massung *et al.*, 2012; Sidi-Boumedine & Rousset, 2011). Unlike rickettsiae,
87 *C. burnetii* produces a small, dense, highly resistant spore-like form (~~Coleman *et al.,* 2004~~ Heinzen *et al.,* 1999;
88 Minnick & Raghavan, 2012). This ability has been attributed to the existence of *C. burnetii* developmental cycle
89 variants described from *in-vitro* studies: large-cell variants (LCV), small-cell variants (SCV), and small dense cells
90 (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.,*~~
91 ~~2004;~~ Heinzen *et al.,* 1999; Minnick & Raghavan, 2012). The SDC and SCV represent the small morphological
92 variants of the bacteria likely to survive extracellularly as infectious particles, a trait that is important for persistence in
93 the environment and transmission (ECDC, 2010; EFSA, 2010; Kersh *et al.* 2010).

94 Another essential characteristic is that *C. burnetii* has two antigenic forms: the pathogenic phase I, isolated from
95 infected animals or humans, and the attenuated phase II, obtained by repeated *in-ovo* or *in-vitro* passages. An LPS
96 (lipopolysaccharide) change occurs during serial passages: phase I cells, with full-length LPS O-chains, change to
97 intermediate phases with decreasing LPS O-chain lengths and then to phase II, with truncated LPS. Thus, the long
98 phase I LPS contains the phase II part. The latter has been described as a major immunogenic determinant.
99 Currently available commercial tests allow the detection of at least the anti-*C. burnetii* phase II antibodies, which
100 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 and 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 retain 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; Rousset *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; Rousset *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 1.1.5 *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. Moreover, the culling of pregnant animals, a temporary breeding ban, ~~stamping out,~~ identifying and culling ~~shedders~~ shedding herds or flocks and controlling animal movements ~~are considered as suitable options~~ may have a role in the ~~case~~ face of 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 biorisk 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 coverage 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 immune status of the laboratory personnel.

B. DIAGNOSTIC TECHNIQUES

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

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification[1]						
PCR	+++	n/a	+++	+++	++	n/a
Culture	±	n/a	±	–	±	n/a
Staining	±	n/a	±	±	±	n/a
Genotyping	n/a	n/a	n/a	n/a	++	n/a
Detection of immune response[2]						
ELISA	+++	n/a	+++	+++	+++	+++
IFA	++	n/a	++	++	++	++
CFT	–	n/a	–	–	±	±

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 (Horigan *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~~ abortion (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

293   also be used to detect the presence of *C. burnetii* <u>and to monitor the process of isolation</u>. Further
294   passages may be required to obtain an isolate in pure culture.

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

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

1.2. Staining

329   In the case of an abortion having a suspected infectious origin, smears of placental cotyledon are
330   prepared on microscope slides. <u>Spleen,</u> lung, liver and abomasal contents of the aborted fetus or
331   vaginal discharge may be used in the same manner. These could be stained according to several
332   methods: Stamp, Gimenez, Macchiavello, Giemsa and modified Koster (Gimenez, 1964; Quinn *et al.,*
333   1994; Samuel & Hendrix, 2009). The first three techniques give the best results. These methods are
334   close to the modified Ziehl–Neelsen method involving basic fuchsin to stain bacteria. For example, the
335   Stamp staining method is performed with 0.4% basic fuchsin solution, followed by rapid decolouration
336   with 0.5% acetic acid solution, and counterstaining with 1% methylene blue or malachite green
337   solution. The smears are examined microscopically with an oil-immersion objective lens (×500 or
338   more). The Stamp method is preferred in veterinary laboratories while the Gimenez method is
339   widespread for monitoring infected cultural cells in research laboratories. Gimenez is fastest because
340   an acidic solution is not included for differentiation. *Coxiella burnetii* are characterised by a very large
341   number of thin, pink-stained coccobacillary bacteria against a blue or green background. They may
342   sometimes be difficult to detect because of their small size, but this is compensated for by their large
343   numbers; often inclusions within the host cells appear as red masses against the blue or green
344   background. <u>The staining method is rapid. The limit of detection is high (>10^5 bacteria/ml) and
345   appropriate to the clinical diagnostic purpose as high levels of bacteria are present in samples found
346   positive.</u> Attention must be taken in the interpretation of the results as, microscopically, *C. burnetii* can
347   be confused with *Chlamydophila abortus* or *Brucella* spp. However, using the same staining procedure,
348   *Chlamydophila* have sharper outlines, are round, small and may resemble globules. *Brucella* spp. are
349   larger (0.6–1.5 µm long × 0.5–0.7 µm wide), more clearly defined and stain more intensely. Control
350   positive slides of *C. burnetii*, *Chlamydophila abortus* and *Brucella* must be used for comparison.
351   Diagnosis <u>of clinical cases</u> made on the basis of microscopy, coupled with positive serological results,

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3   ~~Sterilin, Bibby Sterilin Ltd, Stone, Staffordshire ST15 O5A, United Kingdom.~~

352   is usually adequate for routine purposes. When biological staining is inconclusive, one of the other
353   specific methods may be used as a confirmatory test. PCR methods are preferred.

1.3. Specific detection methods

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

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

366   PCR methods have been used successfully to detect *C. burnetii* DNA in cell cultures and biological
367   samples. ~~The PCR methods of *C. burnetii* detection are generally performed for the health~~
368   ~~investigations of ruminant herds or flocks prone to abortions (Sidi-Boumedine *et al.*, 2010).~~
369   ~~Nevertheless, as the number of *C. burnetii* is likely to be lower in milk, colostrums and faeces than in~~
370   ~~abortion material, PCR can be used for analysis of this large diversity of samples.~~ Before performing
371   the PCR, biological samples can be inactivated, for ensuring the safety of laboratory personnel, by
372   heating at 90°C for 30–60 minutes, depending of the samples' nature, their size or their weight. The
373   inactivation process must be checked and validated before any use to ensure the safety of personnel.
374   The PCR technique can be performed in suitably equipped laboratories using primers derived from
375   various targets, such as multicopy insertion sequence IS1111 (accession number M80806), the most
376   largely employed (Berri *et al.,* 2000). The use of these primers for the amplification of this sequence
377   allows the sensitivity of the test to be increased and this because of the presence of several copies in
378   the *Coxiella* genomes. The other target genes reported to be used in the PCR for specific *C. burnetii*
379   identification are: superoxide dismutase (sodB) gene (accession number M74242); com1 encoding a
380   27 kDa outer membrane protein (accession number AB004712); heat shock operon encoding two heat
381   shock proteins (htpA and htpB) (accession number M20482); isocitrate dehydrogenase (icd)
382   (accession number AF069035); and macrophage infectivity potentiator protein (cbmip) (accession
383   number U14170). Some primer and probe sequences can be obtained on the web site of the French
384   national reference centre for human Q fever[4].

385   The real-time PCR provides an additional means of detection and quantification (~~Kim *et al.,* 2005;~~ Klee
386   *et al.,* 2006; Stemmler & Meyer, 2002). As with the conventional PCR, various target genes are used:
387   IS1111; IS30; com1; and icd. To quantify the bacteria in biological samples using the real-time PCR, it
388   is recommended to amplify a unique and specific sequence. Indeed, recent data show that the number
389   of the insertion sequence (IS1111) varied widely (between 7 and 110) depending on the isolate (Klee
390   *et al.,* 2006). Whereas the use of this sequence could increase the sensitivity of the test, it may not be
391   accurate for quantification when different strains are involved. It is nevertheless sufficiently informative
392   and accurate for high quantities of bacteria (i.e. >10^4 per vaginal swab) for abortive diagnosis (Sidi-
393   Boumedine *et al.*, 2010). Regarding complex matrices, the DNA eluates should be evaluated for their
394   ability to inhibit a PCR by adding an internal DNA control (such as a GAPDH sequence target) or an
395   external control.

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

403   ~~However~~ For the future, there is an urgent need for the development of a molecular method for the
404   assessment of bacterial viability, especially ~~in milk~~ to assess ~~samples and~~ environmental samples as
405   well as disinfectants and inactivation processes. Isolation is a way to show the viability of the bacteria
406   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

407 enumerate viable bacteria. The development of a multiplex PCR or microarray would provide a useful
408 screening method for all infectious abortive agents in a single assay.

1.4. Genotyping methods

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

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

427 To date, MLVA and MST are considered to be the most discriminating methods for *C. burnetii*, allowing
428 the identification of up to 36 distinct genotypes. Moreover, databases have been
429 established http://mlva.u-psud.fr/MLVAnet/ and http://ifr48.timone.univ-mrs.fr, respectively for MLVA
430 and MST. The availability of such databases allows interlaboratory comparisons to be made easily and
431 this will lead to a better understanding of the propagation of the *C. burnetii* isolates or to identify new
432 emerging strains. Furthermore, their use in the characterisation of field samples or isolates is
433 increasing (Chmielewski *et al.,* 2009; Klaassen *et al.,* 2009) and efforts to produce a standardised
434 scheme for MLVA, based on common decisions for allele calling and marker panels to be used, are in
435 progress and should be encouraged so that they can be made available in the near future (Massung *et*
436 *al.,* 2012; Sidi-Boumedine & Rousset, 2011; Sidi-Boumdedine *et al.*, 2009).

2. Serological tests

438 Among the various techniques that can be employed, The three most commonly used serological tests are the
439 IFA, the ELISA and the CFT. Older serological tests that are no longer used in routine diagnosis include the
440 microagglutination technique, the capillary agglutination test and the indirect haemolysis test. Overall, ELISAs are
441 preferred for practical reasons. Currently, no IFA is commercially available for ruminants. The production of the
442 commercial antigen for CFT will probably cease in the future. Numerous reports showed a weak sensitivity of CFT
443 compared with other methods (EFSA, 2010; Kittelberger *et al.,* 2009; Rousset *et al.,* 2007; 2009a). Serological
444 tools allowing specific antibody detection in sera from different animal species (not only ruminants) should be
445 developed (Jaspers *et al.*, 1994; Soliman *et al.,* 1992).

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

2.1. Enzyme-linked immunosorbent assay (ELISA)

455 This technique has a high sensitivity and a good specificity according to comparative evaluations
456 between methods (Emery *et al.,* 2014; Horigan *et al.,* 2011; Kittelberger *et al.,* 2009; Niemczuk *et al.,*
457 2014; Rousset *et al.,* 2007; 2009a). It is easy to perform in laboratories that have the necessary
458 equipment (a spectrophotometer) and reagents. The ELISA is preferred to IFA and CFT, particularly for
459 veterinary diagnosis, because it is convenient for large-scale screening and the most robust and, as it
460 is a reliable technique for demonstrating *C. burnetii* antibody in various animal species (Jaspers *et al.,*
461 1994; Soliman *et al.,* 1992). Ready-to-use kits are commercially available and can detect anti-phase II
462 antibodies or both anti-phase I and II antibodies. The quality control for some ELISA kits was recently

463 improved using an external reference material, available from the French national reference laboratory,
464 showing the standardisation between kit batches.

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

2.1.1. Materials and reagents

475 Microtitre plates with 96 flat-bottomed wells, freshly coated or previously coated with ~~Q fever~~
476 *C. burnetii* antigen; microplate reader (spectrophotometer; 405 and/or 450 and/or 492 nm
477 filters); 37°C humidified incubator; 8-and 12-channel pipettes with disposable plastic tips;
478 microplate shaker (optional).

479 Positive and negative control sera; conjugate (ruminant anti-immunoglobulin or protein A/G
480 labelled with peroxidase); tenfold concentration of diluent (PBS–Tween); distilled water;
481 substrate or chromogen (TMB [tetramethylbenzidine], ABTS [2,2'-azino-bis-(3-ethylbenzo-
482 thiazoline-6-sulphonic acid)] for peroxidase); hydrogen peroxide.

2.1.2. Test procedure

484 i)   Dilute the serum samples, including control sera, to the appropriated dilution (usually
485   1/100) and distribute 0.1 ml per well in duplicate. Control sera are positive and negative
486   sera provided by the manufacturer and an internal positive reference serum from the
487   laboratory in order to compare the titres between different tests.

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

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

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

492 v)   Add 0.1 ml of freshly prepared chromogen substrate solution to each well (for example:
493   TMB in 0.1 M acetic acid and 30% H_2O_2 solution [0.2 µl/ml]; or 0.25 mM ABTS in citrate
494   phosphate buffer, pH 5.0, and 30% H_2O_2 solution [0.1 µl/ml]).

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

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

2.1.3. Interpretation of the results

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

502 For example: calculate the mean absorbance (Ab) of the sample serum and of the positive
503 (Ab_{pos}) and negative (Ab_{neg}) control sera, and for each serum, calculate the percentage

504
$$\frac{Ab - Ab_{neg}}{Ab_{pos} - Ab_{neg}} \times 100$$

505 Interpret the results as follows:
506 $Ab < 30\%$ negative serum
507 $Ab\ 30–40\%$ doubtful serum
508 $Ab > 40\%$ positive serum

509

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. <u>Nevertheless, the interpretation as acute or chronic forms has not been validated for ruminants.</u>

2.2.1. Antigen preparation

<u>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 2–5 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.</u> ~~An infection on mice can require 7–14 days.~~

<u>Phase II *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 (5000 *g*, 15 minutes) resuspended in 1 ml of PBS with 0.1% formaldehyde and incubated for 24 hours at 4°C. After pooling, the remaining cells are broken by sonication. Cellular debris 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.</u>

<u>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 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).</u>

<u>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.</u>

<u>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.</u>

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)[5] or antigen in phase I and II mixture prepared from Nine Mile strain[6].

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 18 hours at 4°C.

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

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. burnetti* 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~~

661   changes that occurred in LPS phase variation are not clearly defined. Even with extensive repeated passage in
662   non-immunologically competent hosts (cultural cells, embryonated eggs), the majority of isolates are non-clonal
663   as growth from a single colony is difficult to establish (Samuel & Hendrix, 2009). The risk of aerosols must be
664   taken into account at all stages when working with viable *C. burnetii*. Sustained serological monitoring of Q fever
665   should be carried out for laboratory personnel.

2.   Diagnostic biologicals

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

672   Phase II *C. burnetii* Nine Mile are grown in confluent layers of Vero or L929 cells in 150 cm^2 culture flasks at 35°C
673   under 5% CO$_2$ with minimal essential medium (MEM) supplemented with 2 mM L-glutamine and 4% fetal bovine
674   serum. The infection is monitored by microscopic examination of intracellular vacuoles or by Gimenez-stained
675   cells collected from the supernatants of the flasks. Recent specific real-time quantitative PCR has been extremely
676   valuable in routine monitoring. When a heavy *C. burnetii* infection is seen, the supernatants of 15 flasks are
677   individually pelleted by centrifugation (5000 *g*, 15 minutes) resuspended in 1 ml of PBS with 0.1% formaldehyde
678   and incubated for 24 hours at 4°C. After pooling, the remaining cells are broken by sonication. Cellular debris are
679   removed by two successive centrifugation steps (100 *g*, 10 minutes each). The 15 ml suspension is then
680   centrifuged through 20 ml of PBS with 25% sucrose (6000 *g*, 30 minutes, without a break). The resulting pellet is
681   washed three times in PBS (6000 *g*, 10 minutes), resuspended in the smallest possible volume of sterile distilled
682   water, and adjusted to 2 mg/ml by UV spectroscopy. An antibacterial preservative, such as sodium azide at a final
683   dilution of 0.1% or thiomersal at 0.01%, is added. Antigen prepared in this manner is frozen at –20°C.

684   In order to obtain phase I antigen, mice are inoculated with *C. burnetii* grown in cells (mainly in phase II). Nine
685   days after infection, the spleens are removed. Each one is ground in 7.5 ml MEM, and inoculated into three
686   75 cm^2 culture flasks containing L929 or Vero cell monolayers (2.5 ml per flask). Amplification of phase I
687   *C. burnetii* is conducted for 4 weeks, with a culture medium change once a week. The infected cells are then
688   harvested and the bacteria are purified as described above (mainly in phase I).

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

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

3.   Vaccine

706   The protective antigen is composed of purified particles in phase I with the nontruncated phase I LPS structure. In
707   some countries, vaccination is practised for occupationally exposed people, such as abattoir workers,
708   veterinarians and laboratory personnel. A vaccine inactivated by formaldehyde (Q-VAX, CSL Ltd, Australia),
709   prepared from the Henzerling strain of phase I *C. burnetii*, received the approval of the Australian authorities in
710   1989. Phase I vaccines are effective, but vaccination is contraindicated for individuals who had seroconverted or
711   had been exposed to *C. burnetii* prior to immunisation.

712   Several vaccines have been developed against animal Q fever. Results converge today towards the use of a
713   phase I vaccine that is helpful against Q fever in combination with other control measures. An inactivated phase I
714   vaccine is commercially available (Coxevac, CEVA, Hungary) for vaccination of ruminants. A review on Q fever in
715   Slovakia suggests that the decrease in the occurrence of human and animal Q fever could be the result of the

716 large-scale vaccination of cattle that was carried out there over a 10-year period, together with improved
717 veterinary control of domestic animal transport within the country (Serbezov *et al.,* 1999). In the Netherlands, a
718 large vaccination programme has been implemented in goat and sheep farms, accompanied by the controlled
719 processing of manure and checks on animal transports, but it is not clear yet whether bacterial shedding by
720 animals is prevented or at least reduced by vaccination. Controlling the epidemic is difficult and can be
721 compromised by the prolonged stability of the bacterium in the environment and the possible role of animal
722 species other than small ruminants (EFSA, 2010).

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

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

737 # REFERENCES

738 ANDERSON A., BIJLMER H., FOURNIER P.E., GRAVES S., HARTZELL J., KERSH G.J., LIMONARD G., MARRIE T.J., MASSUNG
739 R.F., MCQUISTON J.H., NICHOLSON W.L., PADDOCK C.D. & SEXTON D.J. (2013). Diagnosis and management of Q
740 fever – United States, 2013: recommendations from CDC and the Q Fever Working Group. *MMWR Recomm.*
741 *Rep.,* **62** (RR-03), 1–30.

742 ARRICAU-BOUVERY N., HAUCK Y., BEJAOUI A., FRANGOULIDIS D, BODIER C., SOURIAU A. MEYER H., NEUBAUER H.,
743 RODOLAKIS A. & VERGNAUD G. (2006). Molecular characterization of Coxiella burnetii isolates by infrequent
744 restriction site-PCR and MLVA typing. *BMC Microbiol.,* **6**, 38.

745 ARRICAU-BOUVERY N. & RODOLAKIS A. (2005). Is Q fever an emerging or re-emerging zoonosis? *Vet. Res.*, **3**, 327–
746 349.

747 ARRICAU-BOUVERY N., SOURIAU A., BODIER C., DUFOUR P., ROUSSET E. & RODOLAKIS A. (2005). Effect of vaccination
748 with phase I and phase II *Coxiella burnetii* vaccines in pregnant goats. *Vaccine,* **23**, 4392–4402.

749 BEARE P.A., UNSWORTH N., ANDOH M., VOTH D.E., OMSLAND A., GILK S.D., WILLIAMS K.P., SOBRAL B.W., KUPKO J.J.
750 3RD, PORCELLA S.F., SAMUEL J.E. & HEINZEN R.A. (2009). Comparative genomics reveal extensive transposon-
751 mediated genomic plasticity and diversity among potential effector proteins within the genus *Coxiella. Infect.*
752 *Immun.*, **77**, 642–656.

753 BERRI M., LAROUCAU K. & RODOLAKIS A. (2000). The detection of *Coxiella burnetii* from ovine genital swabs, milk
754 and fecal samples by the use of a single touchdown polymerase chain reaction. *Vet. Microbiol.,* **72**, 285–293.

755 CHMIELEWSKI, T., SIDI-BOUMEDINE K., DUQUESNE V., PODSIADLY E., THIÉRY R. & TYLEWSKA-WIERZBANOWSKA S. (2009).
756 Molecular epidemiology of Q fever in Poland. *Pol. J. Microbiol.*, **58** (1), 9–13.

757 COLEMAN S.A., FISCHER E.R., HOWE D., MEAD D.J. & HEINZEN R.A. (2004). Temporal analysis of *Coxiella burnetii*
758 morphological differentiation. *J. Bacteriol.,* **186**, 7344-7352.

759 DRANCOURT M. & RAOULT D. (2005). Genus I. *Coxiella. In:* Bergey's Manual Of Systematic Bacteriology, Volume 2:
760 The *Proteobacteria*, Part B: The *Gammaproteobacteria*, Brenner D.J., Krieg N.R., Staley J.T. & Garrity G.M., eds.
761 Springer-Verlag, East Lansing, MI, USA, 237–241.

762 DE CREMOUX R., ROUSSET E., TOURATIER A., AUDUSSEAU G., NICOLLET P., RIBAUD D., DAVID V. & LE PAPE M. (2012).
763 *Coxiella burnetii* vaginal shedding and antibody responses in dairy goat herds in a context of clinical Q fever
764 outbreaks. *FEMS Immunol. Med. Microbiol.*, **64**,120–122.

ECDC (EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL) (2010). Panel with Representatives from the Netherlands, France, Germany, United Kingdom, United States of America. Risk assessment on Q fever. *ECDC Technical Report,* 40 pp. doi:10.2900/28860. Available online: www.ecdc.europa.eu

EFSA (EUROPEAN FOOD SAFETY AUTHORITY) (2010). Panel on Animal Health and Welfare (AHAW); Scientific Opinion on Q Fever. *EFSA Journal*, **8** (5), 1595, 114 pp. doi:10.2903/j.efsa.2010.1595. Available online: www.efsa.europa.eu

EMERY M.P., OSTLUND E.N., AIT ICHOU M., BALLIN J.D., MCFARLING D. & MCGONIGLE L. (2014). *Coxiella burnetii* serology assays in goat abortion storm. *J. Vet. Diagn. Invest.*, **26**, 141–145.

GIMENEZ D.F. (1964). Staining rickettsiae in yolk-sack cultures. *Stain. Technol.,* **39**, 135–140.

GLAZUNOVA O., ROUX V., FREYLIKMAN O., SEKEYOVA Z., FOURNOUS G., TYCZKA J., TOKAREVICH N., KOVACAVA E., MARRIE T. & RAOULT D. (2005). *Coxiella burnetii* genotyping. *Emerg. Infect. Dis.*, **11**, 1211–1217.

GUATTEO R., BEAUDEAU F., JOLY A. & SEEGERS H. (2007). *Coxiella burnetii* shedding by dairy cows. *Vet. Res.*, **38** (6) 849–860.

GUATTEO R., SEEGERS H., JOLY A. & BEAUDEAU F (2008). Prevention of *Coxiella burnetii* shedding in infected dairy herds using a phase I *C. burnetii* inactivated vaccine. *Vaccine*, **26** (34), 4320–4338.

HEINZEN R.A., HACKSTADT T. & SAMUEL J.E. (1999). Developmental biology of *Coxiella burnettii. Trends Microbiol.,* **7**, 149–154.

HORIGAN M.W., BELL M.M., POLLARD T.R., SAYERS A.R. & PRITCHARD G.C. (2011). Q fever diagnosis in domestic ruminants: comparison between complement fixation and commercial enzyme-linked immunosorbent assays. *J. Vet. Diagn. Invest.*, **23**, 924–931.

HEINZEN R., STIEGLER G.L., WHITING L.L., SCHMITT S.A., MALLAVIA L.P. & FRAZIER M.E. (1990). Use of pulsed field gel electrophoresis to differentiate *Coxiella burnetii* strains. *Ann. N.Y. Acad. Sci.,* **590**, 504–513.

HENDRIX L.R., SAMUEL J.E. & MALLAVIA L.P. (1991). Differentiation of *Coxiella burnetii* isolates by analysis of restriction-endonuclease-digested DNA separated by SDS-PAGE. *J. Gen. Microbiol.,* **137** (2), 269–276.

JAGER C., WILLEMS H., THIELE D. & BALJER G. (1998). Molecular characterization of *Coxiella burnetii* isolates. *Epidemiol. Infect.*, **120** (2), 157–164.

JASPERS U., THIELE D. & KRAUSS H. (1994). Monoclonal antibody based competitive ELISA for the detection of specific antibodies against *Coxiella burnetii* in sera from different animal species. *Zentralbl. Bakteriol.*, **281**, 61–66.

JENSEN T.K., MONTGOMERY D.L., JAEGER P.T., LINDHARDT T., AGERHOLM J.S., BILLE-HANSEN V. & BOYE M. (2007). Application of fluorescent *in situ* hybridisation for demonstration of *Coxiella burnetii* in placentas from ruminant abortions. *APMIS,* **115**, 347–353.

KERSH G.J., WOLFE T.M., FITZPATRICK K.A., CANDEE A.J., OLIVER L.D., PATTERSON N.E., SELF J.S., PRIESTLEY R.A., LOFTIS A.D. & MASSUNG R.F. (2010). Presence of *Coxiella burnetii* DNA in the environment of the United States (2006–2008). *Appl. Environ. Microbiol.*, **76**, 4469–4475. [Epub ahead of print]

KIM S.G., KIM E.H., LAFFERTY C.J. & DUBOVI E. (2005). *Coxiella burnetii* in bulk tank milk samples, United States. *Emerg. Infect. Dis.,* **11**, 619–621.

KITTELBERGER R., MARS J., WIBBERLEY G., STING R., HENNING K., HORNER G.W., GARNETT K.M., HANNAH M.J., JENNER J.A., PIGOTT C.J. & O'KEEFE J.S. (2009). Comparison of the Q fever complement fixation test and two commercial enzyme-linked immunosorbent assays for the detection of serum anibodies against *Coxiella burnetii* (Q-fever) in ruminants: Recommandations for use of serological tests on imported animals in New Zealand. *NZ Vet. J.*, **57** (5), 262–268.

KLAASSEN C.H., NABUURS-FRANSSEN M.H., TILBURG J.J., HAMANS M.A. & HORREVORTS A.M. (2009). Multigenotype Q fever outbreak, the Netherlands. *Emerg. Infect. Dis.*, **15** (4), 613–614.

KLEE S.R., TYCZKA J., ELLERBROK H., FRANZ T., LINKE S., BALJER G. & APPEL B. (2006). Highly sensitive real-time PCR for specific detection and quantification of *Coxiella burnetii. BMC Microbiol.*, **6**, 2.

KRAUSS H. (1989). Clinical aspects and prevention of Q fever in animals. *Eur. J. Epidemiol.,* **5**, 454–455.

812 Lang G.H. (1990). Coxiellosis (Q fever) in animals. *In:* Q Fever. Volume I: The Disease, Marrie T.J., ed. CRC
813 Press, Boca Raton, USA, 23–48.

814 Massung M.F., Cutler S.J. & Frangoulidis D. (2012). Molecular typing of *Coxiella burnetii* (Q fever). *Adv. Exp.*
815 *Med. Biol.,* **984**, 381–396.

816 Maurin M. & Raoult D. (1999). Q fever. *Clin. Microbiol. Rev.*, **12**, 518–553.

817 Minnick R.F. & Raghavan R. (2012). Developmental biology of *Coxiella burnetii. Adv. Exp. Med. Biol.,* **984**, 231–
818 248.

819 Niemczuk K., Szymańska-Czerwińska M., Śmietanka K. & Bocian Ł. (2014). Comparison of diagnostic potential of
820 serological, molecular and cell culture methods for detection of Q fever in ruminants. *Vet. Microbiol.*, **171**, 147–
821 152.

822 O'Neill T.J., Sargeant J.M. & Poljak Z. (2013). A systematic review and meta-analysis of Phase I inactivated
823 vaccines to reduce shedding of *Coxiella burnetii* from sheep and goats from routes of public health importance.
824 *Zoonoses Public Health.* doi: 10.1111/zph.12086. [Epub ahead of print].

825 Quinn P.J., Carter M.E., Markey B. & Carter G.R. (1994). Bacterial pathogens: microscopy, culture and
826 identification. *In:* Clinical Veterinary Microbiology. Wolfe Publishing, Mosby-Year Book Europe Limited, 21–30.

827 Raoult D., Laurent J.C. & Mutillod M. (1994). Monoclonal antibodies to *Coxiella burnetii* for antigenic detection
828 in cell cultures and in paraffin-embedded tissues. *Am. J. Clin. Pathol.,* **101**, 318–320.

829 Raoult D., Vestris G. & Enea M. (1990). Isolation of 16 strains of *Coxiella burnetii* from patients by using a
830 sensitive centrifugation cell culture system and establishment of the strains in HEL cells. *J. Clin. Microbiol.,* **28**,
831 2482–2484.

832 Rousset E., Berri M., Durand B., Dufour P., Prigent M., Delcroix T., Touratier A. & Rodolakis. A. (2009a).
833 *Coxiella burnetii* shedding routes and antibody response after outbreaks of Q fever-induced abortion in dairy goat
834 herds. *Appl. Environ. Microbiol.*, **75**, 428–433.

835 Rousset E., Durand B., Berri M., Dufour P., Prigent M., Russo P., Delcroix T., Touratier A., Rodolakis A. &
836 Aubert M.F. (2007). Comparative diagnostic potential of three serological tests for abortive Q fever in goat herds.
837 *Vet. Microbiol.,* **124**, 286–297.

838 Rousset E., Durand B., Champion J.L., Prigent M., Dufour P., Forfait C., Marois M., Gasnier T., Duquesne V.,
839 Thiery R. & Aubert M.F (2009b). Efficiency of a phase I vaccine for the reduction of vaginal *Coxiella burnetii*
840 shedding in a clinically affected goat herd. *CMI,* **15** (suppl 1), 1–2.

841 Rousset E., Prigent M., Ameziane G., Brugidou R., Martel I., Grob A., Le Gall G., Kerninon S., Delaval J.,
842 Chassin A., Vassiloglou B., Aulagnon S., Valogne A., Ogier M., Audeval C., Colocci F., Perennes S., Cazalis
843 L., Nicollet P., Maingourt C. & Sidi-Boumedine K. (2012). Adoption by a network's laboratories of a validated
844 quantitative real-time PCR method for monitoring Q fever abortions in ruminant livestock. *Euroreference.* **No. 8**,
845 21–27. Available online: https://pro.anses.fr/euroreference/Documents/ER08-Meth-FievreQAvortEN.pdf

846 Samuel J.E. & Hendrix L.R (2009). Laboratory maintenance of *Coxiella burnetii. Curr. Proto. Micriobiol.*, **6C** (suppl.
847 15), 1–16.

848 Scott G.H., Williams J.C. & Stephenson E.H. (1987). Animal models in Q fever: pathological responses of inbred
849 mice to phase I *Coxiella burnetii. J. Gen. Microbiol.,* **133**, 691–700.

850 Serbezov V.S., Kazar J., Novkirishki V., Gatcheva N., Kovacova E. & Voynova V. (1999). Q fever in Bulgaria and
851 Slovakia. *Emerg. Infect. Dis.,* **5**, 388–394.

852 Seshadri R., Paulsen I.T., Eisen J.A., Read T.D., Nelson K.E., Nelson W.C., Ward N.L., Tettelin H., Davidsen
853 T.M., Beanan M.J., Deboy R.T., Daugherty S.C., Brinkac L.M., Madupu R., Dodson R.J., Khouri H.M., Lee K.H.,
854 Carty H.A., Scanlan D., Heinzen R.A., Thompson H.A., Samuel J.E., Fraser C.M. & Heidelberg J.F. (2003).
855 Complete genome sequence of the Q-fever pathogen *Coxiella burnetii. Proc. Natl Acad. Sci. USA,* **100**, 5455–
856 5460.

857 Sidi-Boumedine K., Duquesne V., Rousset E., Cochonneau D., Cutler S.J., Frangoulidis D., Rodolakis A., Roest
858 H.J., Ruuls R., van Rotterdam B., Vincent G. & Thiéry, R. (2009). A multicentre MLVA and MST typing-ring trial
859 for *C. burnetii* genotyping: An approach to standardisation of methods. 5th MedVetNet Annual Scientific
860 Conference. Madrid, Spain.

861 SIDI-BOUMEDINE K. & ROUSSET E. (2011). Molecular epidemiology of Q fever: a review of *Coxiella burnetii*
862 genotyping methods and main achievements. *EuroReference,* **No. 5**, 30–37. Available
863 online: http://www.ansespro.fr/euroreference/numero5/PNB0I0.htm

864 SIDI-BOUMEDINE K., ROUSSET E., HENNING K., ZILLER M., NIEMCZUCK K., ROEST H.I.J. & THIÉRY R. (2010).
865 Development of harmonised schemes for the monitoring and reporting of Q-fever in animals in the European
866 Union. *EFSA Scientific Report on Question No EFSA-Q-2009-00511.*, 48 pp. Available
867 online: www.efsa.europa.eu

868 SOLIMAN A.K., BOTROS B.A. & WATTS D.M. (1992). Evaluation of a competitive immunoassay for detection of
869 *Coxiella burnetii* antibody in animal sera. *J. Clin. Microbiol.*, **30**, 1595–1597.

870 STEMMLER M. & MEYER H. (2002). Rapid and specific detection of *Coxiella burnetii* by LightCycler PCR. *In:*
871 Methods and Applications. Microbiology and Food Analysis, Reisch U., Wittwer C. & Cockerill F., eds. Springer,
872 Berlin, Germany149–154.

873 ~~SVRAKA S., TOMAN R., SKULTETY L., SLABA K. & HOMAN W.L. (2006) Establishment of a genotyping scheme for~~
874 ~~*Coxiella burnetii. FEMS Microbiol. Lett.,* **254**, 268–274.~~

875 THIELE D., KARO M. & KRAUSS H. (1992). Monoclonal antibody based capture ELISA/ELIFA for detection of *Coxiella*
876 *burnetii* in clinical specimens. *Eur. J. Epidemiol.,* **8**, 568–574.

877 TISSOT-DUPONT H., THIRION X. & RAOULT D. (1994). Q fever serology: cutoff determination for
878 microimmunofluorescence. *Clin. Diagn. Lab. Immunol.,* **1**, 189–196.

879 VAN ASSELDONK M.A., PRINS J. & BERGEVOET R.H. (2013). Economic assessment of Q fever in the Netherlands.
880 *Prev. Vet. Med.*, **112**, 27–34.

881 *

882 * *

883 **NB:** There is an OIE Reference Laboratory for Q fever
884 (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date
885 list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
886 Please contact the OIE Reference Laboratories for any further information on
887 diagnostic tests, reagents and vaccines for Q fever

888

889

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 counter immunoelectrophoresis.*

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

A. INTRODUCTION

Vesicular stomatitis (VS) was described in the United States of America (USA) by Oltsky *et al.* (1926) and Cotton (1927) as a vesicular disease of horses, and subsequently of cattle and pigs. Vesicles are caused by virus on the tongue, lips, buccal mucosa, teats, and in the coronary band epithelium of cattle, horses, pigs, and many other species of domestic and wild animals. Natural disease in sheep and goats is rare, although both species can be experimentally infected. Mixed infections of foot and mouth disease (FMD) and VS viruses have occurred in the same herds of cattle and can be induced experimentally. Many species of laboratory animals are also susceptible. The disease is limited to the Americas; however, it was described in France (1915 and 1917) and in South Africa (1886 and 1897) (Hanson, 1952).

45 Influenza-like signs, normally without vesicles, have been observed in humans who are in contact with animals
46 with VS or who handle infective virus. All manipulations involving virus, including infective materials from animals,
47 should be undertaken with using proper biosafety procedures.

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

65 The mechanism of transmission of the virus is unclear. The viruses have been isolated from sandflies,
66 mosquitoes, and other insects (Comer *et al.*, 1992; Francy *et al.*, 1988; Mason, 1978). Experimental transmission
67 of VS NJ has been demonstrated to occur from black flies (*Simulium vittatum*) to domestic swine and cattle (Mead
68 *et al.*, 2004; 2009) There are also hypotheses that the VS virus is a plant virus present in pasture (Mason, 1978)
69 and that animals are the end of the epidemiological chain and, in special circumstances, the virus could undergo
70 an adaptation process to infect animals, followed by direct transmission between susceptible animals. During the
71 1982 epizootic in <u>the</u> western USA, there were a number of cases where there was direct transmission from
72 animal to animal (Sellers & Maarouf, 1990). ~~While VS is not diagnosed in livestock every year in the USA, it
73 is~~ <u>VSV has historically been</u> considered to be endemic in feral pigs on Ossabaw Island, Georgia, USA (Boring &
74 Smith, 1962), <u>but recent evidence suggests it may have disappeared from the island (Killmaster *et al.*, 2011).</u>

75 The incidence of disease can vary widely among affected herds. Usually 10–15% of the animals show clinical
76 signs. Clinical cases are mainly seen in adult animals. Cattle and horses under 1 year of age are rarely affected.
77 Mortality is close to zero in both species. However, high mortality rates in pigs affected by the NJ virus have been
78 observed. Sick animals recover in about 2 weeks. The most common complications of economic importance are
79 mastitis and loss of production in dairy herds (Lauerman *et al.*, 1962). ~~Both NJ and IND-1 serotypes in the 1995,
80 1997 and 1998 US outbreaks primarily caused clinical disease in horses. Although some clinical signs were
81 observed in cattle, the primary finding in cattle was seroconversion.~~ <u>Recent VSV outbreaks in the USA have been
82 associated primarily with horses and the NJ serotype.</u>

B. DIAGNOSTIC TECHNIQUES

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

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

92 Vesicle fluid, epithelium covering unruptured vesicles, epithelial flaps of freshly ruptured vesicles, or swabs of the
93 ruptured vesicles are the best diagnostic samples. These samples can be collected from mouth lesions, as well
94 as from the feet and any other sites of vesicle development. It is recommended that animals should be sedated
95 before samples are collected to avoid injury to helpers and for reasons of animal welfare. Samples from all
96 species should be placed in containers of Tris-buffered tryptose broth with phenol red, pH 7.6. If complement
97 fixation (CF) is to be carried out for antigen detection, samples from all species can be collected in
98 glycerol/phosphate buffer, pH 7.2–7.6. (Note: glycerol is toxic to virus and decreases the sensitivity of virus
99 isolation; it is therefore only recommended for collection of samples for CFT.) Samples should be kept
100 refrigerated and if they can arrive at the laboratory within 48 hours after collection, they should be sent
101 refrigerated. If samples are sent frozen with dry ice, precautions should be taken to protect the sample from
102 contact with any CO_2. There are special packaging requirements for shipping samples with dry ice (see Chapter

103   1.1.1 *Collection and shipment of diagnostic specimens*, for further information on shipping of diagnostic samples).
104   Alternatively, samples can be shipped with commercially available freezer packs that have been frozen in an
105   ultralow freezer (–60°C or colder) if shipping time is of short duration.

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

112   When it is not possible to collect samples for identification of the agent, serum samples ~~from recovered animals~~
113   can be used for detecting and quantifying specific antibodies. Paired sera from the same animals, collected 1–
114   2 weeks apart, ~~are preferred for checking the change in antibody titre~~ may be needed depending on the
115   serological assay being used and prior history of VSV in the country.

116   Specific reagents for VS diagnosis are not commercially available and each laboratory must produce its own or
117   obtain them from a Reference Laboratory. The two OIE Reference Laboratories for vesicular stomatitis (see Table
118   given in Part 4 of this *Terrestrial Manual*), and the Pirbright Institute, UK[1], produce and distribute diagnostic
119   reagents on request.

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

Method	**Purpose**					
	Population freedom from virus circulation	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination***
Agent identification[2]						
Virus isolation*	=	±	=	+++	=	=
IS-ELISA*	=	±	=	+++	=	=
CFT*	=	±	=	++	=	=
RT-PCR*	=	±	=	++	=	=
Detection of immune response[3]						
LP-ELISA**	+++	+++	+++	+++	+++	++
C-ELISA**	+++	+++	=	=	+++	++
VN**	+++	+++	+++	+++	+++	+++
CFT**	=	±	±	+++	±	=

121   Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other
122   factors severely limits its application; – = not appropriate for this purpose.
123   Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that
124   they have been used widely without dubious results, makes them acceptable.
125   IS-ELISA = indirect sandwich enzyme-linked immunosorbent assay; CFT = complement fixation test;
126   RT-PCR = reverse transcriptase polymerase chain reaction; LP-ELISA = liquid-phase blocking ELISA;
127   C-ELISA = competitive ELISA; VN = virus neutralisation
128   *Should only be used on animals demonstrating clinical signs compatible with VSV. A positive result is meaningful. A negative
129   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.

130 the samples were not maintained at appropriate temperatures and received in an appropriate time period following collection for
131 virus isolation (virus inactivated). **The presence of VSV antibodies only indicates prior exposure to VSV. It does not determine
132 whether the antibodies are due to current infection or past infection. Interpretation of results needs to be based on serological
133 results, clinical presentation, and epidemiology. CF antibody duration in an animal is generally less than 1 year. Antibodies
134 detected by the VN assay and competitive ELISAs can be detected for years following infection. The difference in sensitivity of
135 the serological assays has an effect on detection during the acute phase of infection; combination testing, such as C-ELISA and
136 CF or paired sampling showing four-fold titre change (CF, VN, LP-ELISA), is therefore necessary when an animal presents with
137 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

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

1.2. *In-vitro* cultivation

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

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

159 The preferred immunological methods for the identification of the viral antigens in the laboratory are the
160 ELISA (Alonso *et al.*, 1991; Ferris & Donaldson, 1988), the CFT (Alonso *et al.*, 1991; Jenny *et al.*,
161 1958) and fluorescent antibody staining. The virus neutralisation (VN) test, with known positive antisera
162 against the VS virus NJ and IND serotypes, may be used in tissue cultures or embryonated eggs, but it
163 is more time-consuming.

1.3. *In-vivo* testing

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

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

1.4. Virus isolation

1.4.1. Test procedure

176 i) Inoculate cell culture in Leighton tubes and 25 cm^2 flasks with the clarified suspension of
177   tissues or vesicular fluid.

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

179 iii) Discard inoculum and wash cell cultures three times with cell culture medium and replace
180   with cell culture medium containing 2.5% fetal bovine serum (FBS).

181   Iv)   Incubate Leighton tube cell cultures at 33–35°C and observe for CPE.

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

185   vi)   Remaining Leighton tube cultures and 25 cm^2 flask cultures are incubated at 35–37°C for
186   6 more days and observed daily for CPE.

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

189   viii)   If CPE is observed and the FA staining is negative, a second passage is made, as
190   described above, using the cells from the 25 cm^2 flask. Note: First passage cultures with
191   significant CPE may yield false-negative immunofluorescence results. Serial tenfold
192   dilutions may be prepared and inoculated to provide distinct plaques of fluorescing cells.

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

196   x)   Alternatively cell culture in flasks can be inoculated with field samples, incubated at 35–
197   37°C for 48 hours and observed daily for CPE. If no CPE is observed after 48 hours, the
198   flask cultures are frozen and thawed and a sample of the supernatant is inoculated into
199   fresh cell culture. Up to three passages are made, of 48 hours each. To detect the
200   presence of VSV antigen, clarified supernatants of each passage are tested by ELSA or
201   CFT.

202   **1.5. Enzyme-linked immunosorbent assay**

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

209   **1.5.1. Test procedure**

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

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

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

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

227   v)   *Substrate:* H_2O_2-activated substrate is added and left to react at room temperature for
228   15 minutes, followed by the addition of sulphuric acid to stop the reaction. Absorbance
229   values are measured using an ELISA reader.

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

233   vi)   <u>*Interpretation of the results:* Absorbance values of positive and negative antigen controls</u>
234   <u>wells should be within specified values for acceptance. An antiserum giving an absorbance</u>

235 ≥0.3 is considered to be positive for the corresponding virus subtype. Absorbance values
236 <0.3–0.2 are considered suspicious and values <0.2 are considered negative for the
237 corresponding virus subtype. Suspicious and negative samples should be inoculated in
238 cell culture and passages re-tested in ELISA.

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

1.6. Complement fixation test

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

1.6.1. Test procedure

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

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

253 iii) *Complement:* 4 CHU_{50} (50% complement haemolytic units) are added to the serum and
254   antigen. (An alternative is to use 7.5, 10 and 20 CHU_{50} with the goal of reaching 4 CHU_{50}
255   in the test.) The mixture of antisera, test samples and complement is incubated at 37°C
256   for ~~30~~ 60 minutes.

257 iv) *Haemolytic system:* A suspension of sheep red blood cells (SRBC) in VB, sensitised with
258   10 HU_{50} (50% haemolytic units) of rabbit anti-SRBC serum, is added to the wells. The
259   haemolytic system has an absorbance of 0.66 read at 545 nm, in the proportion of two
260   volumes of haemolytic system + three volumes of distilled water. The mixture is incubated
261   for 30 minutes at 37°C. Subsequently, the plates are centrifuged and the reaction is
262   observed visually.

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

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

270 v) *Interpretation of the results:* When controls are as expected, samples with haemolysis
271   <20% for one antiserum in comparison with the other antiserum and controls are
272   considered to be positive for the corresponding type.

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

1.7. Nucleic acid recognition methods

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

282

283 **2. Serological tests**

284 For the identification and quantification of specific antibodies in serum, the ELISA and the VN test are preferable.
285 The CFT may be used for quantification of early antibodies. Antibody can usually be detected between 5 and
286 8 days post-infection; the length of time antibody persists has not been accurately determined for the three tests
287 but is thought to be relatively short for the CF and for extended periods for the VN and ELISA (Katz *et al.*, 1997).

288 **2.1. Liquid phase blocking enzyme-linked immunosorbent assay (a prescribed test for**
289 **international trade)**

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

294 **2.1.1. Test procedure**

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

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

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

305 iv) *Interpretation of the results:* 50% end-point titres are expressed in $\log_{10}$ in reference to the
306 50% reduction of negative serum control OD of the antigen control, according to the
307 Spearmann–Kärber method. Titres of >1.0 (1/10) are considered to be positive.

308 **2.2. Competitive enzyme-linked immunosorbent assay (a prescribed test for international trade)**

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

312 **2.2.1. Test procedure**

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

320 ii) *Liquid phase:* 50 µl of serum diluted 1/8 in 1% nonfat dry milk in PBS is added to each of
321 the duplicate wells for each sample. A positive and negative control serum for each
322 serotype should be included on each ELISA plate. The plates are incubated at 37°C for
323 30 minutes. Without washing, 50 µl of polyclonal ascites fluid is added to each well and
324 plates are incubated at 37°C for 30 minutes.

325 iii) *Detector:* The plates are washed three times, and 50 µl of goat anti-mouse horseradish-
326 peroxidase conjugate diluted in 1% nonfat dry milk with 10% normal goat serum is added
327 to each well. The plates are incubated at 37°C for 30 minutes, washed three times, and
328 50 µl of tetramethyl-benzidine (TMB) substrate solution is added to each well. The plates
329 are incubated at 25°C for 5–10 minutes and then 50 µl of 0.05 M sulphuric acid is added to
330 each well. The plates are read at 450 nm and the optical density of the diluent control wells
331 must be > 1.0.

332 iv) *Interpretation of the results:* A sample is positive if the absorbance is ≤50% of the
333 absorbance of the diluent control. Note that horses naturally infected with New Jersey
334 virus have been known to test positive by this assay for at least 5 8 years following
335 infection.

336 **2.3. Virus neutralisation (a prescribed test for international trade)**

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

341 **2.3.1. Test procedure**

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

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

346 iii) *Virus neutralisation:* Sera are diluted in a two-fold or four-fold dilution series across the
347 plates, starting from 1/4 dilution. Two rows of wells are used per serum. The same volume
348 of NJ or IND VS virus suspension containing about 1000 $TCID_{50}$/25 µl is added and
349 incubated at 37°C for 60 minutes to allow neutralisation to take place. Subsequently, 50 µl
350 of the mixtures is deposited on preformed cell monolayers in microtitre plates or 150 µl of
351 300,000/ml IB-RS-2 or Vero cell suspension is added to each well with the serum/virus
352 mixtures. The plates are covered with loosely fitting lids and incubated for 48–72 hours at
353 37°C in an atmosphere of 5% CO_2 or sealed with pressure-sensitive tape and incubated in
354 a normal atmosphere. (It has been determined that a virus titre of 1000 $TCID_{50}$/25 µl will
355 decrease the nonspecific reactions and maintain a high test sensitivity.)

356 iv) *Interpretation of the results:* Wells without CPE are considered to be positive. End-point
357 titres of test serum titres are determined by the Spearmann–Kärber method when the virus
358 titres are between 750 and 1330 $TCID_{50}$ and when titres of positive and negative standard
359 sera are within twofold of their mean values as estimated from previous titration. The
360 100% neutralisation titres of each serum are expressed at log 10. Sera with values of 1/32
361 or greater are considered to be positive for antibodies against VSV. Note that horses
362 naturally infected with New Jersey virus have been known to test positive by this test
363 method for at least 5̶ 8 years following infection. In an alternative protocol a viral dose of
364 1000 $TCID_{50}$ per millilitre of virus/serum mixture is used and reaction incubated at 37°C for
365 60 minutes to allow neutralisation to take place. Subsequently, 100 µl of the mixtures is
366 deposited on preformed cell monolayers in microtitre plates. The plates are covered with
367 loosely fitting lids and incubated for 48 hours at 37°C in an atmosphere of 5% CO_2. Wells
368 without CPE are considered to be positive. End-point titres of test serum are determined
369 by the Spearmann–Kärber method when the virus titres are between $10^{2\pm0.5}$/100 µl and
370 when titres of positive and negative standard sera are within twofold of their mean values
371 as estimated from previous titration. ~~In an alternative protocol, the end-point titre of the test~~
372 ~~serum is determined when the virus doses are between $10^{2\pm0.5}$/100 µl and when titres of~~
373 ~~positive and negative standard sera are within twofold of their mean values as estimated~~
374 ~~from the previous titration.~~ The 50% neutralisation titre of each serum is expressed as log
375 10. Sera with values of 1.3 (1/20) or greater are considered to be positive for VS
376 antibodies (Allende *et al.*, 1992).

377 **2.4. Complement fixation test (a prescribed test for international trade)**

378 A detailed description of this test is given in Section B.1.6. This is modified as follows. The CFT may be
379 used for quantification of early antibodies, mostly IgM. For this purpose, twofold serum dilutions are
380 mixed with 2 CFU_{50} of known antigen and with 5% normal bovine or calf sera included in 4 CHU_{50} of
381 complement. The mixture is incubated for 3 hours at 37°C or overnight at 4°C. Subsequently, the
382 haemolytic system is added followed by incubation for 30 minutes at 37°C. The serum titre is the
383 highest dilution in which no haemolysis is observed. Titres of 1/5 or greater are considered to be
384 positive. This CF has low sensitivity and is frequently affected by anticomplementary or nonspecific
385 factors.

386

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 being 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.

488 For killed and modified live virus (MLV) vaccines product safety will be based on an
489 absence of adverse reactions such as shock, abscesses at site of inoculation, etc. In the
490 specific case of MLV vaccines, it would not be expected to see clinical signs. If clinical
491 signs of vesicular stomatitis virus are observed, use of the vaccine should be
492 reconsidered. Residual virus should be evaluated for prior to mixing the antigen with
493 adjuvant. Initial safety is evaluated in a few animals for 21 days under close observation to
494 assess for gross safety issues. If the vaccine passes this first safety test, the vaccine is
495 used in the field in a larger number of animals to evaluate if subtle safety issues are
496 present: adverse reactions/swelling, abscesses, shock, etc.

497 ii) Reversion-to-virulence for attenuated/live vaccines

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

503 iii) Environmental consideration

504 Inactivated vesicular stomatitis vaccines probably present no special danger to the user,
505 although accidental inoculation may result in an adverse reaction caused by the adjuvant
506 and secondary components of the vaccine. Modified live virus vaccines may pose a
507 hazard to the user depending on the level of inactivation of the virus.

508 Preservatives should be avoided if possible, and where not possible, should be limited to
509 the lowest concentration possible. Vaccine bottles, syringes, and needles may pose an
510 environmental hazard for vaccines using adjuvants or preservatives and for modified live
511 virus vaccines. Instructions for disposal should be included within the vaccine packaging
512 information and based on current environmental regulations in the country of use.

513 **2.3.2. Efficacy requirements**

514 The gaps in knowledge regarding the pathogenesis of VSV infection and the immune
515 mechanism that affords protection against viral infection are limitations for the development and
516 implementation of a validated protocol for an efficacy test. Ideally vaccine efficacy should be
517 estimated in vaccinated animals directly by evaluating their resistance to live virus challenge.
518 Vaccine efficacy should be established for every strain to be authorised for use in the vaccine.

519 Live reference VSV viruses corresponding to the virus strains circulating in the region are stored
520 at ultralow temperatures. Each challenge virus is prepared as follows. Tongue tissue infected by
521 VSV should be obtained from original field case of VS and received at the Reference Laboratory
522 in glycerol buffer as described in Section B. *Diagnostic Techniques*.

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

526 The preparation of the stock of challenge virus to be aliquoted is prepared starting from lesions
527 collected in two cattle over 6 months of age, previously recognised to be free of VSV antibodies.
528 These animals are tranquillised, for example using xylazine 100 mg/ml (follow instructions for
529 use), then inoculated intradermally (i.d.) in the tongue with the suspension in about 20 sites,
530 0.1 ml each. The vesiculated tongue tissue is harvested at the peak of the lesions,
531 approximately 2 days later.

532 A 2% suspension is prepared as above and filtered through a 0.2 µm filter, aliquoted and frozen
533 in the gas phase of liquid nitrogen, and constitutes the stock of challenge virus. The infective
534 titres of this stock are determined both in cell culture ($TCID_{50}$) and in two cattle (BID_{50}). These
535 two cattle that have been tranquilised using xylazine, are injected intradermally in the tongue
536 with tenfold dilutions (1/10 through 1/10,000), using four sites per dilution (Henderson, 1949).
537 The cattle titrations are read 2 days later. Most frequently, titres are above 10^6 $TCID_{50}$ for 0.1 ml
538 and above 10^5 BID_{50} for 0.1 ml calculated using the Spearmann–Kärber method. The dilution
539 for use in cattle challenge test is 10 000 DIB_{50} in a total volume of 4× 0.1 ml by intralingual
540 injection for both the PD_{50} test and the PGP test (House *et al.,* 2003).

541   i)   Vaccination–challenge method

542 For this experimental method, a group of 12 VSV sero-negative cattle of at least 6 months
543 of age are vaccinated with a bovine dose by the route and in the volume recommended by
544 the manufacturer at day 0 and day 40. These animals and a control group of two non-
545 vaccinated animals are challenged 2 weeks or more after the second vaccination. The
546 challenge strain is a suspension of bovine virus that is fully virulent and appropriate to the
547 virus types in the vaccine under test by inoculating a total of 10,000 BID_{50} intradermally
548 into four sites (0.1 ml per site) on the upper surface of the tongue. Animals are observed at
549 7–8 days after challenge.

550 It was proposed that vaccinated animals showing no lesion on the tongue should be
551 considered fully protected. Vaccinated animals showing lesions at one, two, or three
552 inoculation sites should be considered partially protected, and animals showing lesions at
553 four sites are considered not protected (House *et al.,* 2003). Control animals must develop
554 lesions at four sites. Vaccine should fully protect at least nine animals out of 12 vaccinated
555 (75% protection), the remaining animals being partially or not protected. This test gives a
556 certain measure of the protection following the injection of two commercial bovine doses of
557 vaccine in a limited cattle population.

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

561   ii)   Efficacy in other species

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

2.3.3. Duration of immunity

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

2.3.4. Stability

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

574   i)   For animal production

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

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

588 The duration of immunity and recommended frequency of vaccination of a vaccine should
589 be determined before a product is approved. Initially, such information is acquired directly
590 using host animal vaccination/challenge studies. The period of demonstrated protection,
591 as measured by the ability of vaccinates to withstand challenge in a valid test, can be
592 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

AFSHAR A., SHAKARCHI N.H. & DULAC G.C. (1993). Development of a competitive enzyme linked immunosorbent assay for detection of bovine, equine, ovine and porcine antibodies to vesicular stomatitis virus. *J. Clin. Microbiol.*, **31**, 1860–1865.

ALONSO A., MARTINS M., GOMES M.P.D., ALLENDE R. & SONDAHL M.S. (1991). Development and evaluation of an enzyme-linked immunosorbent assay for detection, typing and subtyping of vesicular stomatitis virus. *J. Vet. Diagn. Invest.,* **3**, 287–292.

ALONSO FERNANDEZ A. & SONDAHL M.S. (1985). Antigenic and immunogenic characterisation of various strains of the Indiana serotype of vesicular stomatitis isolated in Brazil. *Bol. Cen. Panam. Fiebre Aftosa,* **51**, 25–30.

ALLENDE R., SEPULVEDA L., MENDES DA SILVA A., MARTINS M., SONDAHL M.S. & ALONSO FERNANDEZ A. (1992). An enzyme-linked immunosorbent assay for the detection of vesicular stomatitis virus antibodies. *Prev. Vet. Med.,* **14**, 293–301.

BORING W. & SMITH D. (1962). Vesicular Stomatitis Virus: A Survey and Analysis of the Literature. Technical Study No. 43, US Army Biological Laboratories, Fort Detrick, USA.

COMER S.A., CORN J.L., STALLKNECHT D.E., LANDGRAF J.G. & NETTLES V.F. (1992). Titers of vesicular stomatitis virus New Jersey serotype in naturally infected male and female *Lutzomyia shannoni* (Diptera: Psychodidae) in Georgia. *J. Med. Entomol.,* **29**, 368–370.

COTTON W.E. (1927). Vesicular stomatitis. *Vet. Med.,* **22**, 169–175.

FEDERER K.E., BURROWS R. & BROOKSBY J.B. (1967). Vesicular stomatitis virus – the relation between some strains of the Indiana serotype. *Res. Vet. Sci.*, **8**, 103–117.

FERRIS N.P. & DONALDSON A.I. (1988). An enzyme-linked immunosorbent assay for the detection of VSV antigen. *Vet. Microbiol.*, **18**, 243–258.

FRANCY D.B., MOORE C.G., SMITH G.C., TAYLOR S.A. & CALISER C.H. (1988). Epizootic vesicular stomatitis in Colorado, 1982: isolation of virus from insects collected along the northern Colorado Rocky Mountain Front Range. *J. Med. Entomol.,* **25**, 343–347.

HANSON R.P. (1952). The natural history of vesicular stomatitis. *Bacteriol. Rev.,* **16**, 179–204.

HENDERSON W.M. (1949). The quantitative study of foot and mouth disease virus. Agricultural Research Council Report Series No. 8, HMSO, London, UK, page 5.

HOFNER M.C., CARPENTER W.C., FERRIS N.P., KITCHING R.P. & BOTERO F.A. (1994). A hemi-nested PCR assay for the detection and identification of vesicular stomatitis virus nucleic acid. *J. Virol. Methods*, **50**, 11–20.

HOUSE J.A, HOUSE C., DUBOURGET P. & LOMBARD M. (2003). Protective immunity in cattle vaccinated with a commercial scale inactivated, bivalent vesicular stomatitis vaccine. *Vaccine,* **21**, 1932–1937.

639 JENNY E.W., MOTT L.O. & TRAUB E. (1958). Serological studies with the virus of vesicular stomatitis. I. Typing of
640 vesicular stomatitis by complement fixation. *Am. J. Vet. Res.*, **19**, 993–998.

641 KATZ J.B., EERNISSE K.A., LANDGRAF J.G. & SCHMITT B.J. (1997). Comparative performance of four serodiagnostic
642 procedures for detecting bovine and equine vesicular stomatitis virus antibodies. *J. Vet. Diagn. Invest.,* **9**, 329–
643 331.

644 KATZ J.B., SHAFER A.L. & EERNISSE K.A. (1995). Construction and insect larval expression of recombinant vesicular
645 stomatitis nucleocapsid protein and its use in competitive ELISA. *J. Virol. Methods,* **54**, 145–157.

646 KILLMASTER L.F., STALKNECHT D.E., HOWERTH E.W., MOULTON J.K., SMITH P.F. & MEAD D.G. (2011). Apparent
647 disappearance of vesicular stomatitis New Jersey virus from Ossabaw Island, Georgia. *Vector Borne Zoonotic*
648 *Dis.*, **11**, 559–565.

649 LAUERMAN L.H., KUNS M.L. & HANSON R.S. (1962). Field trial of live virus vaccination procedure for prevention of
650 vesicular stomatitis in dairy cattle. I: Preliminary immune response. Proceedings of the 66th Annual Meeting of the
651 United States Animal Health Association, 365–369.

652 MASON J. (1978). The epidemiology of vesicular stomatitis. *Bol. Cen. Panam. Fiebre Aftosa,* **29**–**30**, 35–53.

653 MEAD D.G., LOVETT K.R., MURPHY M.D., PAUSZEK S.J., SMOLIGA G., GRAY E.W., NOBLET R., OVERMYER J. &
654 RODRIGUEZ L.L. (2009). Experimental transmisión of vesicular stomatitis New Jersey virus from *Simulium vittatum*
655 to cattle: clinical outcome is influenced by sie of insect feeding. *J. Med. Entomol.,* **46**, 866–872.

656 MEAD D.G., GRAY E.W., MURPHY M.D., HOWERTH E.W. & STALLKNECHT D.E. (2004). Biological transmission of
657 vesicular stomatitis virus (New Jersey seroype) by *Simulium vittatum* (Diptera: Simuliidae) to domestic swine (*Sus*
658 *scrofa*). *J. Med. Entomol.*, **41**, 78–82.

659 OLTSKY P.K., TRAUM J. & SCHOENING H.W. (1926). Comparative studies on vesicular stomatitis and foot and mouth
660 disease. *J. Am. Vet. Med. Assoc.,* **70**, 147–167.

661 RODRIGUEZ L.L., LETCHWORTH G.J., SPIROPOULOU C.F. & NICHOL S.T. (1993). Rapid detection of vesicular stomatitis
662 virus New Jersey serotype in clinical samples by using polymerase chain reaction. *J. Clin. Microbiol.*, **31**, 2016–
663 2020.

664 SELLERS R.F. & MAAROUF A.R. (1990). Trajectory analysis of winds in vesicular stomatitis in North America.
665 *Epidemiol. Infect.,* **104**, 313–328.

666 WILSON W.C., LETCHWORKTH G.J., JIMENEZ C., HERRERO M.V., NAVARRO R., PAZ P., CORNISH T.E., SMOLIGA G.,
667 PAUSZEK S.J., DORNAK C., GEORGE M. & RODRIGUEZ L.L. (2009). Field evaluation of a multiplex real-time reverse
668 transcription polymerase chain reaction assay for detection of Vesicular stomatitis virus. *J. Vet. Diagn. Invest.*, **21**,
669 179–186.

670   *

671   * *

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

CHAPTER 2.3.9.

FOWL CHOLERA

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 butyraceous. 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

45 exposed by the oral or subcutaneous routes. Other bacterial diseases, including salmonellosis, colibacillosis, and
46 listeriosis in chickens, and pseudotuberculosis, erysipelas, and chlamydiosis in turkeys, may present with clinical
47 signs and lesions similar to fowl cholera. Differentiation is based on isolation and identification, as *P. multocida* is
48 readily cultured from cases of fowl cholera.

49 # B. DIAGNOSTIC TECHNIQUES

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

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

67 Diagnosis depends on isolation and identification of the causative organism.

68 **Table 1.** *Test methods available for the diagnosis of fowl cholera and their purpose*

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification[1]						
Culture	=	=	=	+++	=	=
Detection of immune response						
Serological ELISA	=	=	=	=	=	++

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

74 ## 1. Identification of the agent

75 *Pasteurella multocida* is a facultative anaerobic bacterium that grows best at 35–37°C. Primary isolation is usually
76 accomplished using media such as blood agar, trypticase–soy agar or dextrose starch agar, and isolation may be
77 improved by supplementing these media with 5% heat-inactivated serum. Maintenance media usually do not
78 require supplemental serum. Colonies range from 1 to 3 mm in diameter after 18–24 hours of incubation. They
79 usually are discrete, circular, convex, translucent, and butyraceous. Capsulated organisms usually produce larger
80 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_2S 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* Pasteurella *species and* Riemerella anatipestifer

Test*	*Pasteurella*		*Riemerella*
	multocida	*gallinarum*	*anatipestifer*
Haemolysis on blood agar	–*	–	v
Growth on MacConkey's agar	–	–	–
Indole production	+	–	–
Gelatin liquefaction	–	–	+u
Catalase production	+	+	+
Urease production	–	–	v
Glucose fermentation	+	+	–
Lactose fermentation	–u	–	–
Sucrose fermentation	+	+	–
Maltose fermentation	–u	+	–
Ornithine decarboxylase	+	–	–

*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

120 reagents. To determine the serotype, the laboratory prepares the unknown bacterial culture as antigen for the
121 AGID test and then must test it against all 16 serotype-specific antisera. Antigens present in a single isolate may
122 react with multiple serotype-specific antisera resulting in bi- or trinomial serotypes, as illustrated by the 3, 4 and 3,
123 4, 12 strains (Rimler, 1998; Glisson *et al.*, 2008).

1.1. Somatic typing procedure using the gel diffusion precipitin test

1.1.1. 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 *Hha*I or *Hpa*II endonuclease (Wilson *et al.*, 1992).

2. Serological tests

167 Serological tests for the presence of specific antibodies are not used for diagnosis of fowl cholera. The ease of
168 obtaining a definitive diagnosis by isolation and identification of the causative organism precludes the need for
169 serodiagnosis. Serological tests, such as agglutination, AGID, and passive haemagglutination, have been used
170 experimentally to demonstrate antibody against *P. multocida* in serum from avian hosts; none were highly
171 sensitive. Determinations of antibody titres using enzyme-linked immunosorbent assays have been used with
172 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 aluminium 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 (sse 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

219 volume is prepared. Cultures are harvested when they reach a suitable density, frequently
220 measured by spectrophotometry (optical density).

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

2.2.2. Requirements for ingredients

226 See chapter 1.1.6.

2.2.3. In-process controls

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

2.2.4. Final product batch tests

236 i)   Sterility/purity

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

241 ii)   Identity

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

245 iii) Safety

246   Safety testing is conducted on each bulk or filled vaccine lot ~~Live vaccines are tested~~
247   ~~according to the method described in Section C1.2.3.2.i, except that only one~~
248   ~~representative animal species is required. Bacterins are administered according to label~~
249   ~~recommendations,~~ and ~~the~~ may be assessed in birds ~~are observed~~ vaccinated for ~~14 days;~~
250   ~~at least 18 of 20 birds must show no unfavourable reactions attributable to the~~
251   ~~bacterin~~ batch potency tests.

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

254 iv)   Batch potency

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

258   ~~Bacterins are~~ In the USA, inactivated vaccines are typically tested for batch potency in a
259   vaccination–challenge trial, such as described in 9 CFR Parts 113.116-118 (USDA, 2013).
260   Separate groups of birds (20 vaccinates, 10 controls) ~~must be~~ are challenged with each of
261   the serotypes of *P. multocida* for which protection is claimed. ~~Bacterins~~ Vaccines are
262   administered according to the dose and route recommended on the label ~~Two doses are~~
263   ~~administered 3 weeks apart~~, and all birds are challenged 2 weeks after the second dose.
264   The birds are observed for 14 days after challenge. For a satisfactory test according to
265   9 CFR, at least 14 of 20 vaccinates must survive and at least 8 of 10 controls must die.

266   ~~The mean bacterial count of any vaccine lot at the time of preparation must be sufficiently~~
267   ~~high to ensure that at any time prior to product expiration, the count is at least twice the~~
268   ~~immunogenicity standard. (The European Pharmacopoeia requires a count that is at least~~
269   ~~equal to the immunogenicity standard.)~~

270 In the EU, a serological test or other validated method may be used for batch potency after
271 a batch of minimum permissible potency is initially tested in a vaccination–challenge trial
272 (European Pharmacopoeia, 2008).

273 v) Formaldehyde content

274 Vaccines inactivated with formaldehyde are tested for residual formaldehyde.

2.3. Requirements for authorisation/registration/licensing

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

2.3.1. Manufacturing process

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

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

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

2.3.2. Safety requirements

291 i) Target and non-target animal safety

292 Inactivated vaccines should pose no hazard to non-target species. Safety in target animals
293 may be evaluated according to harmonised requirements in VICH GL44 (VICH, 2009). The
294 EU and USA recommend vaccinating at least 20 non-immune, unexposed birds according
295 to label recommendations and evaluating daily for adverse reactions. The EU monitors for
296 21 days. In the USA, target animal safety is evaluated during the pre-challenge period of
297 the efficacy study, which is typically 5 weeks.

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

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

302 Not applicable.

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

307 iii) Precautions (hazards)

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

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

2.3.3. Efficacy requirements

316 Products prepared from candidate master seeds should be shown to be effective against
317 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

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.

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%.

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 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 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.

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

CARTER G.R. (1955). Studies on *Pasteurella multocida*. I. A hemagglutination test for the identification of serological types. *Am. J. Vet. Res.*, **16**, 481–484.

CARTER G.R. (1972). Improved hemagglutination test for identifying type A strains of *Pasteurella multocida. Appl. Microbiol*, **24**, 162–163.

~~CODE OF FEDERAL REGULATIONS (CFR) (OF THE UNITED STATES OF AMERICA) (2001). Title 9, Animals and Animal Products. US Government Printing Office, Washington D.C., USA.~~

DERIEUX W.T. (1978). Response of young chickens and turkeys to virulent and avirulent *Pasteurella multocida* administered by various routes. *Avian Dis.*, **22**, 131–39.

EUROPEAN PHARMACOPOEIA (2008). Vaccines for Veterinary Use. Fowl Cholera Vaccine Inactivated. 1945. European Directorate for the Quality of Medicines and Health Care (EDQM), Council of Europe, Strasbourg, France.

GLISSON J.R., HOFACRE C.L. & CHRISTENSEN J.P. (2008). Pasteurellosis and other respiratory bacterial infections. *In:* Diseases of Poultry, Twelfth Edition, Saif Y.M., Fadly A.M., Glisson J.R., McDougald L.R., Nolan L.K. & Swayne D.E., eds. Blackwell Publishing Professional, Ames, Iowa, USA, 739–758.

GLISSON J.R., SANDHU T.S., & HOFACRE C.L. (2008). Pasteurellosis, Avibacteriosis, Gallibacteriosis, Riemerellosis, and Pseudotuberculosis. *In:* A Laboratory Manual for the Isolation, Identification, and Characterization of Avian Pathogens, Fifth Edition, Dufour-Zavala L., Swayne D.E., Glisson J.R., Pearson J.E., Reed W.M., Jackwood M.W. & Woolcock P.R., eds. American Association of Avian Pathologists, Athens, Georgia, USA, 12–14.

HEDDLESTON K.L. (1962). Studies on pasteurellosis. V. Two immunogenic types of *Pasteurella multocida* associated with fowl cholera. *Avian Dis.*, **6**, 315–321.

HEDDLESTON K.L., GALLAGHER J.E. & REBERS P.A. (1972). Fowl cholera: Gel diffusion precipitin test for serotyping *Pasteurella multocida* from avian species. *Avian Dis.*, **16**, 925–936.

International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) (2003). Guideline 25: Testing of residual formadehyde. http://www.vichsec.org/pdf/03_2003/Gl25_st7.pdf

International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (2003). Guideline 26: Testing of residual moisture. http://www.vichsec.org/pdf/03_2003/Gl26_st7.pdf

International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (2006). Guideline 40: Target Animal Safety – Examination of Live Veterinary Vaccines in Target Animals for Absence of Reversion to Virulence. http://www.vichsec.org/pdf/0807/GL41-st7.pdf

International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) (2009). Guideline 44: Target Animal Safety – Examination of Live Veterinary Vaccines in Target Animals for Absence of Reversion to Virulence. http://www.vichsec.org/pdf/0807/GL41-st7.pdf

503   RIMLER R.B. (1994). Presumptive identification of *Pasteurella multocida* serogroups A, D, and F by capsule
504   depolymerisation with mucopolysaccharidases. *Vet. Rec.*, **134**, 191–192.

505   RIMLER R.B. & GLISSON J.R. (1997). Fowl cholera. *In:* Diseases of Poultry, Tenth Edition, Calnek B.W., Barnes
506   H.J., Beard C.W., McDougald L.R. & Saif Y.M., eds. Iowa State University Press, Ames, Iowa, USA. 143–159.

507   RIMLER R.B., SANDHU T.S. & GLISSON J.R. (1998). Pasteurellosis, Infectious Serositis, and Pseudotuberculosis. *In:*
508   A Laboratory Manual for the Isolation and Identification of Avian Pathogens, Fourth Edition, Swayne D.E., Glisson
509   J.R., Jackwood, M.W., Pearson, J.E. & Reed W.M., eds. American Association of Avian Pathologists, Kennett
510   Square, Pennsylvania, USA, 17–28.

511   UNITED STATES DEPARTMENT OF AGRICULTURE (USDA) (2013). Code of Federal Regulations, Title 9, Animals and
512   Animal Products. Office of the Federal Register, National Archives and Records Administration. US Government
513   Printing Office, Washington D.C., USA.

514   WILSON M.A., RIMLER R.B. & HOFFMAN L.J. (1992). Comparison of DNA fingerprints and somatic serotypes of
515   serogroups B and E *Pasteurella multocida* isolates. *J. Clin. Microbiol.*, **30**, 1518–1524.

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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 and does not cause clinical disease. The organism is classified in the genus* Anaplasma *belonging to the family* Anaplasmataceae *of the order* Rickettsiales.

Description of the disease: *Anaemia, and jaundice 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*

45 *amplification can occur. Recently, real-time PCR assays with analytical sensitivity equivalent to*
46 *nested conventional PCR have been described.*

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

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

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

A. INTRODUCTION

58

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

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

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

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

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

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

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

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

B. DIAGNOSTIC TECHNIQUES

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

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Microscopic examination	=	±	=	+++	=	=
Agent identification[1]						
PCR	=	+++	=	+++	=	=
Detection of immune response[2]						
CAT	=	=	=	=	+–	±
ELISA	+++	±	+++	=	+++	+++
IFAT	±	=	=	=	++	++
CFT	=	=	=	=	±	±

107 Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other
108 factors severely limits its application; – = not appropriate for this purpose.
109 Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine
110 nature and the fact that they have been used widely without dubious results, makes them acceptable.
111 Agent id. = agent identification; CAT = card agglutination test; CFT = complement fixation test; ELISA = enzyme-linked
112 immunosorbent assay; IFAT = indirect fluorescent antibody test; PCR = polymerase chain reaction.

1. Identification of the agent

1.1. Microscopic examination

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

122 In contrast to *Babesia bovis, A. marginale* does not accumulate in capillaries, so blood drawn from the
123 jugular or other large vessel is satisfactory. Because of the rather indistinctive morphology of
124 *Anaplasma,* it is essential that smears be well prepared and free from foreign matter, as specks of
125 debris can confuse diagnosis. Thick blood films as used for the diagnosis of babesiosis are not
126 appropriate for the diagnosis of anaplasmosis, as *Anaplasma* are difficult to identify once they become
127 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.

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

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

137 Both blood and organ smears can be stained in 10% Giemsa stain for approximately 30 minutes after
138 fixation in absolute methanol for 1 minute. After staining, the smears are rinsed three or four times with
139 tap water to remove excess adhering stain, and are then air-dried. Conditions for Giemsa staining vary
140 from laboratory to laboratory, but distilled water is not recommended for dilution of Giemsa stock.
141 Water should be pH 7.2–7.4 to attain best resolution with Giemsa stain. Commercial stains that give
142 very rapid staining of *Anaplasma* are available in some countries[3]. Smears are examined under oil
143 immersion at a magnification of ×700–1000.

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

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

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

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

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

169 ### 1.2. Polymerase chain reaction

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

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3 Commercial stains include Camco-Quik and Diff-Quik, Baxter Scientific Products, McGaw Park, Illinois, USA, and Hema 3 and Hema-Quik, Curtin-Matheson, Houston, Texas, USA.

181   maintenance, and may be beyond the capabilities of some laboratories. Real-time PCR assays may
182   target one of several genes (Carelli *et al.*, 2007; Decaro *et al.*, 2008), or 16S rRNA (Reinbold *et al.*,
183   2010<u>b</u>), and are reported to achieve a level of analytical sensitivity equivalent to nested conventional
184   PCR (Carelli *et al.*, 2007; Decaro *et al.*, 2008; Reinbold *et al.*, 2010<u>b</u>).

185 ## 2.   Serological tests

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

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

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

206 ### 2.1. Competitive enzyme-linked immunosorbent assay

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

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

225   #### 2.1.1. Kit reagents

226   A 96-well microtitre plate coated with rMSP5 antigen,

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

228   100×MAb/peroxidase conjugate,

229   10× wash solution and ready-to-use conjugate-diluting buffer,

230   Ready-to-use substrate and stop solutions,

231   Positive and negative controls

232   #### 2.1.2. Test procedure

233 234	i)	Add 70 µl of undiluted serum sample to the coated adsorption/transfer plate and incubate at room temperature for 30 minutes.
235 236	ii)	Transfer 50 µl per well of the adsorbed serum to the rMSP5-coated plate and incubate at room temperature for 60 minutes.
237	iii)	Discard the serum and wash the plate twice using diluted wash solution.
238 239	iv)	Add 50 µl per well of the 1× diluted MAb/peroxidase conjugate to the rMSP5-coated plate, and incubate at room temperature for 20 minutes.
240 241	v)	Discard the 1×diluted MAb/peroxidase conjugate and wash the plate four times using diluted wash solution.
242 243	vi)	Add 50 µl per well of the substrate solution, cover the plate with foil, and incubate for 20 minutes at room temperature.
244 245	vii)	Add 50 µl per well of stop solution to the substrate solution already in the wells and gently tap the sides of the plate to mix the wells.
246	viii)	Immediately read the plate in the plate reader at 620 nm.

2.1.3. Test validation

248
249 The mean optical density (OD) of the negative control must range from 0.40 to 2.10. The per cent inhibition of the positive control must be ≥30%.

2.1.4. Interpretation of the results

251 The % inhibition is calculated as follows:

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

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

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255 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

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268 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.

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

2.2.1. Test reagents

271 A 96-well microtitre plate coated with crude *A. marginale* antigen,

272 PBS/Tween buffer, (PBS 0.1 M, pH 7.2, Tween 20 0.05%),

273 Blocking reagent this is usually commercial dry skim milk

274 Tris buffer 0.1M, MgCl2, 0.1M, NaCl .005 M, pH 9.8

275 Substrate *p*-Nitrophenyl phosphate disodium hexahydrate

276 Positive and negative controls.

2.2.2. Test procedure (this test is run in triplicates)

278	i)	Plates can be prepared ahead of time and kept under airtight conditions at –20°C
279 280	ii)	Carefully remove the plastic packaging to use plates being careful not to touch the bottom of them as it can distort the optical density reading.
281 282	iii)	Remove the lid and deposit 200 μl PBST20 solution in each well and incubate for 5 minutes at room temperature (RT).
283	iv)	For one plate, dissolve 1.1 g of skim milk in 22 ml of PBST20.
284 285	v)	Remove the plate contents and deposit in each well, 200 μl of blocking solution put the lid 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;
288 289	viii)	Remove the contents of the plate and deposit 200 μl of diluted serum in each of the three 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.
292 293	xi)	Dilute 1/1000 anti-IgG bovine alkaline phosphatase conjugate in PBST20 solution; Add 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.
295 296	xiii)	Remove the contents of the plate and deposit 195 μl of 0.075% *p*-Nitrophenyl phosphate disodium hexahydrate in Tris buffer and incubate for 60 minutes at 37°C
297 298	ix)	The reaction is quantified by a microplate reader spectrophotometer, adjusted to 405 nm wavelength. The data are expressed in optical density (OD).

2.2.3. Data analysis

Analysis of results should take into account the following parameters.

301	i)	The mean value of the blank wells.
302	ii)	The mean value of the positive wells with their respective standard deviations.
303	iii)	The mean value of negative wells with their respective standard deviations.
304 305	iv)	The mean value of the blank wells is subtracted from the mean of all the other samples if not automatically subtracted by the ELISA reader.
306 307	v)	Control sera are titrated to give optical density values ranging from 0.90 to 1.50 for the 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 the negative and two times the standard deviation.

For purposes of assessing the consistency of the test operator, the error "E" must also be estimated; this is calculated by determining the percentage represented by the standard 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 field, and gives a result within a few minutes. Nonspecific reactions may be a problem, and subjectivity in interpreting assay reactions can result in variability in test interpretation. In addition, the CAT antigen, which is a suspension of *A. marginale* particles, can be difficult to prepare and can vary from batch to batch and laboratory to laboratory. Splenectomised calves are infected by intravenous inoculation with blood containing *Anaplasma*-infected erythrocytes. When the rickettsaemia exceeds 50%, the animal is exsanguinated, the infected erythrocytes are washed, lysed, and the erythrocyte ghosts and *Anaplasma* particles are pelleted. The pellets are sonicated, washed, and then resuspended in a stain solution to produce the antigen suspension.

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

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[4]. 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.*, 2007; 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).

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

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

382 ## 2. Outline of production and minimum requirements for conventional vaccines

383 ### 2.1. Characteristics of the seed

384 #### 2.1.1. Biological characteristics

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

391 *Anaplasma centrale* usually causes benign infections, especially if used in calves under
392 9 months of age. Severe reactions following vaccination have been reported when adult cattle
393 are inoculated. The suitability of an isolate of *A. centrale* as a vaccine can be determined by
394 inoculating susceptible cattle, monitoring the subsequent reactions, and then challenging the
395 animals and susceptible controls with a virulent local strain of *A. marginale.* Both safety and
396 efficacy can be judged by monitoring rickettsaemias in stained blood films and the depression of
397 packed cell volumes of inoculated cattle during the vaccination and challenge reaction periods.

398 Infective material for preparing the vaccine is readily stored as frozen stabilates of infected
399 blood in liquid nitrogen or dry ice. Dimethyl sulphoxide (DMSO) and polyvinylpyrrolidone M.W.
400 40,000 (Bock *et al.*, 2004) are the recommended cryopreservatives, as they allow for
401 intravenous administration after thawing of the stabilate. A detailed account of the freezing
402 technique using DMSO is reported elsewhere (Mellors*et al.,* 1982), but briefly involves the
403 following: infected blood is collected, chilled to 4°C, and cold cryoprotectant (4 M DMSO in
404 PBS) is added slowly with stirring to a final blood:protectant ratio of 1:1, to give a final
405 concentration of 2 M DMSO. The entire dilution procedure is carried out in an ice bath and the
406 diluted blood is dispensed into suitable containers (e.g. 5 ml cryovials), and frozen, as soon as
407 possible, in the vapour phase of a liquid nitrogen container.

408 #### 2.1.2. Quality criteria

409 Evidence of purity of the *A. centrale* isolate can be determined by serological testing of paired
410 sera from the cattle used in the safety test for possible contaminants that may be present (Bock
411 *et al.*, 2004; Pipano, 1997). Donor calves used to expand the seed for vaccine production
412 should be examined for all blood-borne infections prevalent in the vaccine-producing country,
413 including *Babesia*, *Anaplasma*, *Ehrlichia*, *Theileria* and *Trypanosoma*. This can be done by
414 routine examination of stained blood films after splenectomy, and preferably also by serology.
415 Any calves showing evidence of natural infections of any of these agents should be rejected.
416 The absence of other infective agents should also be confirmed. These may include the agents
417 of enzootic bovine leukosis, mucosal disease, infectious bovine rhinotracheitis, ephemeral
418 fever, Akabane disease, bluetongue, foot and mouth disease, and rinderpest. The testing
419 procedures will depend on the diseases prevalent in the country and the availability of tests, but
420 should involve serology of paired sera at the very least and, in some cases, virus isolation,
421 antigen, or DNA/RNA detection (Bock *et al.*, 2004; Pipano, 1981; 1997).

422 ### 2.2. Method of manufacture

423 #### 2.2.1. Procedure

424   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 1×10^8/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 guaranty sterility and obviate the need to prepare glass flask 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 stabilate (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

Infective material for chilled vaccine is prepared in the same way as for frozen vaccine, but it must be issued and used as soon as possible after collection. The infective blood can be diluted to provide 1×10^7 parasites per dose of vaccine. A suitable diluent is 10% sterile bovine serum in a glucose/balanced salt solution containing the following quantities per litre: NaCl (7.00 g), $MgCl_2.6H_2O$ (0.34 g), glucose (1.00 g), Na_2HPO_4 (2.52 g), KH_2PO_4 (0.90 g), and $NaHCO_3$ (0.52 g).

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

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, ephemeral fever, Akabane disease, Aino virus, bluetongue, parainfluenza, foot and mouth disease, lumpy skin disease, rabies, Rift Valley fever, rinderpest, contagious bovine pleuropneumonia, Jembrana disease, heartwater, pathogenic *Theileria* and *Trypanosoma spp.*, *Brucella abortus*, *Coxiella*, and *Leptospira* (Bock *et al.*, 2004; Pipano, 1981; 1997). 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

AL-ADHAMI B., SCANDRETT W.B., LOVANOV V.A. & GAJADHAR A.A. (2011). Serological cross reactivity between *Anaplasma marginale* and *Ehrlichia* species in naturally and experimentally infected cattle. *J. Vet. Diagn. Invest.,* **23**, 1181–1188.

AMERAULT T.E. & ROBY T.O. (1968). A rapid card agglutination test for bovine anaplasmosis. *J. Am. Vet. Med. Assoc.*, **153**, 1828–1834.

AMERAULT T.E., ROSE J.E. & ROBY T.O. (1972). Modified card agglutination test for bovine anaplasmosis: evaluation with serum and plasma from experimental and natural cases of anaplasmosis. *Proc. U.S. Anim. Health Assoc.,* **76**, 736–744.

BARRY D.N., PARKER R.J., DE VOS A.J., DUNSTER P. & RODWELL B.J. (1986). A microplate enzyme-linked immunosorbent assay for measuring antibody to *Anaplasma marginale* in cattle serum. *Aust. Vet. J.,* **63***, 76–79.*

BOCK R., JACKSON L., DE VOS A. & JORGENSEN W. (2004). Babesiosis of cattle. *Parasitology,***129**,Suppl, S247–269.

BOCK R.E. & DE VOS A.J. (2001). Immunity following use of Australian tick fever vaccine: a review of the evidence. *Aust. Vet. J.,* **79**, 832–839.

BRADWAY D.S., TORIONI DE ECHAIDE S., KNOWLES D.P., HENNAGER S.G. & MCELWAIN T.F. (2001). Sensitivity and specificity of the complement fixation test for detection of cattle persistently infected with *Anaplasma marginale. J. Vet. Diagn. Invest.*, **13**, 79–81.

CARELLI G., DECARO N., LORUSSO A., ELIA G., LORUSSO E., MARI V., CECI L. & BUONAVOGLIA C. (2007). Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR. *Vet. Microbiol.*, **124**, 107–114.

COETZEE J.F., SCHMIDT P.L., APLEY M.D., REINBOLD J.B. & KOCAN K.M. (2007). Comparison of the complement fixation test and competitive ELISA for serodiagnosis of *Anaplasma marginale* infection in experimentally infected steers. *Am. J. Vet. Res.*, **68**, 872–878.

DECARO N., CARELLI G., LORUSSO E., LUCENTE M.S., GRECO G., LORUSSO A., RADOGNA A., CECI L. & BUONAVOGLIA C. (2008). Duplex real-time polymerase chain reaction for simultaneous detection and quantification of *Anaplasma marginale* and *Anaplasma centrale. J. Vet. Diagn. Invest.*, **20**, 606–611.

DE VOS A.J. & JORGENSEN W.K. (1992). Protection of cattle against babesiosis in tropical and subtropical countries with a live, frozen vaccine. *In:*Tick Vector Biology, Medical and Veterinary Aspects, Fivaz B.H., PetneyT.N. & Horak I.G., eds. Springer Verlag, Berlin, Germany, 159–174.

DREHER U.M., DE LA FUENTE J., HOFMANN-LEHMANN R., MELI M.K., PUSTERIA N., KOCAN K.M., WOLDEHIWET A., REGULA G. & STAERK K.D.C. (2005). Serologic cross reactivity between *Anaplasma marginale* and *Anaplasma phagocytophilum. Clin. Vaccine. Immunol.,* **12**, 1177–1183.

DUMLER J.S., BARBET A.F., BEKKER C.P., DASCH G.A., PALMER G.H., RAY S.C., RIKIHISA Y. & RURANGIRWA F.R. (2001). Reorganization of genera in the Families Rickettsiaceae and Anaplasmataceae in the order *Rickettsiales:* unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia*, and *Ehrlichia* with *Neorickettsia*, descriptions of five new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila. Int. J. Syst. Evol. Microbiol.*, **51**, 2145–2165.

JORGENSEN W.K., DE VOS A.J. & DALGLIESH R.J. (1989). Infectivity of cryopreserved *Babesia bovis, Babesia bigemina* and *Anaplasma centrale* for cattle after thawing, dilution and incubation at 30°C. *Vet. Parasitol.*, **31**, 243–251.

KOCAN K.M., DE LA FUENTE J., BLOUIN E.F. & GARCIA-GARCIA J.C. (2004). *Anaplasma marginale* (Rickettsiales: Anaplasmataceae): recent advances in defining host-pathogen adaptations of a tick-borne rickettsia. *Parasitology*, **129**, S285–S300.

KOCAN K.M., DE LA FUENTE J., GUGLIELMONE A.A. & MELENDÉZ R.D. (2003). Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. *Clin. Microbiol. Rev.*, **16**, 698–712.

KNOWLES D., TORIONI DE ECHAIDE S., PALMER G., McGUIRE T., STILLER D. & McELWAIN T. (1996). Antibody against an *Anaplasma marginale* MSP5 epitope common to tick and erythrocyte stages identifies persistently infected cattle. *J. Clin. Microbiol.*, **34**, 2225–2230.

KREIER J.P. & RISTIC M. (1963). Anaplasmosis. X Morphological characteristics of the parasites present in the blood of calves infected with the Oregon strain of *Anaplasma marginale. Am. J Vet. Res.,* **24**, 676–687.

HOFMANN-LEHMANN R., MELI M.L., DREHER U.M., GÖNCZI E., DEPLAZES P., BRAUN U., ENGELS M., SCHÜPBACH J., JÖRGER K., THOMA R., GRIOT C., STÄRKK.D.C., WILLI B., SCHMIDT J., KOCAN K.M. & LUTZ H. (2004). Concurrent infections with vector-borne pathogens associated with fatal haemolytic anemia in a cattle herd in Switzerland. *J. Clin. Microbiol.*, **42**, 3775–3780.

McHARDY N. (1984). Immunization against anaplasmosis: a review. *Prev. Vet. Med.,* **2**, 135–146.

MELLORS L.T., DALGLIESH R.J., TIMMS P., RODWELL B.J. & CALLOW L.L. (1982). Preparation and laboratory testing of a frozen vaccine containing *Babesia bovis*, *Babesi abigemina* and *Anaplasma centrale. Res. Vet. Sci.*, **32**, 194–197.

MOLLOY J.B., BOWLES P.M., KNOWLES D.P., McELWAIN T.F., BOCK R.E., KINGSTON T.G., BLIGHT G.W. & DALGLIESH R.J. (1999). Comparison of a competitive inhibition ELISA and the card agglutination test for detection of antibodies to *Anaplasma marginale* and *Anaplasma centrale* in cattle. *Aust. Vet. J.,* **77**, 245–249.

PIPANO E. (1981). Frozen vaccine against tick fevers of cattle. *In:* XI International Congress on Diseases of Cattle, Haifa, Israel. Mayer E., ed. Bregman Press, Haifa, Israel, 678–681.

PIPANO E. (1995). Live vaccines against hemoparasitic diseases in livestock. *Vet. Parasitol.*, **57**, 213–231.

PIPANO E. (1997). Vaccines against hemoparasitic diseases in Israel with special reference to quality assurance. *Trop. Anim. Health Prod.*, **29** (Suppl. 4), 86S–90S.

PIPANO E., KRIGEL Y., FRANK M., MARKOVICS A. & MAYER E. (1986). Frozen *Anaplasma centrale* vaccine against anaplasmosis in cattle. *Br. Vet. J.*, **142**, 553–556.

REINBOLD J.B., COETZEE J.F., HOLLIS L.C., NICKELL J.S., RIEGEL C.M., CHRISTOPHER J.A. & GANTA R.R. (2010a). Comparison of iatrogenic transmission of *Anaplasma marginale* in Holstein steers via needle and needle-free injection techniques. *Am. J. Vet. Res.,* **71**, 1178–1188.

REINBOLD J.B., COETZEE J.F., SIRIGIREDDY K.R. & GANTA R.R. (2010b). Detection of *Anaplasma marginale* and *A. phagocytophilum* in bovine peripheral blood samples by duplex real-time reverse transcriptase PCR assay. *J. Clin. Microbiol.,* **48**, 2424–2432.

ROGERS T.E., HIDALGO R.-J. & DIMOPOULLOS G.T. (1964). Immunology and serology of *Anaplasma marginale*. I. Fractionation of the complement-fixing antigen. *J. Bacteriol.,* **88**, 81–86.

STICH R.W., OLAH G.A., BRAYTON K.A., BROWN W.C., FECHHEIMER M., GREEN-CHURCH K., JITTAPALAPONG S., KOCAN K.M., McGUIRE T.C., RURANGIRWA F.R. & PALMER G.H. (2004).Identification of a novel *Anaplasma marginale* appendage-associated protein that localizes with actin filaments during intraerythrocytic infection. *Infect Immun.,* **72**, 7257–7264.

665 STIK N.I., ALLEMAN A.R., BARBET A.F., SORENSON H.L., WANSLEY H.L., GASCHEN F.P., LUCKSCHANDER N., WONG S.,
666 CHU F., FOLEY J.E., BJOERSDORFF A., STUEN S. & KNOWLES D.P. (2007). Characterization of *Anaplasma*
667 *phagocytophilum* major surface protein 5 and the extent of its cross-reactivity with *A. marginale. Clin. Vaccine*
668 *Immunol.*, **14**, 262–268.

669 TORIONI DE ECHAIDE S., KNOWLES D.P., MCGUIRE T.C., PALMER G.H., SUAREZ C.E. & MCELWAIN T.F. (1998).
670 Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a
671 competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. *J. Clin. Microbiol.,*
672 **36**, 777–782.

673   *

674   *   *

675   **NB:** There is an OIE Reference Laboratory for *Anaplasma* sp.
676   (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date
677   list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
678   Please contact the OIE Reference Laboratory for any further information on
679   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

C H A P T E R   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.

A. INTRODUCTION

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 *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. 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 camelids. 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 Eᴿⁿˢ 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

161	between non-cytopathic biotypes, or mutation of the persistent biotype (Brownlie, 1990). There is
162	usually little need to specifically confirm that a PI animal has succumbed to mucosal disease as this is
163	largely a scientific curiosity and of little practical significance, other than that the animal is PI with
164	BVDV. However, cases of mucosal disease may be the first indication in a herd that BVDV infection is
165	present, and should lead to more in depth investigation and intervention.

3.5. Semen and embryos

166	
167	Bulls that are PI usually have poor quality, highly infective semen and reduced fertility (McGowan &
168	Kirkland, 1995). All bulls used for natural or artificial insemination should be screened for both acute
169	and persistent BVDV infection. A rare event, possibly brought about by acute infection during
170	pubescence, can result in persistent infection of the testes and thus strongly seropositive bulls that
171	intermittently excrete virus in semen (Voges *et al.*, 1998). This phenomenon has also been observed
172	following vaccination with an attenuated virus (Givens *et al.*, 2007). Embryo donor cows that are PI with
173	BVDV also represent a potential source of infection, particularly as there are extremely high
174	concentrations of BVDV in uterine and vaginal fluids. While oocysts without an intact zona pellucida
175	have been shown to be susceptible to infection *in vitro*, the majority of oocysts remain uninfected with
176	BVDV. Normal uninfected progeny have also been 'rescued' from PI animals by the use of extensive
177	washing of embryos and in vitro fertilisation. Female cattle used as embryo recipients should always be
178	screened to confirm that they are not PI, and ideally, are sero-positive or were vaccinated at least
179	4 weeks before first use.

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

4. Approaches to diagnosis and sample collection

185	
186	The diagnosis of BVDV infection can sometimes be complex because of the delay between infection and clinical
187	expression. While detection of PI animals should be readily accomplished using current diagnostic methods, the
188	recognition of acute infections and detection of BVDV in reproductive materials can be more difficult.

4.1. Acute infections

189	
190	Unlike PI animals, acutely infected animals excrete relatively low levels of virus and for a short period
191	of time (usually about 7–10 days) but the clinical signs may occur during the later stages of viraemia,
192	reducing the time to detect the virus even further. In cases of respiratory or enteric disease, samples
193	should be collected from a number of affected animals, preferentially selecting the most recently
194	affected. Swabs should be collected from the nares and conjunctiva of animals with respiratory disease
195	or from rectum and faeces if there are enteric signs. Lung and spleen are preferred from dead animals.
196	Viral RNA may be detected by real-time RT-PCR assays and have the advantages of high sensitivity
197	and being able to detect genome from non-infectious virus. As the virus levels are very low, it is not
198	usually practical to undertake virus isolation unless there is a need to characterise the strain of BVDV
199	involved. Serology undertaken on paired acute and convalescent sera (collected at least 21 days after
200	the acute sample and from 8–10 animals) is worthwhile and gives a high probability of incriminating or
201	excluding BVDV infection.

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

4.2. Persistent infections

215	
216	In the past, identification of PI animals relied heavily on the use of virus isolation in cell cultures.
217	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

Table 1. Test methods available for diagnosis of bovine viral diarrhoea and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification[1]						
Virus isolation	+	+++	++	+++	–	–
Antigen detection by ELISA	++	+++	+++	+++	+++	–
Antigen detection by IHC	–	–	–	++	–	–

---

1   A combination of agent identification methods applied on the same clinical sample is recommended.

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
NA detection by real-time RT-PCR	+++	+++	+++	+++	+++	–
Detection of immune response[2]						
ELISA	+++	++	+++	–	+++	+++
VN	+	+++	++	–	+	+++

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 RNA (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 sero-negative 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 BVDV-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 up to five times (original inoculation plus four passages). Conventional methods for virus isolation are used, with the addition of a final immune-staining step (immunofluorescence or, more frequently, peroxidase staining) to detect growth of non-cytopathic virus. Thus tube cultures should include flying cover-slips, while microplate cultures can be fixed and labelled directly in the plate. Examples are given below. Alternatively, culture supernatant from the final passage can be screened by real-time RT-PCR (see below).

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% CO_2 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 50ul 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 50ul 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 100ul 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.)

f) Incubate at 37°C for 15 minutes.

g) Empty the plate and wash three times in PBST.

h) 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.

i) Empty the plate and wash three times in PBST.

j) Rinse the plate in distilled water. All fluid is tapped out from the plate.

k) Add freshly prepared hydrogen peroxide substrate with a suitable chromogen, e.g. 3-amino-9-ethyl carbazole (AEC).

An alternative substrate can be made, consisting of 9 mg diaminobenzidine tetrahydrochloride and 6 mg sodium perborate tetrahydrate dissolved in 15 ml of

PBS. Though the staining is not quite so intense, these chemicals have the advantage that they can be shipped by air.

l) 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.

456   vi)   Include BVDV negative and positive controls in the test. Special caution must be taken to
457   avoid accidental contamination of test wells by the positive control, for example always
458   handling the positive control last.

459   vii)   Observe plates microscopically to ensure freedom from contamination and cytotoxicity. No
460   cytopathology is expected as a result of BVDV infection but other viruses such as BHV-1
461   could be inadvertently isolated.

462   viii) After 5–7 days, the cultures are frozen at or below approximately –70°C and thawed,
463   clarified by centrifugation, and the supernatant used to inoculate fresh monolayers.

464   ix)   At the end of the second passage, the supernatant from the freeze-thaw preparation
465   should be passaged onto cultures in a suitable system for immunoperoxidase staining or
466   other antigen detection or by real-time RT-PCR after 5 days of culture. This is most readily
467   achieved in 96 well microplates. The sample is considered to be negative, if there is no
468   evidence of viral antigen or BVDV RNA detected.

469   **1.2. Nucleic acid detection**

470   Conventional gel based RT-PCR has in the past been used for the detection of BVD viral RNA for
471   diagnostic purposes. A multiplex RT-PCR has been used for the simultaneous amplification and typing
472   of virus from cell culture, or direct from blood samples. However, gel based RT-PCR has the
473   disadvantage that it is relatively labour intensive, expensive and prone to cross contamination. These
474   problems had been markedly reduced following the introduction of probe-based real-time or
475   quantitative RT-PCR methods . Nevertheless, stringent precautions should still be taken to avoid
476   nucleic acid contamination in the test system and general laboratory areas where samples are handled
477   and prepared (see Chapter 1.1.5 *Principles and methods of validation of diagnostic assays for
478   infectious diseases* and Validation Guideline 3.6.3 *Development and optimisation of nucleic acid
479   assays*). These assays have even higher sensitivity than gel based RT-PCR and can be completed in a
480   few hours. They are in widespread use for the diagnosis of infectious diseases, allowing the direct
481   detection of viral RNA from a wide range of specimens including serum, whole blood, tissues, milk and
482   semen. The high analytical sensitivity allows the adoption of strategies to screen pools of individual
483   samples or testing of bulk tank milk. By using this approach the presence of one or more PI animals
484   can be identified in herds containing several hundred cows. Although slightly more expensive than
485   immunostaining methods, real-time RT-PCR is a quick and reliable method that can also be used to
486   screen culture supernatant from the final passage of cell cultures. While real-time RT-PCR has very
487   high sensitivity and can be applied to the screening of biological materials used for vaccine
488   manufacture, caution is needed in the interpretation of results, as the detection of viral RNA does not
489   imply *per se* that infective virus is present. Real-time RT-PCR assays based on fluorescent-labelled
490   DNA probes can also be used to differentiate pestiviruses (e.g. McGoldrick *et al.*, 1999).

491   Primers for the assay should be selected in highly conserved regions of the genome, ideally the 5'-
492   noncoding region, or the NS3 (p80 gene). There are published assays that are broadly reactive across
493   the pestivirus genus, detecting all BVDV types, CSFV and most of the 'atypical' pestiviruses (e.g.
494   Hoffman *et al.*, 2006). A sensitive broadly reactive assay is recommended for diagnostic applications
495   because interspecies transfer of different pestiviruses is occasionally encountered. When further
496   identification of the specific virus is required, pestivirus species-specific assays can be applied to
497   further type the virus. It is important to thoroughly optimise all aspects of the real-time RT-PCR assay,
498   including the nucleic acid extraction and purification. Optimal concentrations of Mg^{2+}, primers, probe
499   and polymerase, and the cycling parameters need to be determined. However, fully formulated and
500   optimised 'ready to use' 'mastermixes' are now available commercially and only require addition of
501   optimised concentrations of primers and probe. Optimised cycling conditions are often recommended
502   for a particular mastermix.

503   A variety of commercially available nucleic acid purification systems are available in kit form and
504   several can be semi-automated. Systems based on the capture and purification of RNA using magnetic
505   beads are in widespread use and allow rapid processing of large numbers of samples. Specific
506   products should be evaluated to determine the optimal kit for a particular sample type and whether any
507   preliminary sample processing is required. For whole blood samples, the type of anticoagulant and
508   volume of blood in a specimen tube is important. More problems with inhibitors of the PCR reaction are
509   encountered with samples collected into heparin treated blood than EDTA. These differences are also
510   exacerbated if the tube does not contain the recommended volume of blood, thereby increasing the
511   concentration of anticoagulant in the sample. To identify possible false-negative results, it is
512   recommended to spike an exogenous ('internal control') RNA template into the specimen prior to RNA
513   extraction (e.g. Hoffman *et al.*, 2006). By the inclusion of PCR primers and probe specific to the
514   exogenous sequence, the efficiency of both the RNA extraction and also the presence of any PCR
515   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 pestivirurses. 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 immunosorbent 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 to 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 conjugated 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 ERNS) in lysates of peripheral blood leukocytes; the new generation of antigen-capture ELISAs (ERNS 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 ERNS 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 it 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 *et 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.

845 **2. Outline of production and minimum requirements for vaccines**

846 **2.1. Characteristics of the seed**

847 For optimal efficacy, it is considered that there should be a close antigenic match between viruses
848 included in a vaccine and those circulating in the target population. BVDV type 2 strains should be
849 included as appropriate. Due to the regional variations in genotypes and subtypes of BVDV, many
850 vaccines contain more than one strain of BVDV to give acceptable protection. A good appreciation of
851 the antigenic characteristics of individual strains can be obtained by screening with panels of MAbs
852 (Paton *et al.*, 1995).

853 **2.1.1. Biological characteristics of the master seed**

854 Isolates of cytopathic virus are often mixed with the noncytopathic biotype. The separation and
855 purification of the two biotypes from an initial mixed culture is important to maintain the
856 expected characteristics of the seen and depends on several cycles of a limiting dilution
857 technique for the noncytopathic virus, or plaque selection for the cytopathic virus. Purity of the
858 cytopathic virus should be confirmed by at least one additional passage at limiting dilution.
859 When isolates have been cloned, their identity and key antigenic characteristics should be
860 confirmed. The identity of the seed virus should be confirmed by sequencing. Where there are
861 multiple isolates included in the vaccine, each has to be prepared separately.

862 While retaining the desirable antigenic characteristics, the strains selected for the seed should
863 not show any signs of disease when susceptible animals are vaccinated. Live attenuated
864 vaccines should not be transmissible to unvaccinated 'in-contact' animals and should not be
865 able to infect the fetus. Ideally seeds prepared for the production of inactivated vaccines should
866 grow to high titre to minimise the need to concentrate the antigens and there should be a
867 minimal amount of protein from the cell cultures incorporated into the final product. Master
868 stocks for either live or inactivated vaccines should be prepared under a seed lot system
869 involving master and working stocks that can be used for production in such a manner that the
870 number of passages can be limited and minimise antigenic drift. While there are no absolute
871 criteria for this purpose, as a general guide, the seed used for production should not be
872 passaged more than 20 times beyond the master seed and the master seed should be of the
873 lowest passage from the original isolate as is practical.

874 **2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)**

875 It is crucial to ensure that all materials used in the preparation of the bulk antigens have been
876 extensively screened to ensure freedom from extraneous agents. This should include master
877 and working seeds, the cell cultures and all medium supplements such as bovine serum. It is
878 particularly important to ensure that any serum used that is of bovine origin is free of both
879 adventitious BVDV and antibodies against BVDV strains because low levels of either virus or
880 antibody can mask the presence of the other. Materials and vaccine seeds should be tested for
881 sterility and freedom from contamination with other agents, especially viruses as described in
882 the chapter 1.1.6 and chapter 1.1.7.

883 **2.1.3. Validation as a vaccine strain**

884 All vaccines should pass standard tests for efficacy. Tests should include as a minimum the
885 demonstration of a neutralising antibody response following vaccination, a reduction in virus
886 shedding after challenge in vaccinated cattle and ideally a prevention of viraemia. Efficacy tests
887 of BVD vaccines by assessing clinical parameters in non-pregnant cattle can be limited by the
888 difficulty of consistently establishing clinical signs but, when employed, clinical parameters such
889 as a reduction in the rectal temperature response and leukopenia should be monitored.
890 Although it can be difficult by using virus isolation in cell culture to consistently demonstrate the
891 low levels of viraemia associated with an acute infection, real-time PCR could be considered as
892 an alternative method to establish the levels of circulating virus.

893 If a vaccine passes basic tests, the efficacy of vaccination should ultimately be measured by the
894 capacity to prevent transplacental transmission. If there is a substantial reduction and ideally
895 complete prevention of fetal infection, a vaccine would be expected to be highly effective in
896 other situations (for example prevention of respiratory disease). A suitable challenge system
897 can be established by intranasal inoculation of noncytopathic virus into pregnant cows between
898 60 and 90 days of gestation (Brownlie *et al.*, 1995). Usually this system will reliably produce
899 persistently viraemic offspring in non-immune cows. In countries where BVDV type 2 viruses

900 are commonly encountered, efficacy in protecting against BVDV2 infections should be
901 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 inoculty 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

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

BAKER J.C. (1995). The clinical manifestations of bovine viral diarrhea infection. *Vet. Clin. North. Am. – Food Animal Practice*, **11**, 425–445.

BOLIN S.R. & RIDPATH J.F. (1992). Differences in virulence between two noncytopathic bovine viral diarrhea viruses in calves. *Am. J. Vet. Res.,* **53**, 2157–2163.

BOLIN S.R. & RIDPATH J.F. (1995). Assessment of protection from systemic infection or disease afforded by low to intermediate titers of passively acquired neutralizing antibody against bovine viral diarrhea virus in calves. *Am. J. Vet. Res.*, **56**, 755–759.

BROWNLIE J. (1985). Clinical aspects of the bovine virus diarrhoea/mucosal disease complex in cattle. *In Practice,* **7**, 195–202.

BROWNLIE J. (1990). The pathogenesis of bovine virus diarrhoea virus infections. *Rev. sci. tech. Off. int. Epiz.,* **9**, 43–59.

BROWNLIE J., CLARKE M.C., HOOPER L.B. & BELL G.D. (1995). Protection of the bovine fetus from bovine viral diarrhoea virus by means of a new inactivated vaccine. *Vet. Rec.,* **137**, 58–62.

CORNISH T.E., VAN OLPHEN A.L., CAVENDER J.L., EDWARDS J.M., JAEGER P.T., VIEYRA L.L., WOODARD L.F., MILLER D.R. & O'TOOLE D. (2005). Comparison of ear notch immunohistochemistry, ear notch antigen-capture ELISA, and buffy coat virus isolation for detection of calves persistently infected with bovine viral diarrhea virus. *J. Vet. Diagn. Invest.,* **17**, 110–117.

1054 DUFFELL S.J. & HARKNESS J.W. (1985). Bovine virus diarrhoea-mucosal disease infection in cattle. *Vet. Rec.*, **117**,
1055 240–245.

1056 EDWARDS S. (1990). The diagnosis of bovine virus diarrhoea-mucosal disease in cattle. *Rev. sci. tech. Off. int.*
1057 *Epiz.*, **9**, 115–130.

1058 FRAY M.D., MANN G.E., BLEACH E.C.L., KNIGHT P.G., CLARKE M.C. & CHARLESTON B. (2002). Modulation of sex
1059 hormone secretion in cows by acute infection with bovine viral diarrhoea virus. *Reproduction,* **123**, 281-289.

1060 FULTON R.W., SALIKI J.T., BURGE L.J., DOFFAY J.M., BOLIN S.R., MAES R.K., BAKER J.C. & FREY M.L. (1997).
1061 Neutralizing antibodies to type-1 and type-2 bovine viral diarrhea viruses – detection by inhibition of viral
1062 cytopathology and infectivity by immunoperoxidase assay. *Clin. Diagn. Lab. Immunol.,* **4**, 380–383.

1063 GIVENS M.D., RIDDELL K.P., WALZ P.H., RHOADES J., HARLAND R., ZHANG Y., GALIK P.K., BRODERSEN B.W., COCHRAN
1064 A.M., BROCK K.V., CARSON R.L. & STRINGFELLOW D.A. (2007). Noncytopathic bovine viral diarrhea virus can persist
1065 in testicular tissue after vaccination of peri-pubertal bulls but prevents subsequent infection. *Vaccine,* **25**, 867–
1066 876.

1067 HOFFMANN B., DEPNER K., SCHIRRMEIER H. & BEER M. (2006). A universal heterologous internal control system for
1068 duplex real-time RT-PCR assays used in a detection system for pestiviruses *J. Virol. Meth*ods, **136**, 200–209.

1069 HOUE H., BAKER J.C., MAES R.K., RUEGG P.L. & LLOYD J.W. (1995). Application of antibody titers against bovine
1070 viral diarrhea virus (BVDV) as a measure to detect herds with cattle persistently infected with BVDV. *J. Vet.*
1071 *Diagn. Invest.,* **7**, 327–332.

1072 HOWARD C.J., CLARKE M.C. & BROWNLIE J. (1989). Protection against respiratory infection with bovine virus
1073 diarrhoea virus by passively acquired antibody. *Vet. Microbiol.,* **19**, 195–203.

1074 LETELLIER C. & KERKHOFS P. (2003). Real-time PCR for simultaneous detection and genotyping of bovine viral
1075 diarrhea virus. *J. Virol. Methods,* **114**, 21–27.

1076 McGOLDRICK A., BENSAUDE E., IBATA G., SHARP G. & PATON D. J. (1999). Closed one-tube reverse transcription
1077 nested polymerase chain reaction for the detection of pestiviral RNA with fluorescent probes. *J. Virol. Methods,*
1078 **79**, 85–95.

1079 McGOWAN M.R. & KIRKLAND P.D. (1995). Early reproductive loss due to bovine pestivirus infection. *Br. Vet. J.*, **151**,
1080 263–270.

1081 MEYLING A. (1984). Detection of BVD virus in viraemic cattle by an indirect immunoperoxidase technique. *In:*
1082 Recent Advances in Virus Diagnosis (CEC Seminar), McNulty M.S. & McFerran J.B., eds. Martinus Nijhoff,
1083 Belfast, UK, 37–46.

1084 MOENNIG V. & LIESS B. (1995). Pathogenesis of intrauterine infections with bovine viral diarrhea virus. *Vet. Clin.*
1085 *North. Am.,* **11**, 477–487.

1086 MOENNIG V., HOUE H. & LINDBERG A. (2005). BVD control in Europe: current status and perspectives. *Anim. Health*
1087 *Res. Rev.,* **6**, 63–74.

1088 NISKANEN R. (1993). Relationship between the levels of antibodies to bovine viral diarrhoea virus in bulk tank milk
1089 and the prevalence of cows exposed to the virus. *Vet. Rec.,* **133**, 341–344.

1090 PARK B.K. & BOLIN S.R. (1987). Molecular changes of bovine viral diarrhea virus polypeptides treated with binary
1091 ethylenimine, beta-propiolactone and formalin. *Res. Rep. Rural Dev. Admin.* (*L&V*) *Korea,* **29**, 99–103.

1092 PATON D.J., SANDS J.J. LOWINGS J.P., SMITH J.E., IBATA G., EDWARDS S. (1995). A proposed division of the
1093 pestivirus genus using monoclonal antibodies, supported by cross-neutralisation assays and genetic sequencing.
1094 *Vet. Res.,* **26**, 92–109.

1095 VILCEK S., PATON D.J., DURKOVIC B., STROJNY L., IBATA G., MOUSSA A., LOITSCH A., ROSSMANITH W., VEGA S.,
1096 SCICLUNA M.T. & PALFI V. (2001). Bovine viral diarrhea virus genotype 1 can be separated into at least eleven
1097 genetic groups. *Arch. Virol.,* **146**, 99–115.

VOGES H., HORNER G.W., ROWE S. & WELLENBERG G.J. (1998). Persistent bovine pestivirus infection localized in the testes of an immuno-competent, non-viremic bull. *Vet. Microbiol.*, **61**, 165–175.

ZIMMER G., SCHOUSTRA W. & GRAAT E. A.M. (2002). Predictive values of serum and bulk milk sampling for the presence of persistently infected BVDV carriers in dairy herds. *Res. Vet. Sci.,* **72**, 75–82.

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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/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for bovine viral diarrhoea

1 CHAPTER 2.5.9.

2 # EQUINE RHINOPNEUMONITIS
3 # (EQUINE HERPESVIRUS 1 AND 4)

4 **SUMMARY**

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

9 *Primary infection by either EHV-1 or EHV-4 is characterised by upper a primary respiratory tract*
10 *disease of varying severity that is related to the age and immunological status of the infected*
11 *animal. EHV-1 also causes the more serious complications of abortion, perinatal foal death, or*
12 *paralytic neurological disease (equine herpesvirus myeloencephalopathy). EHV-4 has been*
13 *associated with rare sporadic cases of abortion, but not the large outbreaks associated with EHV-1.*
14 *Like other herpesviruses, EHV-1 and 4 induce long-lasting latent infections and can reactivate*
15 *following stress or pregnancy. Most horses will be infected during their lifetime, detection of viral*
16 *DNA or anti-EHV antibodies should therefore be interpreted with care. Infections by EHV-1 in*
17 *particular are capable of progression beyond the respiratory mucosa to cause the more serious*
18 *disease manifestations of abortion, perinatal foal death, or neurological dysfunction.*

19 ***Identification of the agent:*** *The standard method of identification of EHV-1 and EHV-4 the*
20 *herpesviral agents of ER continues to be laboratory isolation of the virus from appropriate clinical or*
21 *necropsy material is polymerase chain reaction (PCR), followed by seroconfirmation of its*
22 *identity. laboratory isolation of the virus in cell culture. Positive identification of viral isolates as*
23 *EHV-1 or EHV-4 can be achieved by type-specific PCR. Viruses can be isolated in equine cell*
24 *culture from nasal or nasopharyngeal swab extracts samples taken from horses during the febrile*
25 *stage of respiratory tract infection, from liver, lung, spleen, or thymus of aborted fetuses and early*
26 *foal deaths, and from the leukocyte fraction of the blood of animals with acute EHV-1 disease.*
27 *Unlike EHV-4, EHV-1 will also grow on other cell types such as RK-13s and this property can be*
28 *used to distinguish between the two viruses. Positive identification of viral isolates as EHV-1 or*
29 *EHV-4 can be achieved by immunofluorescence with type-specific monoclonal antibodies.*

30 *A rapid presumptive diagnosis of EHV-1/4 induced rhinopneumonitis abortion can be achieved by*
31 *direct immunofluorescent detection of viral antigen in cryostat sections of tissues from aborted*
32 *fetuses, using conjugated polyclonal antiserum.*

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

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

39 ***Serological tests:*** *Because Most horses will possess some level of antibody to EHV-1/4, the*
40 *demonstration of specific antibody in the serum collected from a single blood sample is therefore*
41 *not sufficient for a positive diagnosis of recent infection active ER. Paired, acute and convalescent*
42 *sera from animals suspected of being infected with EHV-1 or EHV-4 should can be tested for a*
43 *four-fold or greater rise in virus-specific antibody titre by either virus neutralisation (VN) or enzyme-*
44 *linked immunosorbent assay, or complement fixation (CF). Neither of these assays is type-specific*
45 *but both have proven useful for diagnostic purposes as VN and CF antibodies are relatively short-*

46 *lived. Limited use has also been made of a type-specific enzyme-linked immunosorbent assay*
47 (*Crabb* et al., *1994; Hartley* et al., *2005).*

48 ***Requirements for vaccines and diagnostic biologicals:*** *Both live attenuated and inactivated*
49 *viral vaccines ~~of varying composition are commercially~~ are available for use in assisting in the*
50 *control of EHV-1/4 ~~ER~~. ~~While~~ Vaccination is helpful in reducing the ~~incidence of abortion in mares,~~*
51 *~~and in ameliorating~~ severity of ~~clinical signs of~~ respiratory infection in young horses <u>and the</u>*
52 *<u>incidence of abortion in mares, however current vaccines are not licenced to protect against</u>*
53 *<u>neurological disease. Vaccination</u> should not be considered a substitute ~~for strict adherence to the~~*
54 *~~well-established tenets of~~ sound management practices known to reduce the risk of <u>infection</u>*
55 *~~rhinopneumonitis~~. Revaccination at frequent intervals is recommended with each of the products,*
56 *as the duration of vaccine-induced immunity is relatively short.*

57 *Standards for production and licensing of both attenuated and inactivated EHV<u>-1/4</u> vaccines are*
58 *established by appropriate veterinary regulatory agencies in the countries of vaccine manufacture*
59 *and use. A single set of internationally recognised standards for <u>EHV</u> ~~ER~~ vaccines is not available.*
60 *In each case, however, vaccine production is based on the system of a detailed outline of*
61 *production employing a well characterised cell line and a master seed lot of vaccine virus that has*
62 *been validated with respect to virus identity, safety, virological purity, immunogenicity and the*
63 *absence of extraneous microbial agents.*

A. INTRODUCTION

65 Equine rhinopneumonitis (ER) is an historically derived term that describes a constellation of several disease
66 entities of horses that may include respiratory disease, abortion, neonatal foal pneumonitis, or
67 myeloencephalopathy (Allen & Bryans, 1986; Allen *et al.*, 1999; Bryans & Allen, 1988; Crabb & Studdert, 1995).
68 The disease has been recognised for over 60 years as a threat to the international horse industry, and is caused
69 by either of two members of the *Herpesviridae* family, equid herpesvirus-1 and -4 (EHV-1 and EHV-4). EHV-1 and
70 EHV-4 are closely related alphaherpesviruses of horses with nucleotide sequence identity within individual
71 homologous genes ranging from 55% to 84%, and amino acid sequence identity from 55% to 96% (Telford *et al.*,
72 1992; 1998). The two herpesviruses are enzootic in all countries in which large populations of horses are
73 maintained as part of the cultural tradition or agricultural economy. There is no recorded evidence that the two
74 herpesviruses of ER pose any health risks to humans working with the agents. <u>Infection with EHV-1 is listed by</u>
75 <u>the OIE.</u>

76 ER is highly contagious among susceptible horses, with viral transmission to cohort animals occurring by
77 inhalation of aerosols of virus-laden respiratory secretions. <u>Aborted tissue from infected mares can contain</u>
78 <u>extremely high levels of live virus and represents a major source of infection.</u> Extensive use of vaccines has not
79 eliminated EHV infections, and the world-wide annual financial burden from these equine pathogens is immense.

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

95 <u>Neurological disease, also known equine herpesvirus myeloencephalopathy, remains an infrequent but serious</u>
96 <u>complication of EHV-1 infection. A single mutation in the DNA polymerase gene (ORF30) has been associated</u>
97 <u>with increased risk of neurological disease, however strains without this marker can also cause paralysis (Nugent</u>
98 <u>*et al.*, 2006; Goodman *et al.*, 2007). Strain typing techniques have been employed to identify viruses carrying the</u>
99 <u>neuropathic marker, and it can be useful to be aware of an increased risk of neurological complications. However,</u>
100 <u>for practical purposes strain-typing does not influence the requirement for strict management practices during an</u>
101 <u>outbreak of EHV-1.</u>

B. DIAGNOSTIC TECHNIQUES

Both EHV-1 and EHV-4 are ~~Because ER is a~~ highly contagious ~~disease with~~ viruses and the former has the potential for ~~occurring as~~ 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*

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection - surveillance	Immune status in individual animals or populations post-vaccination
Agent identification[1]						
Virus isolation	=	+++	=	+++	=	=
PCR	=	+++	=	+++	=	=
AGID	=	=	=	=	=	=
CFT	=	=	=	+++	=	=
Detection of immune response[2]						
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

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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.

by ~~swabbing the nasopharyngeal area with a 5 × 5 cm gauze sponge attached to the end of a 50 cm length of flexible, stainless steel wire encased in latex rubber tubing. A guarded uterine swab devise can also be used~~ 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, <u>fetal calf serum</u> 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.

<u>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.</u>

1.2. Virus detection by polymerase chain reaction

<u>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.</u>

<u>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).</u>

<u>PCR technology is evolving rapidly and a variety of assays have been published. The OIE reference laboratories use 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 ORF68 gene (Nugent et al, 2006). 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.</u>

<u>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).</u>

1.2.1. Test procedure

189   i)   *Prepare template DNA from test specimens:* following sample homogenisation and lysis in
190   the presence of a chaotropic salt, nucleic acids bind selectively to silica or cationic resin
191   substrates. Substrate-bound nucleic acids are purified in a series of rapid wash steps
192   followed by recovery with low-salt elution. The reagents for performing such steps for rapid
193   nucleic acid isolation are available in kit format from a number of commercial sources.

194   ii)   *Nested primer sequences specific for EHV-1*

195   BS-1-P1 = 5'-TCT-ACC-CCT-ACG-ACT-CCT-TC-3' (917–936)

196   gB1-R-2 = 5'-ACG-CTG-TCG-ATG-TCG-TAA-AAC-CTG-AGA-G-3' (2390–2363)

197   BS-1-P3 = 5'-CTT-TAG-CGG-TGA-TGT-GGA-AT-3' (1377–1396)

198   gB1-R-a = 5'-AAG-TAG-CGC-TTC-TGA-TTG-AGG-3' (2147–2127)

199   iii) *Nested primer sequences specific for EHV-4*

200   BS-4-P1 = 5'-TCT-ATT-GAG-TTT-GCT-ATG-CT-3' (1705–1724)

201   BS-4-P2 = 5'-TCC-TGG TTG-TTA-TTG-GGT-AT-3' (2656–2637)

202   BS-4-P3 = 5'-TGT-TTC-CGC-CAC-TCT-TGA-CG-3' (1857–1876)

203   BS-4-P4 = 5'-ACT-GCC-TCT-CCC-ACC-TTA-CC-3' (2456–2437)

204   iv)   *PCR conditions for first stage amplification:* specimen template DNA (1–2 µg in 2 µl) is
205   added to a PCR mixture (total volume of 50 µl) containing 1 × PCR buffer (50 mM KCl,
206   10 mM Tris/HCl, pH 9.0, 0.1% Triton X-100), 200 µM of each deoxynucleotide triphosphate
207   (dNTP), 2.5 mM $MgCl_2$, 2.0 µM of each outer primer (BS-1-P1 and gB1-R-2 for EHV-1
208   detection and, in a separate reaction mixture, BS-4-P1 and BS-4-P2 for EHV-4 detection)
209   and 0.5 u Taq DNA polymerase. Cycling parameters are: initial denaturation at 94°C for
210   4 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for
211   90 seconds; with a final extension at 72°C for 10 minutes. Separate reaction mixtures
212   containing either known viral DNA or no DNA (water) should be prepared and amplified as
213   positive and negative controls.

214   v)   *PCR conditions for second stage (nested) amplification:* two µl of a 1/10 dilution of the first
215   amplification product is added to a fresh PCR mixture (total volume of 50 µl) containing 1 ×
216   PCR buffer, 200 µM of each dNTP, 2.5 mM $MgCl_2$, 2.0 µM of each nested primer (BS-1-P3
217   and gB1-R-a for EHV-1 detection and, in a separate reaction mixture, BS-4-P3 and BS-4-
218   P4 for EHV-4 detection) and 0.5 u Taq DNA polymerase. Cycling parameters are: initial
219   denaturation at 94°C for 4 minutes; 40 cycles of 94°C for 30 seconds, 60°C for
220   30 seconds, and 72°C for 1 minute; with a final extension at 72°C for 10 minutes.

221   vi)   *Gel analysis of amplified products:* 10 µl of each final amplified product, including controls,
222   is mixed with 2 µl of 6 × loading dye and electrophoresed on a 1.5% agarose gel in
223   Tris/acetate or Tris-Borate running buffer, along with a 100 base pairs (bp) DNA ladder.
224   Amplified products are detected using a suitable DNA stain, of either 770 bp for EHV-1 or
225   580 bp for EHV-4.

1.3. Virus isolation

227   For efficient primary isolation of EHV-4 from horses with respiratory disease, equine-derived cell
228   cultures must be used. Both EHV-1 and EHV-4 may be isolated from nasopharyngeal samples using
229   primary equine fetal kidney cells or cell strains of equine fibroblasts derived from dermal (E-Derm) or
230   lung tissue. EHV-1 can be isolated on other cell types, as will be discussed later. The nasopharyngeal
231   swab and its accompanying 3 ml of transport medium are transferred into the barrel of a sterile 10 ml
232   syringe. Using the syringe plunger, the fluid is squeezed from the swab into a sterile tube. A portion of
233   the expressed fluid can be is then filtered through a sterile, 0.45 µm membrane syringe filter unit into a
234   second sterile tube. Filtration will decrease if heavy bacterial contamination is expected, but this may
235   also lower virus titre. Recently prepared cell monolayers in 25 cm^2 tissue culture flasks are inoculated
236   with 0.5 ml of the filtered, as well as the unfiltered, nasopharyngeal swab extract. Cell monolayers in
237   multiwell plates incubated in a 5% CO_2 environment may also be used. Virus is allowed to attach by
238   incubating the inoculated monolayers at 37°C on a platform rocker for 1.5–2 hours. Monolayers of
239   uninoculated control cells should be incubated in parallel with sterile transport medium only.

240 At the end of the attachment period, the inocula are removed and the monolayers are rinsed twice with
241 phosphate buffered saline (PBS) to remove virus-neutralising antibody that may be present in the
242 nasopharyngeal secretions. After addition ~~of 5 ml~~ of supplemented maintenance medium (MEM
243 containing 2% fetal calf serum [FCS] and twice the standard concentrations of antibiotics [penicillin,
244 streptomycin, gentamicin, and amphotericin B]), the flasks are incubated at 37°C. The use of positive
245 control virus samples to validate the isolation procedure carries the risk that this may lead to eventual
246 contamination of diagnostic specimens. This risk can be minimised by using routine precautions and
247 good laboratory technique, including the use of biosafety cabinets, inoculating positive controls after
248 the diagnostic specimens, decontaminating the surfaces in the hood while the inoculum is adsorbing
249 and using a positive control of relatively low titre. Inoculated flasks should be inspected daily by
250 microscopy for the appearance of characteristic herpesvirus cytopathic effect (CPE) (focal rounding,
251 increase in refractility, and detachment of cells). Cultures exhibiting no evidence of viral CPE after
252 1 week of incubation should be blind-passaged into freshly prepared monolayers of cells, using small
253 aliquots of both media and cells as the inoculum. Further blind passage is usually not productive.

254 A number of cell types may be used for isolation of EHV-1 ~~from the tissues of aborted fetuses or from~~
255 ~~post-mortem cases of neurological disease~~ (e.g. rabbit kidney [RK-13 (AATC–CCL37)], baby hamster
256 kidney [BHK-21], Madin–Darby bovine kidney [MDBK], pig kidney [PK-15], etc.). It can be useful to
257 inoculate samples into both non-equine and equine cells in parallel to distinguish between EHV-1 and
258 EHV-4, which causes ~~but equine-derived cell cultures are most sensitive and must be used if~~
259 ~~the~~ infrequent cases of ~~EHV-4~~ abortion ~~are to be detected~~. Around 10% (w/v) pooled tissue
260 homogenates of liver, lung, thymus, and spleen (from aborted fetuses) or of CNS tissue (from cases of
261 neurological disease) are used for virus isolation. These are prepared by first mincing small samples of
262 tissue into 1 mm cubes in a sterile Petri dish with dissecting scissors, followed by macerating the tissue
263 cubes further in serum-free culture medium with antibiotics using a homogeniser or mechanical tissue
264 grinder ~~(e.g. Ten-Broeck or Stomacher)~~. After centrifugation at 1200 *g* for 10 minutes, the supernatant
265 is removed and 0.5 ml is inoculated into duplicate cell monolayers in ~~25 cm²~~ tissue culture flasks.
266 Following incubation of the inoculated cells at 37°C for 1.5–2 hours, the inocula are removed and the
267 monolayers are rinsed twice with PBS or media. After addition of 5 ml of supplemented maintenance
268 medium, the flasks are incubated at 37°C for up to 1 week or until viral CPE is observed.

269 Culture of ~~peripheral blood leukocytes~~ PBMC for the presence of EHV-1 can be attempted from horses
270 during the early stages of ~~myeloencephanlopathy~~ neurological disease. Buffy coats may be prepared
271 from unclotted blood by centrifugation at 600 *g* for 15 minutes, and the buffy coat is taken after the
272 plasma has been carefully removed. The buffy coat is then layered onto ~~Ficoll 1,090~~ a PBMC
273 separating solution (density 1,077 g/ml, commercially available) and centrifuged at 400 *g* for
274 20 minutes ~~and the leukocyte-rich interface is then layered onto Ficoll 1.077 and centrifuged in the~~
275 ~~same way~~. The PBMC interface (without most granulocytes) is washed twice in PBS (300 *g* for
276 10 minutes) and resuspended in 1 ml of MEM containing 2% FCS. ~~Then, 0.5 ml~~ As a quicker
277 alternative method, PBMC may be collected by centrifugation directly from plasma. An aliquot of the
278 rinsed cell suspension is added to each of the duplicate monolayers of equine fibroblast, equine fetal or
279 RK-13 cell monolayers in 25 cm² flasks containing 8–10 ml freshly added maintenance medium. The
280 flasks are incubated at 37°C for 7 days; either with or without removal of the inoculum. If PBMCs are
281 not removed prior to incubation, CPE may be difficult to detect in the presence of the massive inoculum
282 of leukocytes: each flask of cells is freeze–thawed after 7 days of incubation and the contents
283 centrifuged at 300 g for 10 minutes. Finally, 0.5 ml of the cell-free, culture medium supernatant is
284 transferred to freshly made cell monolayers that are just subconfluent. These are incubated and
285 observed for viral CPE for at least 5–6 days before discarding as negative.

286 Virus isolates from positive cultures should be submitted to an OIE reference laboratory to maintain a
287 geographically diverse archive. Further strain characterisation for surveillance purposes or detection of
288 the neurological marker can be completed at some laboratories.

289 ~~c)~~ ~~**Seroconfirmation of virus identity**~~

290 ~~The basis for identification of any herpesvirus isolate recovered from specimens submitted from suspected~~
291 ~~cases of ER is its immunoreactivity with specific antisera. Specific identification of an isolate as EHV-1 or~~
292 ~~EHV-4 can be quickly and simply accomplished by immunofluorescent (FA) detection of viral antigen in the~~
293 ~~infected cell culture using type-specific monoclonal antibodies (MAbs), which are available from OIE~~
294 ~~Reference Laboratories for equine rhinopneumonitis. The test, which is type-specific and accurate, can be~~
295 ~~performed on a small aliquot of infected cells from the same container inoculated with clinical or post-~~
296 ~~mortem material. An isolate made in a laboratory that lacks MAbs or FA capability can be confirmed as EHV-~~
297 ~~1/4 by virus neutralization using a virus-specific polyclonal antiserum or by the PCR (see section B.1.f).~~

298 ~~Cell monolayers infected with the isolate are removed by scraping from the flask when at least 75% CPE is~~
299 ~~evident. The cells are pelleted from the culture medium and resuspended in 0.5 ml of PBS. 50 µl of the cell~~

300 ~~suspension is placed into two wells of a multiwell microscope slide, air-dried, and fixed for 10 minutes with~~
301 ~~100% acetone. Control cell suspensions (uninfected, EHV-1 infected, or EHV-4 infected) are also spotted~~
302 ~~into each of two wells of the same slide. Control cells may be prepared in advance and stored frozen in~~
303 ~~small aliquots. A drop of an appropriate dilution of MAb specific for EHV-1 is added to one well of each cell~~
304 ~~pair, and a drop of MAb specific for EHV-4 is added to each of the other wells. After 30 minutes' incubation~~
305 ~~at 37°C in a humid chamber, unreacted antibody is removed by two 10-minute washes with PBS. MAbs~~
306 ~~bound to viral antigen can be detected with goat anti-mouse IgG conjugated with fluorescein isothiocyanate~~
307 ~~(FITC). A drop of diluted conjugate is added to each well and, after 30 minutes at 37°C, the wells are again~~
308 ~~washed twice with PBS. Cells are examined with a fluorescence microscope, and positive fluorescence with~~
309 ~~the antibody of appropriate specificity indicates the virus type.~~

310 **1.4. Virus detection by direct immunofluorescence**

311 Direct immunofluorescent detection of EHV~~-~~4 antigens in samples of post-mortem tissues collected
312 from aborted equine fetuses <u>and placenta</u> provides an indispensable method to the veterinary
313 diagnostic laboratory for making a rapid preliminary diagnosis of herpesvirus abortion (Gunn, 1992).
314 Side-by-side comparisons of the immunofluorescent and cell culture isolation techniques on more than
315 100 cases of equine abortion have provided evidence that the diagnostic reliability of direct
316 immunofluorescent staining of fetal tissues obtained at necropsy approaches that of virus isolation
317 attempts from the same tissues.

318 In the United States of America (USA), ~~specific and~~ potent polyclonal antiserum to EHV-1, prepared in
319 swine and conjugated with FITC, is provided to veterinary diagnostic laboratories for this purpose by
320 the National Veterinary Services Laboratories of the United States Department of Agriculture (USDA).
321 The antiserum cross-reacts with EHV-4 and hence is not useful for serotyping, <u>however, this can be</u>
322 <u>conducted on any virus positive specimens by PCR</u>.

323 Freshly dissected samples (5 × 5 mm pieces) of fetal tissue (lung, liver, thymus, and spleen) are
324 frozen, sectioned on a cryostat at –20°C, mounted on to microscope slides, and fixed with 100%
325 acetone. After air-drying, the sections are incubated at 37°C in a humid atmosphere for 30 minutes with
326 an appropriate dilution of the conjugated swine antibody to EHV-1. Unreacted antibody is removed by
327 two washes in PBS, and the tissue sections are then covered with aqueous mounting media and a
328 cover-slip, and examined for fluorescent cells indicating the presence of EHV antigen. Each test should
329 include a positive and negative control consisting of sections from known EHV-1 infected and
330 uninfected fetal tissue.

331 **1.5. Virus detection by immunoperoxidase staining**

332 ~~Enzyme~~ Immunohistochemical (IH) staining methods, such as immunoperoxidase, have been
333 developed ~~recently as procedures~~ for detecting EHV-1 antigen in ~~paraffin-embedded~~ <u>fixed</u> tissues of
334 aborted equine fetuses, <u>placental tissues</u> or neurologically affected horses (Schultheiss *et al.*, 1993;
335 Whitwell *et al.*, 1992). Such ~~ancillary IH~~ techniques ~~for antigen detection may facilitate identification of~~
336 ~~the virus in~~ <u>can be used as an alternative to immunofluorescence described above and can also be</u>
337 <u>readily applied to</u> archival tissue samples ~~or in clinical cases in which traditional laboratory methods for~~
338 ~~EHV-1 detection have been unsuccessful. Immunoenzymatic.~~ <u>Immunohistochemical</u> staining for EHV-1
339 is particularly useful for the simultaneous evaluation of morphological lesions and the identification of
340 the <u>virus</u> ~~infectious agent~~. Immunoperoxidase staining for EHV-1/4 may also be carried out on infected
341 cell monolayers (van Maanen *et al.*, 2000). Adequate controls must be included with each
342 immunoperoxidase test run for evaluation of both the method specificity and antibody specificity. <u>In one</u>
343 <u>OIE reference laboratory, this method is used routinely for frozen or fixed tissue, using rabbit polyclonal</u>
344 <u>sera raised against EHV-1. This staining method is not type-specific and therefore needs to be</u>
345 <u>combined with virus isolation or PCR to discriminate between EHV-1 and 4, however it provides a</u>
346 <u>useful method for rapid diagnosis of EHV-induced abortion.</u>

347 **f)** ~~**Virus detection by polymerase chain reaction**~~

348 ~~The PCR can be used for rapid amplification and diagnostic detection of nucleic acids of EHV-1 and -4 in~~
349 ~~clinical specimens, paraffin-embedded archival tissue, or inoculated cell cultures (Borchers & Slater, 1993;~~
350 ~~Lawrence *et al.*, 1994; O'Keefe *et al.*, 1994; Varrasso *et al.*, 2001; Wagner *et al.*, 1992). A variety of type-~~
351 ~~specific PCR primers have been designed to distinguish between the presence of EHV-1 and EHV-4. The~~
352 ~~correlation between PCR and virus isolation techniques for diagnosis of EHV-1 or EHV-4 is high (Varrasso~~
353 ~~*et al.*, 2001). Diagnosis of ER by PCR is rapid, sensitive, and does not depend on the presence of infectious~~
354 ~~virus in the clinical sample. It now forms an integral part of a range of diagnostic tests currently available for~~
355 ~~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):

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* (Borchers & Slater, 1993):

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 ug 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 MgCl$_2$, 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 MgCl$_2$, 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. 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. Serological testing can, however, be a useful adjunct procedure for assisting in the diagnosis of ER in 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~~ may prove 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 5×10^5/ml. Note that RK-13 cells can be used with EHV-1 but do not give clear CPE with EHV-4. Antibody positive and negative control equine sera and controls for cell viability, virus infectivity, and test serum cytotoxicity, must be included in each assay. End-point VN titres of antibody are calculated by determining the reciprocal of the highest serum dilution that protects 100% of the cell monolayer from virus destruction in both of the replicate wells.

464		**2.1.1. Test procedure**

465 A suitable test procedure is as follows:

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

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

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

473 iv) Add 25 µl of the appropriately diluted EHV-1 or EHV-4 virus stock to each well
474 (100 $TCID_{50}$/well) except those of row A, which are the serum control wells for monitoring
475 serum toxicity for the indicator cells. Note that the final serum dilutions, after addition of
476 virus, run from 1/4 to 1/256.

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

480 vi) Incubate the plates for 1 hour at 37°C in 5% CO_2 atmosphere.

481 vii) Add 50 µl of the prepared E-Derm or RK-13 cell suspension (5 × 10^5 cells/ml) in
482 MEM/10% FCS to each well.

483 viii) Incubate the plates for 4–5 days at 37°C in an atmosphere of 5% CO_2 in air.

484 ix) Examine the plates microscopically for CPE and record the results on a worksheet.
485 Alternatively, the cell monolayers can be scored for CPE after fixing and staining as
486 follows: after removal of the culture fluid, immerse the plates for 15 minutes in a solution
487 containing 2 mg/ml crystal violet, 10% formalin, 45% methanol, and 45% water. Then,
488 rinse the plates vigorously under a stream of running tap water.

489 x) Wells containing intact cell monolayers stain blue, while monolayers destroyed by virus do
490 not stain. Verify that the cell control, positive serum control, and serum cytotoxicity control
491 wells stain blue, that the virus control and negative serum control wells are not stained,
492 and that the actual amount of virus added to each well is between $10^{1.5}$ and $10^{2.5}$ $TCID_{50}$.

493 Wells are scored as positive for neutralisation of virus if 100% of the cell monolayer
494 remains intact. The highest dilution of serum resulting in complete neutralisation of virus
495 (no CPE) in both duplicate wells is the end-point titre for that serum.

496 xi) Calculate the neutralisation titre for each test serum, and compare acute and convalescent
497 phase serum titres from each animal for a four-fold or greater increase.

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

498

499 <mark>**NB: SECTION C IS "UNDER STUDY". THIS IS THE LAST ADOPTED VERSION PUBLISHED IN 2008**</mark>

500 Both live attenuated and inactivated vaccines are available as licensed, commercially prepared products for use
501 as prophylactic aids in reducing the burden of disease in horses caused by EHV-1/4 infection. Clinical experience
502 has demonstrated that none of the vaccine preparations should be relied on to provide an absolute degree of
503 protection from ER. Multiple doses repeated annually, of each of the currently marketed ER vaccines are
504 recommended by their respective manufacturers. Vaccination schedules vary with the particular vaccine.

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

508 At least sixteen vaccine products for ER, each containing different permutations of EHV-1, EHV-4, and the two
509 subtypes of equine influenza virus, are currently marketed by five veterinary biologicals manufacturers.

510 The clinical indications stated on the product label for use of the several available vaccines for ER are either
511 herpesvirus-associated respiratory disease, abortion, or both. Only four vaccine products have met the regulatory
512 requirements for claiming efficacy in providing protection from herpesvirus abortion as a result of successful

513 vaccination and challenge experiments in pregnant mares. None of the vaccine products has been conclusively
514 demonstrated to prevent the occurrence of neurological disease sometimes associated with EHV-1 infection.

515 **1. Seed management**

516 **1.1. Characteristics and culture**

517   The master seed virus (MSV) for ER vaccines must be prepared from strains of EHV-1 and/or EHV-4
518 that have been positively and unequivocally identified by both serological and genetic tests. Seed virus
519 must be propagated in a cell line approved for equine vaccine production by the appropriate regulatory
520 agency. A complete record of original source, passage history, medium used for propagation, etc.,
521 shall be kept for the master seed preparations of both the virus(es) and cell stock(s) intended for use in
522 vaccine production. Permanently stored stocks of both MSV and master cell stock (MCS) used for
523 vaccine production must be demonstrated to be pure, safe and, in the case of MSV, also immunogenic.
524 Generally, the fifth passage from the MSV and the twentieth passage from the MCS are the highest
525 allowed for vaccine production. Results of all quality control tests on master seeds must be recorded
526 and made a part of the licensee's permanent records.

527 **1.2. Validation as a vaccine**

528 **1.2.1. Purity**

529   Tests for master seed purity include prescribed procedures that demonstrate the virus and cell
530 seed stocks to be free from bacteria, fungi, mycoplasmas, and extraneous viruses. Special tests
531 must be performed to confirm the absence of equine arteritis virus, equine infectious anaemia
532 virus, equine influenza virus, equine herpesvirus-2, -3, and -5, equine rhinovirus, the
533 alphaviruses of equine encephalomyelitis, bovine viral diarrhoea virus (BVDV – common
534 contaminant of bovine serum), and porcine parvovirus (PPV – potential contaminant of porcine
535 trypsin). The purity check should also include the exclusion of the presence of EHV-1 from
536 EHV-4 MSV and *vice versa*.

537 **1.2.2. Safety**

538   Samples of each lot of MSV to be used for preparation of live attenuated ER vaccines must be
539 tested for safety in horses determined to be susceptible to the virulent wild-type virus, including
540 pregnant mares in the last 4 months of gestation. Vaccine safety must be demonstrated in a
541 'safety field trial' in horses of various ages from three different geographical areas. The safety
542 trial should be conducted by independent veterinarians using a prelicensing batch of vaccine.
543 EHV-1 vaccines making a claim for efficacy in controlling abortion must be tested for safety in a
544 significant number of late gestation pregnant mares, using the vaccination schedule that will be
545 recommended by the manufacturer for the final vaccine product.

546 **1.2.3. Immunogenicity**

547   Tests for immunogenicity of the EHV-1/4 MSV stocks should be performed in horses on an
548 experimental test vaccine prepared from the highest passage level of the MSV allowed for use
549 in vaccine production. The test for MSV immunogenicity consists of vaccination of horses with
550 low antibody titres to EHV-1/4, with doses of the test vaccine that will be recommended on the
551 final product label. Second serum samples should be obtained and tested for significant
552 increases in neutralising antibody titre against the virus, 21 days after the final dose.

553 **1.2.4. Efficacy**

554   An important part of the validation process is the capacity of a prelicensing lot of the ER vaccine
555 to provide a significant level of clinical protection in horses from the particular disease
556 manifestation of EHV-1/4 infection for which the vaccine is offered, when used under the
557 conditions recommended by the manufacturer's product label. Serological data are not
558 acceptable for establishing the efficacy of vaccines for ER. Efficacy studies must be designed to
559 ensure appropriate randomisation of test animals to treatment groups, blinding of the recording
560 of clinical observations, and the use of sufficient numbers of animals to permit statistical
561 evaluation for effectiveness in prevention or reduction of the specified clinical disease. The
562 studies should be performed on fully formulated experimental vaccine products (a) produced in
563 accordance with, (b) at or below the minimum antigenic potency specified in, and, (c) produced
564 with the highest passage of MSV and MCS allowed by the approved 'Outline of Production' (see
565 Section C.2). Vaccine efficacy is demonstrated by vaccinating a minimum of 20 EHV-1/4-
566 susceptible horses possessing serum neutralising antibody titres ≤32, followed by challenge of
567 the vaccinates and ten nonvaccinated control horses with virulent virus. A significant difference

568 in the clinical signs of ER must be demonstrated between vaccinates and nonvaccinated control
569 horses. The vaccination and challenge study must be performed on an identical number of
570 pregnant mares and scored for abortion if the vaccine product will make a label usage claim 'for
571 prevention of' or 'as an aid in the prevention of' abortion caused by EHV-1.

572 ## 2. Method of manufacture

573 A detailed protocol of the methods of manufacture to be followed in the preparation of vaccines for ER must be
574 compiled, approved, and filed as an Outline of Production with the appropriate licensing agency. Specifics of the
575 methods of manufacture for ER vaccines will differ with the type (live or inactivated) and composition (EHV-1 only,
576 EHV-1 and EHV-4, EHV-4 and equine influenza viruses, etc.) of each individual product, and also with the
577 manufacturer.

578 ## 3. In-process control

579 Cells, virus, culture medium, and medium supplements of animal origin that are used for the preparation of
580 production lots of vaccine must be derived from bulk stocks that have passed the prescribed tests for bacterial,
581 fungal, and mycoplasma sterility; nontumorgenicity; and absence of extraneous viral agents.

582 ## 4. Batch control

583 Each bulk production lot of ER vaccine must pass tests for sterility, safety, and immunogenic potency.

584 ### 4.1. Sterility

585 Samples taken from each batch of completed vaccine are tested for bacteria, fungi, and mycoplasma
586 contamination. Procedures to establish that the vaccine is free from extraneous viruses are also
587 required; such tests should include inoculation of cell cultures that allow detection of the common
588 equine viruses, as well as techniques for the detection of BVDV and PPV in ingredients of animal origin
589 used in the production of the batch of vaccine.

590 ### 4.2. Safety

591 Tests to assure safety of each production batch of ER vaccine must demonstrate complete inactivation
592 of virus (for inactivated vaccines) as well as a level of residual virus-killing agent that does not exceed
593 the maximal allowable limit (e.g. 0.2% for formaldehyde). Safety testing in laboratory animals is also
594 required.

595 ### 4.3. Potency

596 Batch control of antigenic potency for EHV-1 vaccines only may be tested by measuring the ability of
597 dilutions of the vaccine to protect hamsters from challenge with a lethal dose of hamster-adapted EHV-
598 1 virus. Although potency testing on production batches of ER vaccine may also be performed by
599 vaccination of susceptible horses followed by either viral challenge or assay for seroconversion the
600 recent availability of virus type-specific MAbs has permitted development of less costly and more rapid
601 *in-vitro* immunoassays for antigenic potency. The basis for such *in-vitro* assays for ER vaccine potency
602 is the determination, by use of the specific MAb, of the presence of at least the minimal amount of viral
603 antigen within each batch of vaccine that correlates with the required level of protection (or
604 seroconversion rate) in a standard animal test for potency.

605 ### 4.4. Duration of immunity

606 Tests to establish the duration of immunity to EHV-1/4 achieved by immunisation with each batch of
607 vaccine are not required. The results of many reported observations indicate that vaccination-induced
608 immunity to EHV-1/4 is not more than a few months in duration; these observations are reflected in the
609 frequency of revaccination recommended on ER vaccine product labels.

610 ### 4.5. Stability

611 At least three production batches of vaccine should be tested for shelf life before reaching a conclusion
612 on the vaccine's stability. When stored at 4°C, inactivated vaccine products generally maintain their
613 original antigenic potency for at least 1 year. Lyophilised preparations of the live virus vaccine are also

614   stable during storage for 1 year at 4°C. Following reconstitution, live virus vaccine is unstable and
615   cannot be stored without loss of potency.

616 ## 5. Tests on the final product

617 Before release for labelling, packaging, and commercial distribution, randomly selected filled vials of the final
618 vaccine product must be tested by prescribed methods for freedom from contamination and safety in laboratory
619 test animals.

620   ### 5.1. Safety

621   See Section C.4.2.

622   ### 5.2. Potency

623   See Section C.4.3.

624 <div align="center">**REFERENCES**</div>

625 ALLEN G.P. (2007). Development of a real-time polymerase chain reaction assay for rapid diagnosis of
626 neuropathogenic strains of equine herpesvirus-1. *J Vet. Diagn. Invest.,* **19**, 69–72.

627 ALLEN G.P. & BRYANS J.T. (1986). Molecular epidemiology, pathogenesis and prophylaxis of equine herpesvirus-1
628 infections. *In:* Progress in Veterinary Microbiology and Immunology, Vol. 2, Pandey R., ed. Karger, Basel,
629 Switzerland & New York, USA, 78–144.

630 ALLEN G.P., KYDD J.H., SLATER J.D. & SMITH K.C. (1999). Recent advances in understanding the pathogenesis,
631 epidemiology, and immunological control of equid herpesvirus-1 (EHV-1) abortion. *Equine Infect. Dis.*, **8,** 129–
632 146.

633 ALLEN G.P., KYDD J.H., SLATER J.D. & SMITH K.C. (2004). Equid herpesvirus-1 (EHV-1) and -4 (EHV-4) infections.
634 *In:* Infectious Diseases of Livestock, Coetzer J.A.W., Thomson G.R. & Tustin R.C., eds. Oxford University Press,
635 Cape Town, South Africa.

636 BORCHERS K. & SLATER J. (1993). A nested PCR for the detection and differentiation of EHV-1 and EHV-4. *J. Virol.*
637 *Methods*, **45**, 331–336.

638 BRYANS J.T. & ALLEN G.P. (1988). Herpesviral diseases of the horse. *In:* Herpesvirus *Diseases* of Animals,
639 Wittman G., *ed.* Kluwer, Boston, USA, 176–229.

640 CRABB B.S., MACPHERSON C.M., REUBEL G.H., BROWNING G.F., STUDDERT M.J. & DRUMMER H.E. (1995). A type-
641 specific serological test to distinguish antibodies to equine herpesviruses 4 and 1. *Arch. Virol.*, **140**, 245–258.

642 CRABB B.S. & STUDDERT M.J. (1995). Equine herpesviruses 4 (equine rhinopneumonitis virus) and 1 (equine
643 abortion virus). *Adv. Virus Res.*, **45**, 153–190.

644 DUTTA S.K., TALBOT N.C. & MYRUP A.C. (1983). Detection of equine herpesvirus-1 antigen and the specific
645 antibody by enzyme-linked immunosorbent assay. *Am. J. Vet. Res.*, **44**, 1930–1934.

646 FRITSCHE A.K. & BORCHERS K. (2011). Detection of neuropathogenic strains of equid herpesvirus 1 (EHV-1)
647 associated with abortions in Germany. *Vet. Microbiol.,* **147**, 176-180.

648 GUNN H.M. (1992). A direct fluorescent antibody technique to diagnose abortion caused by equine herpesvirus.
649 *Irish Vet. J.*, **44**, 37–40.

650 GOODMAN L.B., LOREGIAN A., PERKINS G.A., NUGENT J., BUCKLES E.L., MERCORELLI B., KYDD J.H., PALÙ G., SMITH
651 K.C., OSTERRIEDER N. & DAVIS-POYNTER N. (2007). A point mutation in a herpesvirus polymerase determines
652 neuropathogenicity. *PLoS Pathog.,* **3** (11), e160.

653 LAWRENCE G.L., GILKERSON J., LOVE D.N., SABINE M. & WHALLEY J.M. (1994). Rapid, single-step differentiation of
654 equid herpesvirus 1 and 4 from clinical material using the polymerase chain reaction and virus-specific primers. *J.*
655 *Virol. Methods*, **47**, 59–72.

NUGENT J., BIRCH-MACHIN I., SMITH K.C., MUMFORD J.A., SWANN Z., NEWTON J.R., BOWDEN R.J., ALLEN G.P. & DAVIS-POYNTER N. (2006). Analysis of equid herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic versus nonneuropathogenic disease outbreaks. *J. Virol.,* **80**, 4047–4060.

O'KEEFE J.S., JULIAN A., MORIARTY K., MURRAY A. & WILKS C.R. (1994). A comparison of the polymerase chain reaction with standard laboratory methods for the detection of EHV-1 and EHV-4 in archival tissue samples. *N.Z. Vet. J.*, **42**, 93–96.

SCHULTHEISS P.C., COLLINS J.K. & CARMAN J. (1993). Use of an immunoperoxidase technique to detect equine herpesvirus-1 antigen in formalin-fixed paraffin-embedded equine fetal tissues. *J. Vet. Diagn. Invest.*, **5**, 12–15.

SMITH K.L., LI Y., BREHENY P., COOK R.F., HENNEY P.J., SELLS S., PRONOST S., LU Z., CROSSLEY B.M., TIMONEY P.J. & BALASURIYA U.B. (2012). Development and validation of a new and improved allelic discrimination real-time PCR assay for the detection of equine herpesvirus-1 (EHV-1) and differentiation of A2254 from G2254 strains in clinical specimens. *J. Clin. Microbiol.*, **50**, 1981–1988.

TELFORD E.A.R., WATSON M.S., MCBRIDE K. & DAVISON A.J. (1992). The DNA sequence of equine herpesvirus-1. *Virology*, **189**, 304–316.

TELFORD E.A.R., WATSON M.S., PERRY J., CULLINANE A.A. & DAVISON A.J. (1998). The DNA sequence of equine herpesvirus 4. *J. Gen. Virol.,* **79**, 1197–1203.

THOMSON G.R., MUMFORD J.A., CAMPBELL J., GRIFFITHS L. & CLAPHAM P. (1976). Serological detection of equid herpesvirus 1 infections of the respiratory tract. *Equine Vet. J.*, **8**, 58–65.

VAN MAANEN C., VREESWIJK J., MOONEN P., BRINKHOF J. DE BOER-LUIJTZE E. & TERPSTRA C. (2000). Differentiation and genomic and antigenic variation among fetal, respiratory, and neurological isolates from EHV1 and EHV4 infections in The Netherlands. *Vet. Q.*, **22** (2): 88–93.

VARRASSO A., DYNON K., FICORILLI N., HARTLEY C.A., STUDDERT M.J. & DRUMMER H.E. (2001). Identification of equine herpesviruses 1 and 4 by polymerase chain reaction. *Aust. Vet. J.,* **79**, 563–569.

~~WAGNER W.N., BOGDAN J., HAINES D., TOWNSEND H.G.G. & MISRA V. (1992). Detection of equine herpesvirus and differentiation of equine herpesvirus type 1 from type 4 by the polymerase chain reaction.~~ *~~Can. J. Microbiol.~~*~~,~~ **~~38~~**~~, 1193–1196.~~

WELCH H.M., BRIDGES C.G., LYON A.M., GRIFFITHS L. & EDINGTON N. (1992). Latent equid herpesviruses 1 and 4: detection and distinction using the polymerase chain reaction and cocultivation from lymphoid tissues. *J. Gen. Virol.*, **73**, 261–268.

WHITWELL K.E., GOWER S.M. & SMITH K.C. (1992). An immunoperosidase method applied to the diagnosis of equine herpesvirus abortion, using conventional and rapid microwave techniques. *Equine Vet. J.*, **24**, 10–12.

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* *

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

48 occur. In the skin form ('farcy'), the lymphatics are enlarged and 0.5–2.5 cm sized nodular abscesses ('buds')
49 develop, which ulcerate and discharge yellow oily pus. Dry ulcers may also develop. Pyogranulomatous nodules
50 are sometimes found in the liver and spleen (Wernery *et al.*, 2012). Discharges from the respiratory tract and skin
51 are infective, and transmission between animals, which is facilitated by close contact, inhalation, ingestion of
52 contaminated material (e.g. from infected feed and water troughs), or by inoculation (e.g. via a harness) is
53 common. The incubation period can range from a few days to many months (Wittig *et al.*, 2006).

54 Glanders is transmissible to humans by direct contact with diseased animals or with infected/contaminated
55 material. In the untreated acute disease, the mortality rate can reach 95% within 3 weeks (Neubauer *et al.*, 1997).
56 However, survival is possible if the infected person is treated early and aggressively with multiple systemic
57 antibiotic therapies. A chronic form with abscessation can occur (Neubauer *et al.*, 1997). When handling suspect
58 or known infected animals or fomites, stringent precautions must be taken to prevent self-infection or transmission
59 of the bacterium. Laboratory samples must be securely packaged, kept cool (not frozen) and shipped as outlined
60 in Chapter 1.1.2 *Transport of specimens of animal origin*. All manipulations with potentially infected/contaminated
61 material must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see
62 Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities*).

63 Glanders has been eradicated from many countries by statutory testing, culling of infected animals, and import
64 restrictions. It persists in numerous Asian, African and South American countries and can be considered a re-
65 emerging disease. Glanders can be introduced into glanders-free areas by leisure or racing equids (Neubauer *et*
66 *al.*, 2005).

1.   Diagnostic pathway to confirm a case of glanders

67

68 i)   *Burkholderia mallei* has been isolated and identified in a sample from an equid or a product derived
69   from that equid; or

70 ii)   antigen or genetic material specific to *B. mallei* has been identified in samples from an equid, whether
71   showing clinical or pathological signs consistent with glanders or not, or is epidemiologically linked to a
72   confirmed or suspected outbreak of glanders, or is giving cause for suspicion of previous contact with
73   *B. mallei*; or

74 iii) antibodies have been identified by an appropriate testing regime:

75   a)   a horse, whether showing clinical or pathological signs or not, subjected with positive result at a
76   serum dilution of 1 in 5 to a complement fixation test (CFT), confirmed by a second test with equal
77   or higher sensitivity and higher specificity, e.g. *B. mallei*-specific lipopolysaccharide (LPS)-
78   western blot, I-ELISA (indirect enzyme-linked immunosorbent assay) (based on a recombinant
79   protein from type VI secretion system) or C-ELISA (competitive ELISA) (based on *B. mallei*-
80   specific monoclonal antibodies);

81   b)   a mule, hinny or donkey, whether showing clinical or pathological signs or not, by one of the
82   following tests: *B. mallei*-specific LPS-western blot, I-ELISA (based on a recombinant protein from
83   type VI secretion system) or C-ELISA (based on *B. mallei*-specific monoclonal antibodies).

84

B. DIAGNOSTIC TECHNIQUES

85   ***Table 1.*** *Test methods available for the diagnosis of glanders and their purpose*

Method	Purpose				
	Population freedom from infection	Individual animal freedom from infection	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance
Agent identification[1]					
PCR	–	–	–	+	–
Culture	–	–	–	+	–
~~**Animal inoculation**~~	~~–~~	~~–~~	~~–~~	~~+~~	~~–~~

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1   A combination of agent identification methods applied on the same clinical sample is recommended.

Method	Purpose				
	Population freedom from infection	Individual animal freedom from infection	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance
Detection of immune response[2]					
Complement fixation	+	++[3]	+++	+	+++
ELISA	+	+	++	+	++
Malleinisation	+	+	+	+	+
Western blotting	+	+	++	+	++

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 immunosorbent 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 (*Sporotrichium* 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.

Burkholderia selective agars enable the growth of *B. mallei* (Glass *et al.*, 2009). Even in fresh samples obtained under sterile conditions *B. mallei* is often overgrown by other bacteria, which makes isolation extremely difficult (Wernery, 2009).

Growth characteristics may alter *in vitro*, so fresh isolates should be used for identification reactions. The positive biochemical reactions include reduction of nitrates, utilisation of arginine by arginine dihydrolase, assimilation of glucose, N-acetyl glucosamine and gluconate. Strain to strain variation is observed in the assimilation reactions of arabinose, fructose, mannose, mannitol, adipic acid, malate, trisodium citrate, phenyl acetic acid and VP reaction, which needs an incubation time of 48 hours. Indole is not produced, horse blood is not haemolysed and no diffusible pigments are produced in cultures. Commercially available laboratory biochemical identification systems can be used for easy confirmation that an organism belongs to the *Pseudomonas* group. In general, however, commercially available systems are not suitable for unambiguous identification of members of the steadily growing number of species within the genus *Burkholderia* (Glass & Popovic, 2005). Lack of motility is therefore of special relevance. A bacteriophage specific for *B. mallei* is available.

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

In contaminated samples, supplementation of media with substances that inhibit the growth of Gram-positive organisms (e.g. crystal violet, proflavine) has proven to be useful, as well as pre-treatment with penicillin (1000 units/ml for 3 hours at 37°C). A semi-selective medium (Xie *et al.*, 1980) composed of polymyxin E (1000 units), bacitracin (250 units), and actidione (0.25 mg) incorporated into nutrient agar (100 ml) containing glycerine (4%), donkey or horse serum (10%), and ovine haemoglobin or tryptone agar (0.1%) has been developed. Heavily contaminated samples should also be streaked onto stiff blood agar (3% agar) which inhibits the growth of *Proteus* spp., and onto Sabouraud dextrose agar which inhibits the growth of many Gram-positive and Gram-negative bacteria in glanders samples. These samples should also be streaked onto normal blood agar and incubated for 24 hours anaerobically to inhibit the growth of obligate aerobes. Isolation of *B. mallei* from the anaerobic plates needs a further 24 hours' incubation at 37°C. PCR methods may also prove useful for testing contaminated samples.

Outside the body, the organism shows little resistance to drying, heat, light or chemicals, so that survival beyond 2 weeks is unlikely (Neubauer *et al.*, 1997). Under favourable conditions, however, it can probably survive a few months. *Burkholderia mallei* can remain viable in tap water for at least 1 month. For disinfection, benzalkonium chloride (1/2,000), sodium hypochlorite (500 ppm available chlorine), iodine, mercuric chloride in alcohol, and potassium permanganate have been shown to be highly effective. Phenolic disinfectants are less effective (St. Georgiev, 2008). The guidelines for handling and application of disinfectants in the respective countries must be observed.

1.3. Identification of *Burkholderia mallei* 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; Tomaso *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

181   instructions. DNA is eluted with 80 µl dH$_2$O or as appropriate for the kit used 4 µl eluate is used
182   as template.

1.3.2. PCR assay (Scholz *et al.,* 2006)

184   The assay may have to be adapted to the PCR instrument used with minor modifications to the
185   cycle conditions and the concentration of the reagents used.

186   The oligonucleotides used by Scholz *et al.,* (2006) are based on the differences between the *fliP*
187   sequences from *B. mallei* ATCC 23344^T (accession numbers NC_006350, NC_006351) and
188   *B. pseudomallei* K96243 (accession numbers NC_006348, NC_006349). Primers Bma-IS407-
189   flip-f (5'-TCA-GGT-TTG-TAT-GTC-GCT-CGG-3') and Bma- IS407-flip-r (5'-CTA-GGT-GAA-
190   GCT-CTG-CGC-GAG-3') are used to amplify a 989 bp fragment. The PCR uses 50 µl ready-to-
191   go master mix and 15 pmol of each primer. Thermal cycling conditions are 94°C for 30 seconds
192   and 35 cycles at 65°C for 30 seconds and 72°C for 60 seconds and succeeded by a final
193   elongation step at 72°C for 7 minutes. Visualisation of the products takes place under UV light
194   after agarose gel (1% w/v in TAE buffer) electrophoresis and staining with ethidium bromide. No
195   template controls containing PCR-grade water instead of template and positive controls
196   containing *B. mallei* DNA have to be included in each run to detect contamination by amplicons
197   of former runs or amplification failure.

198   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)

200   The assay should be adapted to the real-time PCR instrument used, e.g. the cycling vials
201   should be chosen according to the manufacturer's recommendations, the concentration of the
202   oligonucleotides may have to be increased, or the labelling of the probes altered.

203   The oligonucleotides used in Tomaso *et al.* (2006) are based on differences in the *fliP*
204   sequences of *B. mallei* ATCC 23344^T (accession numbers NC_006350, NC_006351) and
205   *B. pseudomallei* K96243 (accession numbers NC_006348, NC_006349). The fluorogenic probe
206   is synthesised with 6-carboxy-fluorescein (FAM) at the 5'-end and black hole quencher 1
207   (BHQ1) at the 3'-end. Oligonucleotides used were Bma-flip-f (5'-CCC-ATT-GGC-CCT-ATC-
208   GAA-G-3'), Bma-flip-r (5'-GCC-CGA-CGA-GCA-CCT-GAT-T-3') and probe Bma-flip (5'-6FAM-
209   CAG-GTC-AAC-GAG-CTT-CAC-GCG-GAT-C-BHQ1-3').

210   The 25 µl reaction mixture consists of 12.5 µl 2× master mix, 0.1 µl of each primer (10 pmol/µl),
211   0.1 µl of the probe (10 pmol/µl) and 4 µl sample. Thermal cycling conditions are 50°C for
212   2 minutes; 95°C for 10 minutes; 45 cycles at 95°C for 25 seconds and 63°C for 1 minute.
213   Possible contaminations with amplification products from former reactions are inactivated by an
214   initial incubation step using uracil *N'*-glycosilase.

215   The authors suggest including an internal inhibition control based on a bacteriophage lambda
216   gene target (Lambda-F [5'-ATG-CCA-CGT-AAG-CGA-AAC-A-3] Lambda-R [5'-GCA-TAA-ACG-
217   AAG-CAG-TCG-AGT-3'], Lam-YAK [5'-YAK-ACC-TTA-CCG-AAA-TCG-GTA-CGG-ATA-CCG-
218   C-DB-3']), which can be titrated to provide reproducible cycle threshold values. However,
219   depending on the sample material a house keeping gene targeting PCR may be used
220   additionally or as an alternative. No template controls containing 4 µl of PCR-grade water
221   instead and positive controls containing DNA of *B. mallei* have to be included in each run to
222   detect amplicon contamination or amplification failure.

223   The linear range of the assay was determined to cover concentrations from 240 pg to 70 fg
224   bacterial DNA/reaction. The lower limit of detection defined as the lowest amount of DNA that
225   was consistently detectable in three runs with eight measurements each is 60 fg DNA or four
226   genome equivalents (95% probability). The intra-assay variability of the *fliP* PCR assay for
227   35 pg DNA/reaction is 0.68% (based on Ct values) and for 875 fg 1.34%, respectively. The
228   inter-assay variability for 35 pg DNA/reaction is 0.89% (based on Ct values) and for 875 fg DNA
229   2.76%, respectively.

230   To date, a positive result confirms the diagnosis '*Burkholderia mallei'* for an isolate and the
231   diagnosis 'glanders' in clinical cases. It has to be kept in mind, however, that future genetic
232   evolution may well result in *B. mallei* clones that can no longer be detected by these standard
233   PCRs. The sensitivity of the PCR assays for clinical samples is unknown. A negative result
234   therefore, is no proof of the absence of *B. mallei* in the sample and other diagnostic means
235   must be applied for confirmation.

236 ~~**1.4. Laboratory animal inoculation**~~

237 ~~Animal inoculation is not recommended, because of welfare concerns. If isolation in a laboratory animal~~
238 ~~is considered unavoidable, suspected material is inoculated intraperitoneally into male guinea-pigs. As~~
239 ~~this technique has a sensitivity of only 20%, the inoculation of at least five animals is recommended~~
240 ~~(Neubauer *et al.*, 1997). Positive material will cause a severe localised peritonitis and orchitis (the~~
241 ~~Strauss reaction). The number of organisms and their virulence determines the severity of lesions.~~
242 ~~Additional pre-treatment steps have to be used if the test material is heavily contaminated. The Strauss~~
243 ~~reaction is not specific for glanders and can be provoked by other organisms, therefore *B. mallei* must~~
244 ~~be cultured from the infected testes.~~

245 **1.5. Other methods**

246 Molecular typing techniques such as PCR-restriction fragment length polymorphism (Tanpiboonsak *et*
247 *al.*, 2004), pulsed field gel electrophoresis (Chantratita *et al.*, 2006), ribotyping (Harvey & Minter, 2005)
248 or multilocus sequence typing (MLST) (Godoy *et al.*, 2003) are only appropriate for use in specialised
249 laboratories

250 ## 2. Serological tests

251 **2.1. Complement fixation test in horses, donkeys, and mules (a prescribed test for international**
252 **trade)**

253 The CFT is an accurate serological test that has been used for many years for diagnosing glanders. It
254 will deliver positive results within 1 week post-infection and will also recognise sera from exacerbated
255 chronic cases. Application of rigorous quality control in the formulation of CFT antigens, complement
256 and haemolytic systems are crucial for the performance of this test as its specificity and sensitivity are
257 critically dependent on the antigen used (Elschner *et al.*, 2011; Khan *et al.*, 2011). Recently, however,
258 the specificity of CF testing has been questioned (Neubauer *et al.*, 2005). The CFT is valid for horses,
259 mules and camels; if used in donkeys particular care is needed to avoid misdiagnosis.

260 **2.1.1. Antigen preparation**

261 i)   The stock culture strain of B.mallei (Dubai 7) stored at –80°C is revived by plating onto
262   sheep blood agar and incubated at 37°C for 48 hours to get a confluent growth.

263 ii) From this 48 hours culture, a loopful (0.5 mm diameter) is inoculated to 5 ml of brain–heart
264   infusion (BHI) broth with 3% glycerol and incubated at 37°C for 24 hours.

265 iii) 1 ml from the above culture broth is further inoculated to 100 ml BHI broth with 3% glycerol
266   and incubated at 37°C for 48 hours with gentle agitation.

267 iv) The cultures are inactivated by exposing the flasks to flowing steam (100°C) for
268   60 minutes.

269 v   The clear supernatant is decanted and filtered. The filtrate is heated again by exposure to
270   live steam for 1 hour, and clarified by centrifugation at 3000 rpm for 10 minutes.

271 vi) The clarified product is stored as concentrated antigen in brown glass bottles to shield
272   from light and stored at 4°C. Antigen has been shown to be stable for at least 10 years in
273   this concentrated state.

274 vii) Aliquots of antigen are prepared by diluting the concentrated antigen 1/20 with sterile
275   physiological saline containing 0.5% phenol. The diluted antigen is dispensed into brown-
276   glass vials and stored at 4°C. The final working dilution is determined by a block titration.
277   The final working dilution for the CFT is prepared when performing the test.

278 The resulting antigen consists primarily of lipopolysaccharides (LPS). An alternative procedure
279 is to use young cultures by growing the organism on glycerol–agar slopes for up to 48 hours
280 and washing them off with normal saline. A suspension of the culture is heated for 1 hour at
281 70°C and the heat-treated bacterial suspension is used as antigen. The disadvantage of this
282 antigen preparation method is that the antigen contains all the bacterial cell components. The
283 antigen should be safety tested by inoculating blood agar plates.

284

2.1.2. CFT procedure

285		

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°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% (CH_{50}) 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 quarantine 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 glanderous 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[4]. 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 decantedand 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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427 **REFERENCES**

428 ~~ALTEMANN D. (in preparation). Pathogenesis and diagnosis of glanders in experimentally infected donkey using~~
429 ~~different infection routes and doses, Thesis Veterinary Faculty Giessen, Germany.~~

430 CHANTRATITA N., VESARATCHAVEST M., WUTHIEKANUN V., TIYAWISUTSRI R., ULZIITOGTOKH T., AKCAY E., DAY N.P. &
431 PEACOCK S.J. (2006). Pulsed-field gel electrophoresis as a discriminatory typing technique for the biothreat agent
432 *Burkholderia mallei. Am. J. Trop. Med. Hyg.*, **74**, 345–347.

433 ELSCHNER M.C., SCHOLZ H.C., MELZER F., SAQIB M., MARTEN P., RASSBACH A., DIETZSCH M., SCHMOOCK G., DE ASSIS
434 SANTANA V.L., DE SOUZA M.M., WERNERY R., WERNERY U. & NEUBAUER H. (2011). Use of a Western blot technique
435 for the serodiagnosis of glanders. *BMC Vet. Res.*, **7**, 4.

436 GLASS M.B., BEESLEY C.A., WILKINS P.P. & HOFFMASTER A.R. (2009). Comparison of four selective media for the
437 isolation of *Burkholderia mallei* and *Burkholderia pseudomallei. Am. J. Trop. Med. Hyg.*, **80** (6), 1023–1028.

438 GLASS M.B. & POPOVIC T. (2005). Preliminary evaluation of the API 20NE and RapID NF plus systems for rapid
439 identification of *Burkholderia pseudomallei* and *B. mallei. J. Clin. Microbiol.*, **43**, 479–483.

440 GODOY D., RANDLE G., SIMPSON A.J., AANENSEN D.M., PITT T.L., KINOSHITA R. & SPRATT B.G. (2003). Multilocus
441 sequence typing and evolutionary relationships among the causative agents of melioidosis and glanders,
442 *Burkholderia pseudomallei* and *Burkholderia mallei. J. Clin. Microbiol.*, **41**, 2068–2079.

443 HARVEY S.P. & MINTER J.M. (2005). Ribotyping of *Burkholderia mallei* isolates. *FEMS Immunol. Med. Microbiol.*,
444 **44**, 91–97.

445 KATZ J.B., CHIEVES., HENNAGER S.G., NICHOLSON J.M., FISHER T.A. & BYERS P.E. (1999). Serodiagnosis of equine
446 piroplasmosis, dourine and glanders using an arrayed immunoblotting method. *J. Vet. Diagn. Invest.*, **11**, 292–
447 294.

448 KATZ J., DEWALD R. & NICHOLSON J. (2000). Procedurally similar competitive immunoassay systems for the
449 serodiagnosis of *Babesia equi, Babesia caballi, Trypanosoma equiperdum, and Burkholderia mallei* infection in
450 horses. *J. Vet. Diagn. Invest.*, **12**, 46–50.

451 KHAN I., WIELER L.H., MELZER F., GWIDA M., SANTANA V.L., DE SOUZA M.M., SAQIB M., ELSCHNER M.C. & NEUBAUER H.
452 (2011). Comparative evaluation of three commercially available complement fixation test antigens for the
453 diagnosis of glanders. *Vet. Rec.*, **169** (19), 495.

454 KHAN I., WIELER L.H., MELZER F., ELSCHNER M.C., MUHAMMAD G., ALI S., SPRAGUE L.D., NEUBAUER H. & SAQIB M.
455 (2012). Glanders in animals: a review on epidemiology, clinical presentation, diagnosis and
456 countermeasures. *Transbound. Emerg. Dis.*, doi: 10.1111/j.1865-1682.2012.01342.x

457 KUMAR S., MALIK P., VERMA S.K., PAL V., GAUTAM V., MUKHOPADHYAY C. & RAI G.P. (2011). Use of a recombinant
458 *Burkholderia* intracellular motility a protein for immunodiagnosis of glanders. *Clin. Vaccine Immunol.,* **18** (9),
459 1456–1461.

460 LEE M.A., WANG D. & YAP E.H. (2005). Detection and differentiation of *Burkholderia pseudomallei*, *Burkholderia
461 mallei* and *Burkholderia thailandensis* by multiplex PCR. *FEMS Immunol. Med. Microbiol.*, **43**, 413–417.

462 NAUREEN A., SAQIB M., MUHAMMAD G., HUSSAIN M.H. & ASI M.N. (2007). Comparative evaluation of Rose Bengal
463 plate agglutination test, mallein test, and some conventional serological tests for diagnosis of equine glanders. *J.
464 Vet. Diagn. Invest.,* **19** (4), 362–367.

465 NEUBAUER H., FINKE E.-J. & MEYER H. (1997). Human glanders. *International Review of the Armed Forces Medical
466 Services*, **LXX**, 10/11/12, 258–265.

467 NEUBAUER H., SPRAGUE L.D., ZACHARIA R., TOMASO H., AL DAHOUK S., WERNERY R., WERNERY U. & SCHOLZ H.C.
468 (2005). Serodiagnosis of *Burkholderia mallei* infections in horses: state-of-the-art and perspectives. *J. Vet. Med.
469 [B] Infect. Dis. Vet. Public Health.*, **52**, 201–205.

470 SCHOLZ H.C., JOSEPH M., TOMASO H., AL DAHOUK S., WITTE A., KINNE J., HAGEN R.M., WERNERY R., WERNERY U. &
471 NEUBAUER H. (2006). Detection of the reemerging agent *Burkholderia mallei* in a recent outbreak of glanders in the
472 United Arab Emirates by a newly developed fliP-based polymerase chain reaction assay. *Diagn. Microbiol. Infect.
473 Dis.,* **54**, 241–247.

SPRAGUE L.D. & NEUBAUER H. (2004). A review on animal melioidosis with special respect to epizootiology, clinical presentation and diagnostics. *J. Vet. Med. B Infect. Dis. Vet. Public Health*, **51**, 305–320.

SPRAGUE L.D., ZACHARIAH R., NEUBAUER H., WERNERY R., JOSEPH M., SCHOLZ H.C. & WERNERY U. (2009) Prevalence-dependent use of serological tests for diagnosing glanders in horses. *BMC Vet. Res.*, **5**, 32.

SPRAGUE L.D., ZYSK G., HAGEN R.M., MEYER H., ELLIS J., ANUNTAGOOL N., GAUTHIER Y. & NEUBAUER H. (2002). A possible pitfall in the identification of *Burkholderia mallei* using molecular identification systems based on the sequence of the flagellin fliC gene. *FEMS Immunol. Med. Microbiol.*, **34**, 231–236.

ST. GEORGIEV V. (2008). Glanders. *In:* National Institute of Allergy and Infectious Diseases, NIH: Impact on Global Health. Humana Press (Part of Springer Science+Business Media), New York, USA, 239–241.

TANPIBOONSAK S., PAEMANEE A., BUNYARATAPHAN S. & TUNGPRADABKUL S. (2004). PCR-RFLP based differentiation of *Burkholderia mallei* and *Burkholderia pseudomallei. Mol. Cell. Probes.*, **18**, 97–101.

THIBAULT F.M., VALADE E. & VIDAL D.R. (2004). Identification and discrimination of *Burkholderia pseudomallei*, *B. mallei*, and *B. thailandensis* by real-time PCR targeting type III secretion system genes. *J. Clin. Microbiol.*, **42**, 5871–5874.

TOMASO H., SCHOLZ H.C., AL DAHOUK S., EICKHOFF M., TREU T.M., WERNERY R., WERNERY U. & NEUBAUER H. (2006). Development of a 5'-nuclease real-time PCR assay targeting fliP for the rapid identification of *Burkholderia mallei* in clinical samples. *Clin. Chem.*, **52**, 307–310.

ULRICH M.P., NORWOOD D.A., CHRISTENSEN D.R. & ULRICH R.L. (2006). Using real-time PCR to specifically detect *Burkholderia mallei. J. Med. Microbiol.*, **55**, 551–559.

U'REN J.M. VAN ERT M.N., SCHUPP J.M., EASTERDAY W.R., SIMONSON T.S., OKINAKA R.T., PEARSON T. & KEIM P. (2005). Use of a real-time PCR TaqMan assay for rapid identification and differentiation of *Burkholderia pseudomallei* and *Burkholderia mallei. J. Clin. Microbiol.,* **43**, 5771–5774.

WERNERY U. (2009). Glanders. *In:* Infectious Diseases of the Horse, Mair T.S. & Hutchinson R.E., eds. Equine Veterinary Journal Ltd, Cambridgeshire, UK, 253–260.

WITTIG M.B., WOHLSEIN P., HAGEN R.M., AL DAHOUK S., TOMASO H., SCHOLZ H.C., NIKOLAOU K., WERNERY R., WERNERY U., KINNE J., ELSCHNER M. & NEUBAUER H. (2006). Glanders – a comprehensive review. *Dtsch. Tierarztl. Wochenschr.,* **113**, 323–230.

XIE X., XU F., XU B., DUAN X. & GONG R. (1980). A New Selective Medium for Isolation of Glanders Bacilli. Collected papers of veterinary research. Control Institute of Veterinary Biologics, Ministry of Agriculture, Peking, China (People's Rep. of), **6**, 83–90.

YABUUCHI E., KOSAKO Y., OYAIZU H., YANO I., HOTTA H., HASHIMOTO Y., EZAKI T. & ARAKAWA M. (1992). Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol. Immunol.*, **36**, 1251–1275.

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*   *

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

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

CHAPTER 2.7.9.

OVINE EPIDIDYMITIS
(*Brucella ovis*)

SUMMARY

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.*

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* 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.*

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* 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 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* 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.*

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* B. melitensis *Rev.1 vaccine, administered subcutaneously or, better, conjunctivally, can be used safely and effectively in rams, for the prevention of* 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* Brucella ovis]).

A. INTRODUCTION

1. Definition of the disease

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

Brucella ovis and *B. canis* are the two presently known *Brucella* species naturally in the rough phase. *Brucella ovis* is similar to the other *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 *Brucella* species and biovars are given in detail in the Chapter 2.4.3 *Bovine brucellosis.*

41 ## 3. Description of the disease

42 *Brucella ovis* infects sheep causing genital lesions (epididymitis and orchi-epididymitis) and infertility in rams,
43 placentitis, abortions and infertility in ewes, and increased perinatal mortality in lambs. *Brucella ovis* is usually
44 excreted in semen in infected rams. Passive venereal transmission via the ewe appears to be the most frequent
45 route of infection, but ram-to-ram transmission is also very common (Blasco, 1990; 2010). Under the semi-
46 extensive production systems (most common in European Mediterranean countries), rams are usually housed
47 together. Direct ram-to-ram transmission during non-breeding periods is thus quite frequent and has been
48 suggested to take place by several routes, including anal intercourse and, more frequently, through oral-genital
49 contact (preputial licking).

50 Moreover, infected ewes may excrete *B. ovis* in vaginal discharges and milk and, accordingly, ewe-to-ram and
51 lactating ewe-to-lamb transmission could also be determinant mechanisms of infection. Accordingly, ewes should
52 be considered relevant in the epidemiology of infection, and this should be taken into account for the effective
53 eradication of *B. ovis* in infected flocks (Blasco, 2010; Grilló *et al.,* 1999).

54 The disease has been reported in American and European countries as well as Australia, New Zealand and
55 South Africa, but probably occurs in most sheep-raising countries.

56 The demonstration of genital lesions (unilateral or bilateral epididymitis and orchi-epididymitis) by palpating the
57 testicles of rams may suggest the presence of this infection in a given flock. However, clinical diagnosis lacks
58 sensitivity because not all rams infected with *B. ovis* present palpable genital lesions (Blasco, 1990). Moreover,
59 clinical diagnosis lacks specificity since many other bacteria may cause genital lesions in rams. The most
60 frequently reported pathogens such lesions in rams include Actinobacillus seminis, A. actinomycetemcomitans,
61 *Histophilus ovis*, *Haemophilus* spp., *Corynebacterium pseudotuberculosis ovis*, *B. melitensis*, *Chlamydia abortus*
62 and *Pasteurella* spp. (Bulgin & Anderson, 1983; Garcia-Pastor *et al.,* 2009; Livingstone & Hardy, 1964).
63 Moreover, it must be emphasised that many palpable testicular lesions in rams are sterile, trauma-induced
64 spermatic granulomas.

65 Although cattle, goats and deer have been proved susceptible to *B. ovis* in artificial transmission experiments,
66 natural cases have been reported only in red deer reared in close contact with infected rams (Ridler *et al.,* 2012).

67 ## 4. Zoonotic risk and biosafety requirements

68 To date, no human cases have been reported, and *B. ovis* is considered to be non-zoonotic. However, in areas
69 where *B. melitensis* infection co-exists with *B. ovis*, special care is required when handling samples, which should
70 be transported to the laboratory in leak-proof containers (for further details see Chapter 2.7.2 *Caprine and ovine*
71 *brucellosis* [*excluding* B. ovis] and Chapter 1.1.2 *Transport of specimens of animal origin*). All laboratory
72 manipulations with live cultures or potentially infected/contaminated material must be performed at an appropriate
73 biosafety and containment level determined by biorisk analysis (Chapter 1.1.3 *Biosafety and biosecurity in the*
74 *veterinary microbiology laboratory and animal facilities*).

75 # B. DIAGNOSTIC TECHNIQUES

76 ***Table 1.*** *Test methods available for the diagnosis of infection with* Brucella ovis

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement[a]	Contribute to eradication policies[b]	Confirmation of clinical cases[c]	Confirmation of suspect cases[d]	Herd/flock prevalence of infection – Surveillance
Agent identification[1]						
Staining methods	–	–	–	+	–	–
Culture	–	–	–	+++	+/++[d]	–
PCR[e]	–	–	–	+/++	+/++	–

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1   A combination of agent identification methods applied on the same clinical sample is recommended.

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement[a]	Contribute to eradication policies[b]	Confirmation of clinical cases[c]	Confirmation of suspect cases[d]	Herd/flock prevalence of infection – Surveillance
Detection of immune response[2]						
CFT	+++	+++	+++	++	++	++
I-ELISA	+++	+++	+++	++	++	+++
AGID	++	++	+++	++	++	++

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.
[a]This applies only to herds/flocks, countries or zones free from infection with *Brucella ovis*.
[b]To 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;
[c]In 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.
[d]In 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).
[e]False-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 electro-ejaculation. 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% CO_2. 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% sterile ovine or bovine serum, 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 (C_2H_6OS, 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

174   both mTM and Farrell's media for isolating all smooth *Brucella* species from field samples, and is
175   therefore the selective medium of choice for general *Brucella* isolation (De Miguel *et al.,* 2011).

176   All culture media used should be subjected to quality control with reference strains, to demonstrate that
177   it performs properly.

1.4. Identification and typing

179   *Brucella ovis* colonies are not haemolytic. They are circular, convex, have unbroken edges, are always
180   of the rough type when examined by oblique illumination, and test positive in the acriflavine test (Alton
181   *et al.,* 1988). For growth, *B. ovis* needs an incubating atmosphere containing 5–10% CO_2. It lacks
182   urease activity, fails to reduce nitrate to nitrite, and is catalase and oxidase negative. It does not
183   produce H_2S and, although it does not grow in the presence of methyl violet, it usually grows in the
184   presence of standard concentrations of basic fuchsin and thionin. The cultures are not lysed by
185   *Brucella*-phages of the Tbilisi (Tb), Weybridge (Wb) and Izatnagar (Iz_1) groups at the routine test
186   dilution (RTD) or 10^4 RTD, while they are lysed by phage R/C (Alton *et al.,* 1988). Most laboratories are
187   not equipped enough for a complete identification of *Brucella* at species and biovar levels, and a
188   practical schedule for presumptive identification is needed. Most *B. ovis* isolates can be correctly
189   identified on the basis of growth characteristics, direct observation using obliquely reflected light, Gram
190   or Stamp's staining, catalase, oxidase, urease and acriflavine tests. However, it is recommended that
191   the definitive identification be carried out by reference laboratories with experience in identification and
192   typing of *Brucella.*

193   The polymerase chain reaction (PCR) and other recently developed molecular methods provide
194   additional means of detection and identification of *Brucella* sp. (see Chapter 2.4.3), and are becoming
195   routine in many diagnostic laboratories. The existence of semen samples heavily contaminated with
196   overgrowing organisms or containing dead *B. ovis*, could also justify the use of PCR as a
197   supplementary direct diagnostic test. In fact, several PCR procedures have been reported to have
198   similar sensitivity to standard bacteriological culture when applied to semen samples from *B. ovis*
199   infected rams (Xavier *et al.,* 2010). However, the sensitivity and specificity of these PCR-based direct
200   diagnostic procedures remain to be properly determined on other clinical samples and, for the moment,
201   classical bacteriology should be considered the method of choice for the bacteriological diagnosis of
202   *B. ovis*. By contrast, the use of the Bruce-ladder multiplex PCR (see Chapter 2.4.3) on DNA samples
203   extracted from culture plate colonies is a rapid and highly specific procedure for the proper
204   identification of all *Brucella* species including *B. ovis*.

2.   Serological tests

206 The most efficient and widely used tests are the complement fixation test (CFT), the double agar gel
207 immunodiffusion (AGID) test and the indirect enzyme-linked immunosorbent assay (I-ELISA). Several countries
208 have adopted various standard diagnostic techniques for *B. ovis*, but the only test that has been prescribed up to
209 now by the OIE and the European Union (EU) for international trade was the CFT. However, it has been
210 demonstrated that the AGID test shows similar sensitivity to the CFT, and it is a simpler test to perform. Moreover,
211 although international standardisation is lacking, numerous independent validation studies have shown that the I-
212 ELISA is more sensitive than either the CFT or AGID test. AGID test and I-ELISA have been reported as more
213 sensitive than the CFT. Conversely I-ELISA was sometimes reported as a less specific method, but this greatly
214 depends on the protocol used (Estein *et al.,* 2002; Nielsen *et al.,* 2004; Praud *et al.,* 2012).

215 The International Standard anti-*Brucella ovis* Serum (ISaBoS, International Standard 1985[3]) is the one against
216 which all other standards are compared and calibrated. This reference standard is available to national reference
217 laboratories and should be used to establish secondary or national standards against which working standards
218 can be prepared and used in the diagnostic laboratory for daily routine use.

219 HS antigens for use in serological tests should be prepared from. *Brucella ovis* strain REO 198[4] is CO_2- and
220 serum-independent.

2.1. Antigens

222   When rough *Brucella* cells are heat-extracted with saline (hot-saline method, HS), they yield water-
223   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.

224 & Bosseray, 1973; Myers *et al.,* 1972). For this reason, the HS extract has been referred to as the
225 'rough-specific antigen' or, when obtained from *B. ovis*, as the *'B. ovis*-specific antigen'. However,
226 chemical characterisation of the HS extract from *B. ovis* has shown that it is enriched in rough
227 lipopolysaccharide (R-LPS), group 3 outer membrane proteins and other outer membrane components
228 (Riezu-Boj *et al.,* 1986). Thus, the HS extract contains LPS determinants specific for *B. ovis*, but also
229 additional antigenic epitopes, some of them being shared by rough and smooth *Brucella* (Santos *et al.,*
230 1984). Such epitopes account for the cross-reactivity that is sometimes observed with the HS method
231 and sera of sheep infected with *B. melitensis* or vaccinated with *B. melitensis* Rev.1 (Riezu-Boj *et al.,*
232 1986). The HS extract is the most widely and currently used for the serological diagnosis of *B. ovis*
233 infection. Its water solubility and high content in relevant cell surface epitopes explain its good
234 performance in *B. ovis* serological tests. However, in areas where *B. melitensis* infection also exists or
235 vaccination with *B. melitensis* Rev.1 is applied in sheep, the specificity of the diagnosis with regard to
236 *B. ovis* has to be carefully interpreted taking into account the results of serological tests for smooth
237 *Brucella* (Blasco, 2010).

238 Solid basal non-selective media described in Section B.1.3 are satisfactory for the growth of *B. ovis*
239 REO 198.

2.1.1. Preparation of HS antigen

241 i) Exponentially grow the REO 198 *B. ovis* strain in one of the following ways: for 48 hours in
242 trypticase–soy broth flasks in an orbital incubator at 37 C ± 2 C and 150 rpm; or in Roux
243 bottles of trypticase–soy agar, or other suitable medium; or in a batch-type fermenter as
244 described for *B. abortus.* Addition of 5% serum to the medium is optional as the REO 198
245 *B. ovis* strain is serum-independent.

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

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

249 iv) After cooling, the suspension is centrifuged (15,000 *g*, 5°C ± 3°C, 15 minutes) and the
250 supernatant fluid is filtered and dialysed against purified water using 100 times the volume
251 of the suspension, at 4°C; the water should be changed three times over a minimum of
252 2 days.

253 v) The dialysed fluid can be ultracentrifuged (100,000 *g*, 4°C, 6–8 hours), and the sediment is
254 resuspended in a small amount of purified water and freeze-dried. When produced to be
255 used in the CFT, the addition of control process serum replacement II (CPSRII) prior to
256 freeze-drying may assist in stability and anti-complementary activity.

257 HS is then resuspended either in purified water (for use in the AGID test), veronal buffered saline (for
258 use in the CFT), or carbonate/bicarbonate buffer (for use in the I-ELISA) and titrated accordingly.

259 If it is to be used in the AGID test, the resuspended HS may be kept at 5 C ± 3 C adding optionally
260 0.5% phenol as preservative. Freezing and thawing of antigen suspensions should be avoided (Diaz &
261 Bosseray, 1973).

262 The CFT antigen should be standardised against the ISaBoS to give 50% fixation at a 1/100 serum
263 dilution. It must be emphasised that each CFT antigen batch must be titrated with the CFT procedure
264 that is to be followed for the routine test. Therefore before using a CFT antigen (commercial or in-
265 house) in a particular CFT procedure, the laboratory should ensure that the antigen titre has been
266 established with the same CFT procedure.

267 In the absence of well-established standardisation rules, the I-ELISA and AGID antigens should be
268 titrated against a set of appropriate positive and negative sera.

2.1.2. Standardisation of the I-ELISA

270 The following criteria for standardisation of the I-ELISA have been used in a recent work in
271 which the I-ELISA has been validated in comparison with the CFT (Praud *et al.,* 2012):

272 i) A 1/64 pre-dilution of the ISaBoS made up in a negative serum (or in a negative pool of
273 sera) must give a positive reaction;

274 ii) A 1/256 pre-dilution of the ISaBoS made up in a negative serum (or in a negative pool of
275 sera) must give a negative reaction.

276 These criteria still need to be validated through an international ring-trial.

277   In any case, I-ELISA commercial or in-house kits must have been validated according to Chapter 1.1.5
278   *Principles and methods of validation of diagnostic assays for infectious diseases.*

2.2. Complement fixation test (the prescribed test for international trade)

280   There is no standardised method for the CFT, but the test is most conveniently carried out using the
281   microtitration method. Some evidence shows that cold fixation is more sensitive than warm fixation (Ris *et*
282   *al.*, 1984), but that it is less specific. Anticomplementary reactions, common with sheep serum, are,
283   however, more frequent with cold fixation.

284   Several methods have been proposed for the CFT using different concentrations of fresh sheep red blood
285   cells (SRBCs) (a 2–3% suspension is usually recommended) sensitised with an equal volume of rabbit anti-
286   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
289   determine the amount of complement required to produce either 50% or 100% lysis of sensitised SRBCs in
290   a unit volume of a standardised suspension; these are defined as the 50% or 100% haemolytic unit of
291   complement ($C'H_{50}$ or $C'H_{100}$), respectively. It is generally recommended to titrate the complement before
292   each set of tests, a macromethod being preferred for an optimal determination of $C'H_{50}$. Usually, 1.25–
293   2 $C'H_{100}$ or 5–6 $C'H_{50}$ are used in the test.

294   Barbital (veronal) buffered saline (VBS) is the standard diluent for the CFT. This is prepared from tablets
295   available commercially, otherwise it may be prepared according to the formula described elsewhere (see
296   Chapter 2.4.3). The test sera should be inactivated for 30 minutes in a water bath at 60–63°C, and then
297   diluted (doubling dilutions) in VBS. The stock solution of HS antigen (2.5–20 mg/ml in VBS) is diluted in VBS
298   as previously determined by titration (checkerboard titration). Usually, only one serum dilution is tested
299   (generally 1/10).

2.2.1. Test procedure

301   Using standard 96-well microtitre plates with round (U) bottom, the technique is usually
302   performed as follows:

303   i)   Volumes of 25 µl of diluted inactivated test serum are placed in the well of the first and
304   second rows. The first row is an anti–complementary control for each serum. Volumes of
305   25 µl of VBS are added to the wells of the first row (anti–complementary controls) to
306   compensate for lack of antigen. Volumes of 25 µl of VBS are added to all other wells
307   except those of the second row. Serial doubling dilutions are then made by transferring
308   25 µl volumes of serum from the third row onwards; 25 µl of the resulting mixture in the
309   last row are discarded

310   ii)   Volumes of 25 µl of antigen, diluted to working strength, are added to each well except
311   wells in the first row.

312   iii)   Volumes of 25 µl of complement, diluted to the number of units required, are added to
313   each well.

314   iv)   Control wells are set up to contain 75 µl total volume in each case; the wells contain

315   a)   diluent only,

316   b)   complement + diluent,

317   c)   antigen + complement + diluent,.

318   A control serum that gives a minimum positive reaction should be tested in each set of
319   tests to verify the sensitivity of test conditions.

320   v)   The plates are incubated at 37°C ± 2°C for 30 minutes or at 5°C ± 3°C overnight, and a
321   volume (25 or 50 µl according to the techniques) of sensitised SRBCs is added to each
322   well. The plates are reincubated at 37°C ± 2°C for 30 minutes.

323   vi)   The results are read after the plates have been centrifuged at 1000 *g* for 10 minutes at
324   5°C ± 3°C or left to stand at 5°C ± 3°C for 2–3 hours at least to allow unlysed cells to
325   settle. The degree of haemolysis is compared with standards corresponding to 0, 25, 50,
326   75 and 100% lysis. The titre of the serum under test is the highest dilution in which there is
327   50% or less haemolysis.

328

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_1–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_2O_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 $NaHCO_3$ in 10 ml purified water.

b) Solution B: 1.06 g Na_2CO_3 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: Na_2HPO_4: 10.96 g in 150 ml purified water

Solution B: NaH_2PO_4 (H_2O): 3.15 g in 150 ml purified water (3.5 g in 150 ml purified water if using NaH_2PO_4 2(H_2O)

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[6] 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% H_2O_2):

Citrate buffer (0.05 M, pH 4 ± 0.2):

a) Solution A: 22.97 g citric acid ($C_6H_8O_7.H_2O$) in 1000 ml purified water.

b) Solution B: 29.41 g sodium citrate ($Na_3C_6H_5O_7.2H_2O$) 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 H_2O_2.

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.

470 Comparative studies have shown that the I-ELISA has a better sensitivity than either the AGID
471 test or the CFT (Blasco, 2010; Gall *et al.,* 2003; Praud *et al.,* 2012; Ris *et al.,* 1984). However,
472 due to the existence of some I-ELISA-negative but AGID (or CFT) positive sera and vice versa,
473 the parallel combination of the AGID (or CFT) and I-ELISA results usually in optimal sensitivity
474 and may be helpful in eradication programmes in infected zones or flocks (Blasco, 2010; Praud
475 *et al.,* 2012).

476 Moreover, the CFT has other important disadvantages such as complexity, obligatory serum
477 inactivation, anti-complementary activity of some sera, the difficulty of performing it with
478 haemolysed sera, and prozone phenomena. Because of their sensitivity, simplicity and easy
479 interpretation, both the I-ELISA and AGID test are therefore preferred for surveillance in free or
480 almost-free zones.

481 Little is known about the existence of false positive results in *B. ovis* serological tests as a
482 consequence of infections due to bacteria showing cross-reacting epitopes with *B. ovis*. The
483 foot rot agent (*Dichelobacter nodosus*) has been described as responsible for serological cross-
484 reactions with *B. ovis*, but the extent and practical consequences of this cross-reactivity in
485 *B. ovis* diagnostic tests is not well understood. In addition, *Arcanobacterium pyogenes* and
486 *Corynebacterium ovis*, whose soluble extracts cross-react with sera from *B. ovis* infected rams,
487 have been isolated from several lymph nodes of rams giving strong positive responses in both
488 *B. ovis* AGID and I-ELISA tests (Blasco, 2010; Blasco & Moriyon, unpublished results).

489 ## C. REQUIREMENTS FOR VACCINES

490 As both rams and ewes can play a role in the transmission of infection (Blasco, 2010; Grilló *et al.,* 1999),
491 vaccination of both rams and ewes is probably the most economical and practical means for medium-term control
492 of *B. ovis* in areas with a high prevalence of infection. For long-term control, consideration should be given to the
493 effect of vaccination on serological testing, and *B. ovis*-free accreditation programmes have to be implemented.

494 There is no specific vaccine for *B. ovis* however live *B. melitensis* strain Rev.1 (described in Chapter 2.7.2) is
495 suitable to stimulate immunity against *B. ovis* infection (Blasco, 1990). A single standard dose A single standard
496 dose (10^9 colony-forming units) of Rev.1 administered subcutaneously (in a 1 ml volume) or, better, conjunctivally
497 (in a 25–30 µl volume), to 3–5 month-old animals confers adequate immunity against *B. ovis*. Conjunctival
498 vaccination has the advantage of minimising the intense and long-lasting serological response evoked by
499 subcutaneous vaccination, thereby improving the specificity of serological tests (Blasco, 1990), and facilitating the
500 interpretation of serological results after vaccination. When used in both young and adult males, the safety of the
501 Rev.1 vaccine is adequate enough and side-effects appear to be very rare (Marin *et al.,* 1990; Muñoz *et al.,*
502 2008). Therefore, in countries with extensive management and high levels of prevalence, it would be advisable to
503 vaccinate both young and healthy adult animals (see Chapter 2.7.2). In countries affected by *B. ovis* but free of
504 *B. melitensis,* before using the Rev.1 vaccine account should be taken of possible serological interferences and
505 the conjunctival route should be preferred to minimise this problem. The *B. abortus* RB51 vaccine has not been
506 proven successful against *B. ovis* in sheep (Jiménez De Bagües *et al.,* 1995), and despite the promising results
507 obtained with new generation subcellular vaccines (Cassataro *et al.,* 2007; Da Costa Martins *et al.,* 2010; Estein
508 *et al.,* 2009; Muñoz *et al.,* 2006), no alternative vaccines to Rev.1 exist currently.

509 ## REFERENCES

510 ALTON G.G., JONES L.M., ANGUS R.D. & VERGER J.M. (1988). Techniques for the Brucellosis Laboratory. INRA,
511 Paris, France.

512 BLASCO J.M. (1990). *Brucella ovis. In:* Animal Brucellosis, Nielsen K. & Duncan J.R., eds. CRC Press, Boca
513 Raton, Florida, USA, 351–378.

514 BLASCO J.M. (2010). *Brucella ovis* infection. *In:* Infectious and Parasitic Diseases of Livestock, Lefèvre PC,
515 Blancou J, Chermette R & Uilenberg G. eds. Lavoisier, Paris, France. Vol. 2:1047–1063.

516 BULGIN M.S. & ANDERSON B.C. (1983). Association of sexual experience with isolation of various bacteria in cases
517 of ovine epididymitis. *J. Am. Vet. Med. Assoc.*, **182**, 372–374.

518 CASSATARO J., PASQUEVICH K.A., ESTEIN S.M., LAPLAGNE D.A., ZWERDLING A., DE LA BARRERA S., BOWDEN R.,
519 FOSSATI C.A., GIAMBARTOLOMEI G.H. & GOLDBAUM F.A. (2007). A DNA vaccine coding for the chimera BLSOmp31

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.

DA COSTA MARTINS R., IRACHE J.M., BLASCO J.M., MUÑOZ M.P, MARÍN C.M., JESÚS GRILLO M., JESÚS DE MIGUEL M., BARBERÁN M. & GAMAZO C. (2010). Evaluation of particulate acellular vaccines against *Brucella ovis* infection in rams. *Vaccine*, **28**, 3038–3046.

DE MIGUEL M.J., MARÍN C.M., MUÑOZ P.M., DIESTE L., GRILLÓ M.J. & BLASCO J.M. (2011). Development of a selective culture medium for primary isolation of the main *Brucella* species. *J. Clin. Microbiol.*, **49**, 1458–1463.

DIAZ R. & BOSSERAY N. (1973). Identification d'un composé antigénique spécifique de la phase rugueuse (R) des *Brucella. Ann. Rech. Vet.,* **4**, 283–292.

ESTEIN S.M., BALDI P.C. & BOWDEN R.A. (2002). Comparison of serological tests based on outer membrane or internal antigens for detecting antibodies to *Brucella ovis* in infected flocks. *J. Vet. Diagn. Invest.*, **14**, 407-411.

GALL D., NIELSEN K., VIGLIOCCO A., SMITH P., PEREZ B., ROJAS X. & ROBLES C. (2003). Evaluation of an indirect enzyme-linked immunoassay for presumptive serodiagnosis of *Brucella ovis* in sheep. *Small Rumin. Res.*, **48**, 173–179.

GARCÍA-PASTOR L., BLASCO J.M. & BARBERÁN M. (2009). Pasteurellosis as a cause of genital lesions in rams. A descriptive study. *Small Rumin. Res.*, **87**, 111–115.

GRILLÓ M.J., MARÍN C., BARBERÁN M. & BLASCO J.M (1999). Experimental *Brucella ovis* infection in pregnant ewes. *Vet. Rec.*, **144**, 555–558.

JIMÉNEZ DE BAGÜES M.P., BARBERÁN M., MARÍN C.M. & BLASCO J.M. (1995). The *Brucella abortus* RB51 vaccine does not confer protection against *Brucella ovis* in rams. *Vaccine*, **13**, 301–304.

LIVINGSTONE C.W. & HARDY W.T. (1964). Isolation of *Actinobacillus seminis* from ovine epididymitis. *Am. J. Vet. Res.,* **25**, 660–663.

MARÍN C.M., ALABART J.L. & BLASCO J.M. (1996). Effect of antibiotics contained in two *Brucella* selective media on growth of *B. abortus, B. melitensis* and *B. ovis. J. Clin. Microbiol.*, **34**, 426–428.

MARÍN C.M., ALONSO-URMENETA B., MORIYÓN I., PEREZ S. & BLASCO J.M. (1998). Comparison of polyclonal, monoclonal and protein G peroxidase conjugates in an enzyme-linked immunosorbent assay for the diagnosis of *Brucella ovis* in sheep. *Vet. Rec.,* **143**, 390–394.

MARÍN C.M., BARBERÃN M., JIMÉNEZ DE BAGÜES M.P. & BLASCO J.M. (1990). Comparison of subcutaneous and conjunctival routes of Rev. 1 vaccination for the prophylaxis of *Brucella ovis* infection in rams. *Res. Vet. Sci.,* **48**, 209–215.

MUÑOZ P., DE MIGUEL M.J., GRILLÓ M.J., MARÍN C.M., BARBERÁN M. & BLASCO J.M. (2008). Immunopathological responses and kinetics of *B. melitensis* Rev 1 infection after subcutaneous or conjunctival vaccination in rams. *Vaccine*, **26**, 2562–2569.

MUÑOZ P.M., ESTEBAN M., MARÍN, C.M., DE MIGUEL M.J., GRILLÓ M.J., BARBERÁN M., IRACHE J.M., BLASCO J.M. & GAMAZO C. (2006). *Brucella* outer membrane complex-loaded microparticles as a vaccine against *Brucella ovis* in rams. *Vaccine*, **24**, 1897–1905.

MYERS D.M., JONES L.M. & VARELA-DIAZ V. (1972). Studies of antigens for complement fixation and gel diffusion tests in the diagnosis of infections caused by *Brucella ovis* and other *Brucella. Appl. Microbiol.,* **23**, 894–902.

NIELSEN K., SMITH P., CONDE S., DRAGHI de BENITEZ G., GALL D., HALBERT G., KENNY K., MASSENGILL C., MUENKS Q., ROJAS X, PEREZ B., SAMARTINO L., SILVA P., TOLLERSRUD T. & JOLLEY M. (2004). Rough lipopolysaccharide of *Brucella abortus* RB51 as a common antigen for serological detection of *B. ovis*, *B. canis*, and *B. abortus* RB51 exposure using indirect enzyme immunoassay and fluorescence polarization assay. *J. Immunoassay Immunochem.*, **25**, 171–182.

PRAUD A., CHAMPION J.L., CORDE Y., DRAPEAU A., MEYER L. & GARIN-BASTUJI B. (2012). Assessment of the diagnostic sensitivity and specificity of an indirect ELISA kit for the diagnosis of *Brucella ovis* infection in rams. *BMC Vet. Res.*, **8**:68.

566   RIDLER A.L., WEST D.M. & COLETT M.G. (2012). Pathology of *Brucella ovis* infection in red deer stags (*Cervus*
567   *elaphus*). *N. Z. Vet. J.*, **60**, 146–149.

568   RIEZU-BOJ J.I., MORIYÓN I., BLASCO J.M., MARÍN C.M. & DIAZ R. (1986). Comparison of lipopolysaccharide and outer
569   membrane protein-lipopolysaccharide extracts in an enzyme-linked immunosorbent assay for the diagnosis of
570   *Brucella ovis* infection. *J. Clin. Microbiol.,* **23**, 938–942.

571   RIS D.R., HAMEL K.L. & LONG D.L. (1984). Comparison of an enzyme-linked immunospecific assay (ELISA) with the
572   cold complement fixation test for the serodiagnosis of *Brucella ovis* infection. *N.Z. Vet. J.,* **32**, 18–20.

573   SANTOS J.M., VERSTREATE D.R., PERERA V.Y. & WINTER A.J. (1984). Outer membrane proteins from rough strains of
574   four *Brucella* species. *Infect. Immun.,* **46**, 188–194.

575   VIGLIOCCO A.M., SILVA PAULO P.S., MESTRE J., BRIONES G.C., DRAGHI G., TOSSI M. & NIELSEN K. (1997).
576   Development and validation of an indirect enzyme immunoassay for detection of ovine antibody to *Brucella ovis.*
577   *Vet. Microbiol.*, **54**, 357–368.

578   XAVIER M.N., SILVA T.M.A., COSTA E.A., PAIXAO T.A., MOUSTACAS V.S., CARVALHO C.A. Jr., SANT'ANNA F.M.,
579   ROBLES C.A., GOUVEIA A.M.G., LAGE A.P., TSOLIS R.M. & SANTOS R.L. (2010). Development and evaluation of a
580   species-specific PCR assay for the detection of *Brucella ovis* infection in rams. *Vet. Microbiol.*, **145**, 158–164.

581   *

582   *   *

583   **NB:** There are OIE Reference Laboratories for Ovine epididymitis (*Brucella ovis*)
584   (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date
585   list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
586   Please contact the OIE Reference Laboratories for any further information on
587   diagnostic tests, reagents and vaccines for ovine epididymitis (*Brucella ovis*)

588

589

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 *are differentiated 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 mosquitos). 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 PRRS*V *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 antisera* 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

48   *pregnant sows and gilts and in boars.* Vaccination <u>with modified live virus may result in shedding of</u>
49   <u>vaccinal virus in semen and vertical and horizontal transmission between sows and piglets and</u>
50   <u>between vaccinated and non vaccinated pigs. Subsequent vaccine-virus-induced adverse signs</u>
51   <u>have been reported.</u> *Modified live virus vaccines can persist in vaccinated* ~~animals~~ <u>herds.</u> ~~and~~
52   ~~transmission to nonvaccinated animals and subsequent vaccine-virus-induced disease have been~~
53   ~~reported.~~ <u>Whole virus inactivated vaccines are becoming available too but their efficacy is</u>
54   <u>questioned.</u>

A. INTRODUCTION

56   Porcine reproductive and respiratory syndrome (PRRS) is characterised by reproductive failure of sows and
57   respiratory disease in pigs (~~Benfield *et al.,* 1999~~ <u>Zimmerman *et al.,* 2012</u>). ~~The disease~~ <u>PRRS</u> was first
58   recognised in 1987 in the United States of America, and within a few years it became a pandemic. ~~PRRS~~ <u>The</u>
59   <u>disease</u> is caused by the PRRS virus (PRRSV). It was discovered in 1991 in The Netherlands (<u>Zimmerman *et al.,*</u>
60   <u>2012)</u> (~~Wensvoort *et al.,* 1993~~) and is classified as a member of the order *Nidovirales*, family *Arteriviridae*, genus
61   *Arterivirus* (<u>Faaberg *et al.,* 2012</u>). PRRSV is a single-stranded positive-sense RNA virus and the biology of the
62   virus has been well characterised. <u>Apart from domestic pigs, feral swine and wild boars, no other species are</u>
63   <u>known to be naturally infected with PRRS virus (PRRSV). The virus does not pose a zoonotic risk and it is not</u>
64   <u>infectious for human or for cells of human origin.</u> Soon after the discovery of the virus it became apparent that ~~the~~
65   ~~North American (NA,~~ Type 2 and ~~European (EU,~~ Type 1 PRRSV isolates represent~~ed~~ two genotypes with
66   antigenic differences (~~Larochelle & Magar, 1997c; Magar *et al.,* 1997~~ <u>Zimmerman *et al.,* 2012</u>). Additional
67   investigations have demonstrated regional differences within each continent. These differences are now
68   becoming blurred as Type 2 PRRSV has been introduced into Europe (in part through the use of a modified live
69   vaccine based on a North American isolate) and Type 1 virus has been discovered in North America. Most
70   PRRSV isolates from South America and much of Asia are of Type 2 and it is assumed these viruses were
71   introduced through the movement of swine and/or semen. <u>Most</u> ~~A reportedly~~ highly virulent ~~strain of~~ Type 2
72   viruses in South-East Asia (<u>highly pathogenic PRRSV</u>) are characterised by ~~a discontinuous 30~~ amino acid
73   deletions in the NSP2 region of the genome. <u>However, there is good experimental evidence that these deletions</u>
74   <u>do not determine virulence (Shi *et al.,* 2010a; Zhou *et al.,* 2010).</u>

75   There is an increasing diversity among strains of the two genotypes, which has been attributed to the high error
76   rate inherent in PRRSV replication (~~Chang *et al.,* 2002~~) and recombinations between strains (<u>Murtaugh *et al.,*</u>
77   <u>2010</u> ~~Van Vugt *et al.,* 2001~~). There have also been ~~recent~~ descriptions of east European strains of Type 1 PRRSV
78   with a high degree of polymorphism, providing further insights into the emergence of the relatively new pathogen
79   of pigs. <u>It was proposed to distinguish subtypes 1, 2 and 3 within Type 1. Moreover, mounting evidence indicates</u>
80   <u>that an additional subtype (subtype 4) might exist</u> (Stadejek *et al.,* ~~2006;~~ 2008; 2013). The effects of such diversity
81   on diagnostics and vaccines are largely unknown, but do raise concerns and should be considered. <u>Subtype 3</u>
82   <u>Lena and subtype 2 Bor strains have been shown to have higher virulence than subtype 1 strains (Karniychuk *et*</u>
83   <u>*al.,* 2010; Stadejek *et al.,* unpublished observations). Trus *et al.* (2014) showed that subtype 1 modified live</u>
84   <u>vaccine partially protects against challenge with subtype 3 Lena strain. The overall level of diversity within Type 2</u>
85   <u>does not exceed the one observed for subtype 1, although nine different genetic lineages were identified (Shi *et*</u>
86   <u>*al.,* 2010b; Stadejek *et al.,* 2013).</u>

87   The reproductive syndrome is recognised by late-gestation abortions and early or delayed farrowings that contain
88   dead and mummified fetuses, stillborn pigs, and weak-born pigs. An increase in repeat breeders during the acute
89   phase of the epizootic is commonly reported. Infrequently, there are reports of early- to mid-gestation reproductive
90   failure. <u>Most probably the cause of PRRSV-related reproductive disorders are virus-induced damage to the</u>
91   <u>placenta and endometrium (Karniychuk & Nauwynck, 2013).</u> In boars and unbred replacement gilts and sows,
92   transient fever and anorexia may be observed. The respiratory syndrome is recognised by dyspnoea (thumping),
93   fever, anorexia, and listlessness. Younger pigs are more affected than older animals with boars and sows
94   (unbred) frequently having subclinical infection. An increase in secondary infections is common and mortality can
95   be high. In PRRSV-infected boars and boars that have been vaccinated with live attenuated vaccine, PRRSV can
96   be shed in semen, and changes in sperm morphology and function have been described (Christopher-Hennings
97   *et al.,* 1997). The virus is primarily transmitted directly via <u>contact with</u> infected pigs but also with faeces, urine
98   and semen. It can also be spread indirectly, presumably via aerosol routes, leading to chronic re-infections of
99   herds in swine dense areas, and possibly by mechanical vectors. Gross and microscopic lesions consistent with
100   PRRSV infection have been well described (~~Halbur *et al.,* 1995~~ <u>Zimmerman *et al.,* 2012</u>). In general, the lesions
101   are more severe in younger animals than older ones. Differences in virulence between PRRSV isolates within a
102   genotype and between genotypes ~~are believed~~ <u>were proved</u> to exist based on field observations
103   and ~~some~~ experimental studies (~~Halbur *et al.,* 1995~~ <u>Karniychuk *et al.,* 2010; Weesendorp *et al.,* 2013</u>). This
104   variability has been reinforced with the emergence in 2006 of a PRRSV lineage in South-East Asia associated
105   with porcine high fever disease, a syndrome causing high mortality in all ages of swine (~~Tian *et al.,* 2007~~ <u>Xiao *et*</u>
106   <u>*al.,* 2014</u>). Although there is now an extensive body of research completed since the discovery of PRRSV, there

107 are still many gaps in the knowledge base about the apparent link between PRRSV and other diseases as well as
108 understanding the PRRSV immune response.

109 # B. DIAGNOSTIC TECHNIQUES

110 ***Table 1.*** *Test methods available for diagnosis of porcine reproductive and respiratory syndrome and their purpose*

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification[1]						
Virus isolation	=	++	=	+++	=	=
RT-PCR	+++	+++	+++	+++	++	=
IHC	=	=	=	++	=	=
ISH	=	=	=	++	=	=
Detection of immune response[2]						
ELISA	+++	++	+++	++	+++	++
IPMA	++	++	++	±	++	+++
IFA	++	++	++	±	++	+++

111 Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability,
112 or other factors severely limits its application; – = not appropriate for this purpose.
113 Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature
114 and the fact that they have been used widely without dubious results, makes them acceptable.
115 RT-PCR = reverse-transcription polymerase chain reaction; IHC = immunohistochemistry method,
116 ISH = *in-situ* hybridisation, ELISA = enzyme-linked immunosorbent assay; IPMA = immunoperoxidase monolayer assay,
117 IFA = immunofluorescence assay.

118 ## 1. Identification of the agent

119 Identification of PRRSV can be accomplished by virus isolation, the detection of nucleic acids, and the detection
120 of viral proteins. Following infection, swine develop a viraemia and lung infection that can persist for weeks in
121 young pigs and days in adult animals making serum and bronchoalveolar lung lavage ideal samples to collect for
122 detection of PRRSV. Isolation of PRRSV can be difficult as not all virus isolates (especially Type 1 viruses) can
123 easily infect MARC-145 cells and CL-2621, a cell line clones derived from the MA-104 monkey kidney cell line
124 (Kim *et al.,* 1993 Provost *et al.,* 2012; Zimmerman *et al.,* 2012). Interestingly, this continuous cell culture system
125 has been the only one reported to sustain a PRRSV infection. Recent findings show that porcine monocyte-
126 derived macrophages can also be used for PRRSV isolation and propagation in cell culture (García-Nicolás *et al.,*
127 2014). These can be differentiated *in vitro* from porcine peripheral blood mononucleated cells (PBMCs) without
128 slaughtering animals, as opposed to collection of the lung for porcine alveolar macrophage (PAM) preparations.
129 Moreover, several genetically modified cell lines supporting PRRSV replication have been developed including
130 immortalised PAM cell line expressing CD163, immortalised porcine monomyeloid cells, PK-15 expressing CD163
131 or CD163 and sialoadhesin as well as porcine, feline and baby hamster kidney cells expressing CD163 (Delrue *et*
132 *al.,* 2010; Provost *et al.,* 2012). Other, non-recombined cell lines permissive for PRRSV infection have also been
133 described (Feng *et al.,* 2013; Provost *et al.,* 2012). PAM will support replication of most, if not all PRRSV isolates.
134 However, the collection of PAM is not an easy task as only pigs of high health status and less than 8 weeks of
135 age should be used as the PAM source (Wensvoort *et al.,* 1993 Feng *et al.,* 2013). Different batches of PAM are
136 not always equally susceptible to PRRSV; it is thus necessary to test each batch before use. PAM can be stored
137 in liquid nitrogen until needed as described below. Isolation of PRRSV using PAM is a technique that can be

---

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

138 performed in most diagnostic laboratories. This technique should be sensitive for isolation of all PRRSV strains
139 and will be explained in detail. <u>Samples for virus isolation should be stored at 4°C for not more than 48 hours</u>
140 <u>because of the sensitivity of the virus to pH and temperature, otherwise freezing at –70°C is recommended.</u>

141 <u>One of the most commonly used diagnostic techniques is</u> detection of PRRSV nucleic acid ~~can be~~
142 ~~accomplished~~ with reverse-transcription polymerase chain reaction (RT-PCR), nested set RT-PCR, and real-time
143 RT-PCR (~~Drew, 1995b;~~ Kleiboeker *et al.,* 2005; ~~Larochelle & Magar, 1997a; Mardassi *et al.,* 1994; Wasilk *et al.,*~~
144 ~~2004~~ <u>Wernike *et al.,* 2012a; 2012b). The advantages of RT-PCR are high specificity and sensitivity as well as</u>
145 <u>rapid evaluation of a current infection status. However, inactivated virus cannot be differentiated from infectious</u>
146 <u>virus using this technique. RT-PCR-based</u> ~~These~~ tests are commonly used to detect nucleic acid in tissues and
147 serum. <u>It has been suggested that oral fluids testing also give reliable results for pen-based diagnosis</u>
148 <u>(Kittawornrat *et al.,* 2010). The above-mentioned assays</u> ~~They~~ are also useful when virus isolation is problematic,
149 such as when testing semen (Christopher-Hennings *et al.,* 1997) and when testing tissues partially degraded by
150 autolysis or by heat during transport of specimens for virus isolation. <u>Most of the in-house protocols and currently</u>
151 <u>available commercial kits provide the possibility</u> ~~A multiplex PCR assay has been designed~~ of differentiating
152 isolates of Types 1 and 2 (<u>Kleiboeker *et al.,* 2005;</u> ~~Gilbert *et al.,* 1997;~~ <u>Wernike *et al.,* 2012a; 2012b). False-</u>
153 <u>negative results related to high genetic diversity, and primer and probe mismatches are the major concern when</u>
154 <u>using RT-PCR. Currently, no single RT-PCR assay is capable of detecting all PRRSV strains, especially within</u>
155 <u>highly diverse east European subtypes of Type 1. The technique is also prone to contamination. Therefore, for</u>
156 <u>interpretation, RT-PCR results should be carefully evaluated and continuous validation based on recently</u>
157 <u>circulating PRRSV strains is strongly recommended (Wernike *et al.,* 2012a). Reverse-transcription – loop-</u>
158 <u>mediated isothermal amplification (RT-LAMP) is an alternative technique not requiring advanced equipment unlike</u>
159 <u>the real-time RT-PCR (Zimmerman *et al.,* 2012). All of these nucleic acid tests are more rapid than virus isolation</u>
160 <u>and do not require cell culture infrastructure.</u>

161 Restriction fragment length polymorphism analysis of PCR-amplified products was developed <u>and used</u> for the
162 differentiation of field and vaccine PRRSV isolates (<u>Zimmerman *et al.,* 2012</u> ~~Wesley *et al.,* 1998~~), and ~~recently~~
163 molecular epidemiological studies of PRRSV strains were performed using phylogenetic analyses of specific
164 structural gene sequences. <u>However, high rates of recombination events observed in the field may influence the</u>
165 <u>results of phylogenetic analysis based on short genome fragments.</u> Although seldom used for diagnostic
166 purposes, *in situ* hybridisation is capable of detecting and differentiating Type 1 and 2 PRRSV ~~genotypes~~ in
167 formalin-fixed tissues ~~(Larochelle & Magar, 1997c)~~. The sensitivity and specificity of these methods for detection
168 of PRRSV genome can be compromised by the very high genetic diversity of PRRSV, especially <u>with</u>in Type 1.
169 Immunohistochemistry can be used to identify viral proteins (~~Halbur *et al.,* 1994; Larochelle & Magar, 1995~~) and
170 when performed on formalin-fixed tissues enables the visualisation of antigen together with histological lesions
171 (<u>Zimmerman *et al.,* 2012</u>).

1.1. Harvesting of alveolar macrophages from lungs

173 Lungs should preferably be obtained from SPF pigs or from a herd of pigs that is proven to be free from
174 PRRSV infection. Best results are obtained with pigs that are under 8 weeks of age. The macrophages
175 should be harvested from the lung on the same day that the pig is slaughtered. The lungs should be
176 washed three or four times with a total volume of approximately 200 ml sterile phosphate buffered
177 saline (PBS). The harvested wash fluid is then centrifuged for 10 minutes at 1000 *g*. The resulting
178 pellet of macrophages is resuspended in PBS and centrifuged (washed) twice more. The final pellet is
179 resuspended in 50 ml PBS, and the number of macrophages is counted to determine the cell
180 concentration. The macrophages can then be used fresh, or can be stored in liquid nitrogen according
181 to standard procedures at a final concentration of approximately 6×10^7 macrophages/1.5 ml.
182 Macrophage batches should not be mixed.

1.2. Batch testing of alveolar macrophages

184 Before a batch of macrophages can be used it should be validated. This should be done by titrating a
185 standard PRRSV with known titre in the new macrophages, and by performing an immunoperoxidase
186 monolayer assay (IPMA) with known positive and negative sera on plates seeded with the new
187 macrophages. The cells are suitable for use only if the standard PRRSV grows to its specified titre,
188 ($TCID_{50}$ or 50% tissue culture infective dose). It is recommended that alveolar macrophages and fetal
189 bovine serum (FBS) to supplement culture medium be pestivirus free.

1.3. Virus isolation on alveolar macrophages

191 Alveolar macrophages are seeded in the wells of flat-bottomed tissue-culture grade microtitre plates.
192 After attachment, the macrophages are infected with the sample. Samples can be sera ~~or ascitic fluids,~~
193 or 10% suspensions of tissues, such as tonsils, lung, lymph nodes, and spleen. In general, the PRRSV
194 gives a cytopathic effect (CPE) in macrophages after 1–2 days of culture, but sometimes viruses are

195 found that give little CPE or give a CPE only after repeat passage. After a period of 1–2 days or once
196 CPE has been observed, the presence of PRRSV needs to be confirmed by immunostaining with a
197 specific antiserum or monoclonal antibody (MAb).

1.3.1. Seeding macrophages in the microtitre plates

199 Defrost one vial containing 6×10^7 macrophages/1.5 ml. Wash the cells once with 50 ml PBS
200 and centrifuge the cell suspension for 10 minutes at 300 *g* (room temperature). Collect the cells
201 in 40 ml RPMI (Rose-Peake Memorial Institute) 1640 medium supplemented with 5% FBS and
202 10% antibiotic mixture (growth medium). Dispense 100 µl of the cell suspension into each well
203 of a microtitre plate (with one vial of cells, four plates can be seeded at a concentration of
204 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

207 Dispense 90 µl of growth medium into each well of a microtitre plate. Add 10 µl samples to the
208 wells of rows A and E (duplicate 1/10 dilution). Shake the plates and transfer 10 µl from rows A
209 and E to rows B and F (1/100 dilution). Shake the plates and transfer 10 µl from rows B and F to
210 rows C and G (1/1000 dilution). Shake the plates and transfer 10 µl from rows C and G to rows
211 D and H (1/10,000 dilution). Shake the plates.

1.3.3. Incubation of samples

213 Transfer 50 µl of the sample dilutions from the dilution plates to the corresponding wells of the
214 plate with macrophages (first passage). Incubate for 2–5 days and observe daily for a CPE. At
215 day 2, seed macrophages in new microtitre plates (see above). Transfer 25 µl of the
216 supernatants from the plates of the first passage to the corresponding wells of the freshly
217 seeded plates (second passage). Incubate for 2–5 days and observe daily for a CPE.

1.3.4. Reading and interpreting the results

219 Wells in which macrophages show CPE in the first passage only are considered to be false
220 positive because of the toxicity of the sample. Wells in which macrophages show CPE in both
221 passages or in the second passage only are considered to be suspect positive. All wells with
222 macrophage monolayers that do not show CPE need to be identified as PRRSV negative by
223 immunostaining with a PRRSV-positive antiserum or MAb. CPE-positive samples need to be
224 identified as PRRSV positive by culturing CPE-positive supernatant samples, or the original
225 sample dilutions, for both 24 and 48 hours in macrophages, followed by immunostaining with a
226 PRRSV-positive antiserum or MAb.

1.3.5. Immunostaining with a PRRSV-positive antiserum or MAb

228 Infect macrophages with 50 µl of supernatant or tissue sample as described in Section B.2.1,
229 and grow the infected cells for 24 and 48 hours. Prepare an appropriate dilution of a PRRSV-
230 positive serum in dilution buffer, and immunostain the macrophages as described in Section
231 B.2.1 or B.2.2.

2. Serological tests

233 A variety of assays for the detection of serum antibodies to PRRSV has been described. Serological diagnosis is,
234 in general, easy to perform, with good specificity and sensitivity, especially on a herd basis. Sera of individual pigs
235 sometimes cause difficulties because of nonspecific reactions, but this problem may be solved by resampling the
236 pig after 2–3 weeks. Serology is generally performed with a binding assay, such as the <u>immunoperoxidase</u>
237 <u>monolayer assay</u> (IPMA), immunofluorescence assay (IFA), or the enzyme-linked immunosorbent assay (ELISA)
238 – of which many varieties are described (~~Albina *et al.* 1992; Cho *et al.,* 1997; Denac *et al.,* 1997; Houben *et al.,*~~
239 ~~1995;~~ Diaz *et al.,* 2012; Jusa *et al.,* 1996; Sorensen *et al.,* 1998; Venteo *et al.,* 2012; ~~Nodelijk *et al.,* 1996;~~
240 ~~Wensvoort *et al.,* 1993;~~ Yoon *et al.,* 1992). These tests are often performed with viral antigen of one antigenic
241 type, which means that antibodies directed against the other, heterologous antigenic type may be detected with
242 less sensitivity. A blocking ELISA has been used extensively in Denmark and has been described as a double
243 ELISA set-up using both Types 1 and 2 virus as antigen and thus it can distinguish between serological reaction
244 to both types (Sorensen *et al.,* 1998). ~~The first live attenuated vaccine for PRRS based on genotype 2 virus has~~
245 ~~been observed to spread to nonvaccinated animals (Botner *et al.,* 1997; Torrison *et al.,* 1996), and subsequent~~
246 ~~development in herds of vaccine-virus-induced PRRS reproductive failures has been reported in Denmark (Botner~~
247 ~~*et al.,* 1997; Madsen *et al.,* 1998).~~ This is of high importance as Type 2 strains circulate in Europe following Type
248 2 modified live vaccine use and independent introduction (~~Botner, Madsen, Balka~~ Stadejek *et al.,* 2013). ~~Reaction~~
249 ~~to genotype 2 vaccine-like PRRSV can be anticipated in countries using or having used this vaccine; European~~

250 countries may therefore observe reactions and isolation of both antigenic types (Botner *et al.,* 1997; Madsen *et*
251 *al., 1998).* The identification of Type 1 strains of PRRSV in the USA and Canada has also been reported (Fang *et*
252 *al., 2004*Kleiboeker *et al.,* 2005), but the prevalence of infections by such strains is not well documented. As both
253 types of PRRSV are globally spread, serological tests should contain antigens from both types. Commercial
254 ELISAs with good sensitivity and specificity are available and have been compared (Diaz *et al.,* 2012, Venteo *et*
255 *al.,* 2012).

256 Antibodies to the virus can be detected by antibody-binding assays as early as 7–14 days after infection, and
257 antibody levels reach maximal titres by 30–50 days. Some pigs may become seronegative within 3–6 months, but
258 others remain seropositive for much longer. Antibodies to PRRSV have also been detected in meat juice muscle
259 transudate and oral fluid. Neutralising antibodies develop slowly and do not reach high titres. They can be
260 detected appear from 3 to 4 weeks after infection and they can persist for 1 year or more or remain undetected.
261 The use of complement to increase the sensitivity of the serum virus neutralisation test has been reported (Dea *et*
262 *al., 1996* Jusa *et al., 1996*). Extensive research into the duration of antibody titres after infection has not yet been
263 done, and the results probably depend on the test used. Maternal antibodies have a half-life of 12–14 days, and
264 maternal antibody titre can, in general, be detected until 4–8 weeks after birth, depending on the antibody titre of
265 the sow at birth and the test used. In an infected environment, pigs born from seropositive females can
266 seroconvert actively from the age of 3–6 weeks.

267 This chapter describes the IPMA in detail as this test can easily be performed in laboratories where virus isolation
268 procedures using macrophages have been established, and can be used with virus of both antigenic types. This
269 assay can also be adapted to the MARC-145 cell line for both the European and North American types (Jusa *et*
270 *al.,* 1996). An indirect immunofluorescence assay (IFA) using MARC-145 cells can also be performed for PRRSV
271 serology and is included in the present chapter. Commercial ELISAs with good sensitivity and specificity are
272 available and have been compared (Drew, 1995a).

2.1. Detection of antibodies with the immunoperoxidase monolayer assay

273

274 Alveolar macrophages are seeded in the wells of microtitre plates. After attachment, the macrophages
275 are infected with PRRSV. The objective is to infect approximately 30–50% of the macrophages in a
276 well so as to be able to distinguish nonspecific sera. After an incubation period, the macrophages are
277 fixed and used as a cell substrate for serology. An alternative method is to use MARC 145 cells instead
278 of macrophage cells. On each plate, 11 sera can be tested in duplicate. Test sera are diluted and
279 incubated on the cell substrate. If antibodies are present in the test serum, they will bind to the antigen
280 in the cytoplasm of the macrophages. In the next incubation step, the bound antibodies will be detected
281 by an anti-species horseradish-peroxidase (HRPO) conjugate. Finally, the cell substrate is incubated
282 with a chromogen/ substrate[3] solution. Reading of the test is done with an inverted microscope.

2.1.1. Seeding macrophages in the microtitre plates

284   i)   Defrost one vial containing 6×10^7 macrophages/1.5 ml.

285   ii)   Wash the cells once with 50 ml of PBS and centrifuge the cell suspension for 10 minutes
286   at 300 *g* (room temperature).

287   iii)   Collect the cells in 40 ml RPMI 1640 medium supplemented with 5% FBS, 100 IU
288   (International Units) penicillin and 100 µg streptomycin (growth medium).

289   iv)   Dispense 100 µl of the cell suspension into each well of a microtitre plate (with one vial of
290   cells, four plates can be seeded at a concentration of 10^5 cells in each well of the plates).

291   v)   Incubate the plates for 18–24 hours at 37°C in a 5% CO_2 incubator, under humid
292   conditions. Alternatively, use HEPES buffer (N-2-hydroxyethylpiperazine, N-2-ethane-
293   sulphonic acid) in the medium.

294

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3 *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% H_2O_2 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% CO_2 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 immuofluorescence 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

395 396		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.
397	iii)	Discard the acetone and dry the plates and slides at room temperature.
398 399	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

401 402 403	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).
404	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

406 407 408 409	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.
410 411 412	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.
413 414	iii)	Add volumes of 50 µl of the negative control serum and positive control serum dilutions in the same manner.
415 416	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.
417 418 419 420	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

422 423 424	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.
425	ii)	Incubate plates or slides with their lids on at 37°C for 30 minutes in a humid atmosphere.
426 427 428 429	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.
430	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
444	

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% CO_2 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% CO_2 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 1/20, 1/80, 1/320, 1/1280) in wells containing viral antigen of columns 2, 3, 4, 5 or 7, 8, 9, 10. For each serum, also dispense 50 µl of dilution 1/16 or 1/20 in control wells of columns 1 and 6. Similarly dispense dilutions of positive and negative control sera in wells of columns 11 and 12.

v) Incubate the plates at 37°C for 30 minutes in a humid chamber. Discard the sera and rinse the plates three times using PBS.

vi) Add 50 µl of appropriately diluted anti-swine IgG conjugated with FITC and incubate plates at 37°C for 30 minutes in a humid chamber. Discard conjugate, rinse plates several times and tap the plates on absorbent material to remove excessive liquid.

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 (Kittawornrat *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).

496 **C. REQUIREMENTS FOR VACCINES**

497 **1. Background**

498 **1.1. Rationale and intended use of the product**

499 <u>Several</u> modified-live (MLV) <u>and inactivated (killed)</u> PRRSV vaccines are licensed and commercially
500 available in many countries for the control of the reproductive and/or respiratory forms of PRRS ~~In the~~
501 ~~USA and Europe, a killed virus vaccine is licensed as an aid in the reduction of abortions and weak~~
502 ~~piglets caused by the reproductive form of PRRS. All PRRS vaccines currently licensed in the USA~~
503 ~~contain the antigenic type 2. In Europe, three MLV vaccines are licensed and available~~
504 ~~commercially~~ (Murtaugh *et al*,. 2011)~~: one is based on antigenic type 2 and two others are type 1.~~ It is
505 assumed the most benefit from vaccination occurs when the vaccine virus is ~~more~~ closely related
506 antigenically to the field virus (~~Larochelle & Magar, 1997b;~~ Scortti *et al.,* 2006). Although vaccination of
507 pigs does not prevent PRRSV infection, it may be helpful in herds experiencing problems with PRRS or
508 herds at high risk of PRRSV infection. <u>The killed vaccines are licensed to be used as an aid in the</u>
509 <u>reduction of abortions and weak piglets caused by the reproductive form of PRRS.</u> MLV vaccines are
510 intended to be used in sows and gilts 3–6 weeks prior to breeding and in piglets from 3 weeks of age or
511 older as an aid in the reduction of diseases caused by PRRS. <u>MLV vaccines are not intended to be</u>
512 <u>used in naive herds, pregnant sows or gilts or boars of breeding age.</u> Vaccine virus can persist in boars
513 and be disseminated through semen (Christopher-Hennings *et al.,* 1997). MLV vaccine virus may be
514 shed and transmitted to non-vaccinated contact pigs (~~Torrison *et al.,* 1996~~ Zimmerman *et al.,* 2012).
515 <u>Vaccines based on biotechnology are under development but not available yet on the market.</u>
516 Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary*
517 *vaccine production.* The guidelines given here and in chapter 1.1.6 are intended to be general in nature
518 and may be supplemented by national and regional requirements.

519 **2. Outline of production and minimum requirements for conventional vaccines**

520 **2.1. Characteristics of the seed**

521 **2.1.1. Biological characteristics**

522 The isolate of PRRSV used for vaccine production must be accompanied by a history
523 describing its origin and passage history. The master seed virus (MSV) must be safe in swine at
524 the intended age of vaccination and provide protection against challenge. Isolates for a MLV
525 vaccine must be shown not to revert to virulence after passage in host animals.

526 **2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)**

527 The MSV should be free from bacteria, fungi and mycoplasma. The MSV must be tested for and
528 free from extraneous viruses, including transmissible gastroenteritis virus, <u>porcine respiratory</u>
529 <u>coronavirus, porcine epidemic diarrhoea virus</u>, porcine adenovirus, porcine circovirus type 1 and
530 2, porcine haemagglutinating encephalitis virus, porcine parvovirus, bovine viral diarrhoea virus,
531 reovirus, and rabies virus by the fluorescent antibody technique. The MSV must be free from
532 extraneous virus by CPE and haemadsorption on the Vero cell line and an embryonic swine cell
533 type.

534 **2.2. Method of manufacture**

535 **2.2.1. Procedure**

536 The PRRSV is propagated in a continuous <u>cell line</u> ~~African green monkey kidney cell line,~~ such
537 as <u>MARC-145 (clone of</u> MA-104) or Vero <u>cells</u>. Viral propagation should not exceed five
538 passages from the master seed virus (MSV) unless further passages prove to provide
539 protection in swine.

540 The ~~African green monkey kidney~~ cell line is seeded into suitable vessels. MEM supplemented
541 with FBS is used as the medium for production. Cell cultures are inoculated directly with PRRS
542 working virus stock, which is generally from 1 to 4 passages from the MSV. Inoculated cultures
543 are incubated for 1–8 days before harvesting the culture medium. During incubation, the
544 cultures are observed daily for CPE and bacterial contamination.

545 Killed virus vaccines are chemically inactivated with either formalin or binary ethylenimine and
546 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 <u>for MLV and for inactivated (killed) virus vaccines</u> 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

594   Field trial studies should be conducted to determine the safety of the vaccine. Non-
595   vaccinated sentinel pigs should be included at each site for monitoring the shed of the
596   attenuated virus.

597   ii)   Reversion-to-virulence for attenuated/live vaccines

598   MSV must be shown not to revert to virulence after several passages in host animals,
599   although the definition of virulence with such a virus is difficult. Attenuated PRRSV isolates
600   are known to cause viraemia and will transmit to susceptible animals. The MSV should be
601   shown to be avirulent in weaned piglets and pregnant animals by five serial passages (up
602   to ten passages depending on country) of the MSV through susceptible swine using the
603   most natural route of infection.

604   iii)   Environmental consideration

605   Not applicable

606   **2.3.2. Efficacy requirements**

607   i)   For animal production

608   In an immunogenicity trial, the MSV at the highest passage level intended for production
609   must protect susceptible swine against a virulent, unrelated challenge strain. For the
610   respiratory form, 3-week-old piglets are vaccinated with the highest passage level of MSV.
611   The piglets are challenged with a virulent isolate of PRRSV 2–16 weeks later to determine
612   protection from respiratory clinical signs of PRRS. To determine protection from the losses
613   caused by the reproductive form of PRRS, vaccinated animals are challenged at
614   approximately 85 days' gestation. A prevented fraction, the proportion of potential PRRS
615   disease occurrence reduced due to vaccination, is calculated to determine if there is
616   acceptable protection, based on the proposed label claims, in the vaccinates from the
617   clinical signs of reproductive disease, including fetal mummification, stillborn piglets and/or
618   weak piglets, when compared with the controls.

619   Duration of immunity studies are conducted before the vaccine receives final approval. For
620   the respiratory form of PRRS, duration should be shown up to the market age in pigs.
621   Duration of immunity for the reproductive form should be shown through weaning of the
622   piglets.

623   ii)   For control and eradication

624   Not applicable

625   **2.3.3. Stability**

626   All vaccines are initially given a shelf life of 24 months before expiry. Real-time stability studies
627   are then conducted to confirm the appropriateness of the expiry date.

628   Multiple batches of MLV vaccines should be re-titrated periodically throughout the shelf-life to
629   determine vaccine variability. The release value should be adjusted if the titres are insufficient
630   or highly variable.

631   Killed vaccines using *in-vivo* potency tests should be retested at expiry to demonstrate stability.
632   Parallel-line assays using ELISA antigen-quantifying techniques should demonstrate the
633   stability of the standard.

634   ## 3.   Vaccines based on biotechnology

635   ### 3.1. Vaccines available and their advantages

636   ~~None~~ Under development but not available yet on the market.

637   ### 3.2. Special requirements for biotechnological vaccines, if any

638   ~~None~~ Not applicable yet.

REFERENCES

ALBINA E., LEFORBAN Y., BARON T., PLANA DURAN J. & VANNIER P. (1992). An enzyme linked immunosorbent assay (ELISA) for the detection of antibodies to the porcine reproductive and respiratory syndrome (PRRS) virus. *Ann. Rech. Vet.,* **23**, 167–176.

BOTNER A., STRANDBYGAARD B., SORENSEN K.J., HAVE P., MADSEN K.G., MADSEN E.S. & ALEXANDERSEN S. (1997). Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. *Vet. Rec.,* **141**, 497–499.

BROWN E., LAWSON S., WELBON C., GNANANDARAJAH J., LI J., MURTAUGH M.P., NELSON E.A., MOLINA R.M., ZIMMERMAN J.J., ROWLAND R.R. & FANG Y. (2009). Antibody response to porcine reproductive and respiratory syndrome virus (PRRSV) nonstructural proteins and implications for diagnostic detection and differentiation of PRRSV types I and II. *Clin. Vaccine Immunol.,* **16**, 628–635.

CHANG C.C., YOON K.J., ZIMMERMAN J.J., HARMON K.M., DIXON P.M., DVORAK C.M. & MURTAUGH M.P. (2002). Evolution of porcine reproductive and respiratory syndrome virus during sequential passages in pigs. *J. Virol.,* **76**, 4750–4763.

CHO H.J., MCNAB B., DUBUC C., JORDAN L., AFSHAR A., MAGAR R., PRINS S. & EERNISSE K. (1997). Comparative study of serological methods for the detection of antibodies to porcine reproductive and respiratory syndrome virus. *Can. J. Vet. Res.,* **61**, 161–166.

CHRISTOPHER-HENNINGS J., NELSON E.A., NELSON J.K. & BENFIELD D.A. (1997). Effects of a modified-live virus vaccine against porcine reproductive and respiratory syndrome in boars. *Am. J. Vet. Res.,* **58**, 40–45.

DEA S., GAGNON C.A., MARDASSI H. & MILANE G. (1996). Slow-reacting and European strains of porcine reproductive and respiratory (PRRS) virus. *J. Vet. Med. Sci.*, **58**, 749–753.

DELRUE I., VAN GORP H., VAN DOORSSELAERE J., DELPUTTE P.L. & NAUWYNCK H.J. (2010). Susceptible cell lines for the production of porcine reproductive and respiratory syndrome virus by stable transfection of sialoadhesin and CD163. *BMC Biotechnol.*, **29**, 48.

DENAC H., MOSER C., TRATSCHIN J.D. & HOFMANN M.A. (1997). An indirect ELISA for the detection of antibodies against porcine reproductive and respiratory syndrome virus using recombinant nucleocapsid protein as antigen. *J. Virol. Methods*, **65**, 169–181.

DÍAZ I., VENTEO A., REBOLLO B., MARTÍN-VALLS G.E., SIMON-GRIFÉ M., SANZ A. & MATEU E. (2012). Comparison of two commercial enzyme-linked immunosorbent assays for the diagnosis of porcine reproductive and respiratory syndrome virus infection. *J. Vet. Diagn. Invest.*, **24**, 344–348.

DREW T.W. (1995A). Comparative serology of porcine reproductive and respiratory syndrome in eight European laboratories, using immunoperoxidase monolayer assay and enzyme-linked immunosorbent assay. *Rev. Sci. Tech. Off. Int. Epiz.*, **14**(3), 761–775.

DREW T.W. (1995B). Comparative serology of porcine reproductive and respiratory syndrome in pigs. *J. Vet. Med. Sci.*, **58**, 941–946.

FAABERG K.S., BALASURIYA U.B., BRINTON M.A., GORBALENYA A.E., LEUNG F.C-C., NAUWYNCK H., SNIJDER E.J., STADEJEK T., YANG H. & YOO D. (2012). Arteriviridae. *In:* Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses, King A.M.Q., Adams M.J., Carstens E.B. & Lefkowitz E.J. Elsevier Academic Press, San Diego, USA, 796–805.

FANG Y., KIM D.Y., ROPP S., STEEN P., CHRISTOPHER-HENNINGS J., NELSON E.A. & ROWLAND R.R.R. (2004). Heterogeneity in Nsp2 of European-like porcine reproductive and respiratory syndrome viruses isolated in the United States. *Virus Res.,* **100** (2), 229–235.

FENG L., ZHANG X., XIA X., LI Y., HE S. & SUN H. (2013). Generation and characterization of a porcine endometrial endothelial cell line susceptible to porcine reproductive and respiratory syndrome virus. *Virus Res.* **171,** 209–215.

GARCÍA-NICOLÁS O., BAUMANN A., VIELLE N.J., GÓMEZ-LAGUNA J., QUEREDA J.J., PALLARÉS F.J., RAMIS G., CARRASCO L. & SUMMERFIELD A. (2014). Virulence and type-associated infectivity of interferon-treated macrophages by porcine reproductive and respiratory syndrome viruses. *Virus Res.* **22**, 204–211.

GILBERT S.A., LAROCHELLE R., MAGAR R., CHO H. J. & DERECT D. (1997). Typing of porcine reproductive and respiratory syndrome viruses by a multiplex PCR assay. *J. Clin. Microbiol.*, **35**, 264–267.

688 HALBUR P.G., ANDREWS J.J. HUFFMAN E.L., PAUL P.S., MENG X.-J. & NIYO Y. (1994). Development of a streptavidin-
689 biotin immunoperoxidase procedure for the detection of porcine reproductive and respiratory syndrome virus
690 antigen in porcine lung. *J. Vet. Diagn. Invest.,* **6**, 254–257.

691 HALBUR P.G., PAUL P.S., FREY M.L., LANDGRAF J., EERNISSE K., MENG X.-J., LUM M.A., ANDREWS J.J. & RATHJE J.A.
692 (1995). Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates
693 with that of the Lelystad virus. *Vet. Pathol.*, **32**, 648–660.

694 HOUBEN S., CALLEBAUT P. & PENSAERT M.B. (1995). Comparative study of a blocking enzyme-linked
695 immunosorbent assay and the immunoperoxidase monolayer assay for the detection of antibodies to the porcine
696 reproductive and respiratory syndrome virus in pigs. *J. Virol. Methods*, **51**, 125–128.

697 JUSA E.R., INABA Y., KOUNO M., HIROSE O., SHIBATA I., KUBOTA M. & YASUHARA H. (1996). Slow-reacting and
698 complement-requiring neutralizing antibody in swine infected with porcine reproductive and respiratory (PRRS)
699 virus. *J. Vet. Med. Sci.*, **58**, 749–753.

700 KARNIYCHUK U.U., GELDHOF M., VANHEE M., VAN DOORSSELAERE J., SAVELEVA T.A. & NAUWYNCK H.J. (2010).
701 Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and
702 respiratory syndrome virus isolate. *BMC Vet. Res.*, **4**, 30.

703 KARNIYCHUK U.U. & NAUWYNCK H.J. (2013). Pathogenesis and prevention of placental and transplacental porcine
704 reproductive and respiratory syndrome virus infection. *Vet. Res.,* **44**, 95.

705 KIM H.S., KWANG J., YOON I.J., JOO H.S. & FREY M.L. (1993). ENHANCED REPLICATION OF PORCINE REPRODUCTIVE AND
706 RESPIRATORY SYNDROME (PRRS) VIRUS IN A HOMOGENEOUS SUBPOPULATION OF MA-104 CELL LINE. *ARCH. VIROL.,* **133**,
707 477–483.

708 KITTAWORNRAT A., PRICKETT J., CHITTICK W., WANG C., ENGLE M., JOHNSON J., PATNAYAK D., SCHWARTZ T., WHITNEY
709 D., OLSEN C., SCHWARTZ K. & ZIMMERMAN J. (2010). Porcine reproductive and respiratory syndrome virus (PRRSV)
710 in serum and oral fluid samples from individual boars: will oral fluid replace serum for PRRSV surveillance? *Virus
711 Res,.* **154**, 170–176.

712 KLEIBOEKER S.B., SCHOMMER S.K., LEE S.M., WATKINS S., CHITTICK W. & POLSON D. (2005). Simultaneous detection
713 of North American and European porcine reproductive and respiratory syndrome virus using real-time quantitative
714 reverse transcriptase-PCR. *J. Vet. Diagn. Invest.*, **17**,165–170.

715 LAROCHELLE R. & MAGAR R. (1995). Production, characterization and reactivity of monoclonal antibodies to porcine
716 reproductive and respiratory syndrome virus. *J. Gen. Virol.*, **76**, 1361–1369.

717 LAROCHELLE R. & MAGAR R. (1997a). Evaluation of the presence of porcine reproductive and respiratory syndrome
718 virus by polymerase chain reaction. *J. Virol. Methods.*, **47**, 273–278.

719 LAROCHELLE R. & MAGAR R. (1997b). Differentiation of North American and European porcine reproductive and
720 respiratory syndrome virus genotypes by *in situ* hybridization. *J. Virol. Methods,* **68**, 161–168.

721 LAROCHELLE R., MAGAR R. (1997c). Typing of porcine reproductive and respiratory syndrome viruses by in situ
722 hybridization. *J. Virol. Methods,* **68**, 161–168.

723 MADSEN K.G., HANSEN C.M., MADSEN E.S., STRANDBYGAARD B., BOTNER A. & SORENSEN K.J. (1998). Sequence
724 analysis of porcine reproductive and respiratory syndrome virus antigen in porcine lung. *J. Vet. Diagn. Invest.,* **6**,
725 254–257.

726 MAGAR R., LAROCHELLE R., NELSON E.A. & CHARREYRE C. (1997). Differential reactivity of a monoclonal antibody
727 directed to the membrane protein of porcine reproductive and respiratory syndrome virus. *Can. J. Vet. Res.,* **61**,
728 69–71.

729 MARDASSI H., WILSON L., MOUNIR S. & DEA S. (1994). DETECTION OF PORCINE REPRODUCTIVE AND RESPIRATORY
730 SYNDROME VIRUS AND EFFICIENT DIFFERENTIATION BETWEEN CANADIAN AND EUROPEAN STRAINS BY REVERSE
731 TRANSCRIPTION AND PCR AMPLIFICATION. *J. CLIN. MICROBIOL.*, **32**, 2197–2203.

732 MURTAUGH M.P. & GENZOW M. (2011). Immunological solutions for treatment and prevention of porcine
733 reproductive and respiratory syndrome (PRRS). *Vaccine,* **26**, 8192–8204.

734 MURTAUGH M.P., STADEJEK T., ABRAHANTE J.E., LAM T.T. & LEUNG F.C. (2010). The ever-expanding diversity of
735 porcine reproductive and respiratory syndrome virus. *Virus Res.,* **154**, 18–30.

736 NODELIJK G., WENSVOORT G., KROESE B., VAN LEENGOED L., COLIJN E. & VERHEIJDEN J. (1996). COMPARISON OF A
737 COMMERCIAL ELISA AND AN IMMUNOPEROXIDASE MONOLAYER ASSAY TO DETECT ANTIBODIES DIRECTED AGAINST PORCINE
738 RESPIRATORY AND REPRODUCTIVE SYNDROME VIRUS. *VET. MICROBIOL.*, **49**, 285–295.

739 PROVOST C., JIA J.J., MUSIC N., LÉVESQUE C., LEBEL M.È., DEL CASTILLO J.R., JACQUES M. & GAGNON C.A. (2012).
740 Identification of a new cell line permissive to porcine reproductive and respiratory syndrome virus infection and
741 replication which is phenotypically distinct from MARC-145 cell line. *Virol. J.*, **13**, 267.

742 SCORTTI M., PRIETO C., MARTINEZ-LOBO F.J., SIMARRO I. & CASTRO J.M. (2006). Effects of two commercial European
743 modified-live vaccines against porcine reproductive and respiratory syndrome viruses in pregnant gilts. *Vet. J.,*
744 **172**, 506–514.

745 SHI M., LAM T.T., HON C.C., HUI R.K., FAABERG K.S., WENNBLOM T., MURTAUGH M.P., STADEJEK T. & LEUNG F.C.
746 (2010a). Molecular epidemiology of PRRSV: a phylogenetic perspective. *Virus Res.,* **154**, 7–17.

747 SHI M., LAM T.T., HON C.C., MURTAUGH M.P., DAVIES P.R., HUI R.K., LI J., WONG L.T., YIP C.W., JIANG J.W. & LEUNG
748 F.C. (2010b). Phylogeny-based evolutionary, demographical, and geographical dissection of North American type
749 2 porcine reproductive and respiratory syndrome viruses. *J. Virol.*, **84**, 8700–8711.

750 SORENSEN K.J., STRANDBYGAARD B., BØTNER A., MADSEN E.S., NIELSEN J. & HAVE P. (1998). Blocking ELISAs for the
751 distinction between antibodies against European and American strains of porcine reproductive and respiratory
752 syndrome virus. *Vet. Microbiol.,* **60**, 169–177.

753 STADEJEK T., OLEKSIEWICZ M.B., POTAPCHUK D. & PODGORSKA K. (2006). PORCINE REPRODUCTIVE AND RESPIRATORY
754 SYNDROME VIRUS STRAINS OF EXCEPTIONAL DIVERSITY IN EASTERN EUROPE SUPPORT THE DEFINITION OF NEW GENETIC SUBTYPES.
755 *J. GEN. VIROL.,* **87**, 1835–1841.

756 STADEJEK T., OLEKSIEWICZ M.B., SCHERBAKOV A.V., TIMINA A.M., KRABBE J.S., CHABROS K. & POTAPCHUK D. (2008).
757 Definition of subtypes in the European genotype of porcine reproductive and respiratory syndrome virus:
758 nucleocapsid characteristics and geographical distribution in Europe. *Arch. Virol.,* **153** (8), 1479–1488.

759 STADEJEK T., STANKEVICIUS A., MURTAUGH M.P. & OLEKSIEWICZ M.B. (2013). Molecular evolution of PRRSV in
760 Europe: current state of play. *Vet. Microbiol.,* **26,** 21-28.

761 TIAN K., YU X., ZHAO T., FENG Y., CAO Z., WANG C., HU Y., CHEN X., HU D., TIAN X., LIU D., ZHANG S., DENG X., DING
762 Y., YANG L., ZHANG Y., XIAO H., QIAO M., WANG B., HOU L., WANG X., YANG X., KANG L., SUN M., JIN P., WANG S.,
763 KITAMURA Y., YAN J. & GAO G.F. (2007). EMERGENCE OF FATAL PRRSV VARIANTS: UNPARALLELED OUTBREAKS OF ATYPICAL
764 PRRS IN CHINA AND MOLECULAR DISSECTION OF THE UNIQUE HALLMARK. *PLOS ONE*, **2**, e526.

765 TORRISON J., KNOLL M. & WISEMAN B. (1996). EVIDENCE OF PIG-TO-PIG TRANSMISSION OF A MODIFIED-LIVE PRRS VIRUS
766 VACCINE. *PROC. AM. ASSOC. SWINE PRACTITIONERS*, 89-91.

767 TRUS I., BONCKAERT C., VAN DER MEULEN K. & NAUWYNCK H.J. (2014). Efficacy of an attenuated European subtype 1
768 porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in pigs upon challenge with the East
769 European subtype 3 PRRSV strain Lena. *Vaccine,* **32** (25), 2995–3003.

770 VAN VUGT J.J.F.A., STORGAARD T., OLEKSIEWICZ M.B. & BOTNER A. (2001). HIGH FREQUENCY RNA RECOMBINATION IN
771 PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS OCCURS PREFERENTIALLY BETWEEN PARENTAL SEQUENCES
772 WITH HIGH SIMILARITY. *J. GEN. VIROL.,* **82**, 2615–2620.

773 VENTEO A., REBOLLO B., SARRASECA J., RODRIGUEZ M.J. & SANZ A.(2012). A novel double recognition enzyme-linked
774 immunosorbent assay based on the nucleocapsid protein for early detection of European porcine reproductive
775 and respiratory syndrome virus infection. *J. Virol. Methods.,* **181**(1), 109–113.

776 WASILK A., CALLAHAN J.D., CHRISTOPHER-HENNINGS J., GAY T.A., FANG Y., DAMMEN M., REOS M.E., TORREMORELL M.,
777 POLSON D., MELLENCAMP M., NELSON E. & NELSON W.M. (2004). DETECTION OF U.S., LELYSTAD, AND EUROPEAN-LIKE
778 PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUSES AND RELATIVE QUANTITATION IN BOAR SEMEN AND SERUM
779 SAMPLES BY REAL-TIME PCR. *J. CLIN. MICROBIOL.*, **42**, 4453–4461.

780 WEESENDORP E., MORGAN S., STOCKHOFE-ZURWIEDEN N., POPMA-DE GRAAF D.J., GRAHAM S.P. & REBEL J.M. (2013).
781 Comparative analysis of immune responses following experimental infection of pigs with European porcine
782 reproductive and respiratory syndrome virus strains of differing virulence. *Vet. Microbiol.,* **12**, 1–12.

783 WENSVOORT G., TERPSTRA C., POL J.M.A., TER LAAK E.A., BLOEMRAAD M., DE KLUYVER E.P., WENSVOORT G. &
784 MOORMANN R.J.M. (1993). LELYSTAD VIRUS, THE ISOLATION OF LELYSTAD VIRUS. *VET. Q.*, **13**, 121–130.

785 WERNIKE K., BONILAURI P., DAUBER M., ERRINGTON J., LEBLANC N., REVILLA-FERNÁNDEZ S., HJULSAGER C., ISAKSSON
786 M., STADEJEK T., BEER M. & HOFFMANN B. (2012a). Porcine reproductive and respiratory syndrome virus:

interlaboratory ring trial to evaluate real-time reverse transcription polymerase chain reaction detection methods. *J. Vet. Diagn. Invest.*, **24** (5), 855–866.

WERNIKE K., HOFFMANN B., DAUBER M., LANGE E., SCHIRRMEIER H. & BEER M. (2012b). Detection and typing of highly pathogenic porcine reproductive and respiratory syndrome virus by multiplex real-time rt-PCR. *PLoS One.,* **7** (6), 38251.

WESLEY R.D., MENGELING W.L., LAGER K.M., CLOUSER D.F., LANDGRAF J.G. & FREY M.L. (1998). DIFFERENTIATION OF A PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS VACCINE STRAIN FROM NORTH AMERICAN FIELD STRAINS BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF ORF 5. *J. VET. DIAGN. INVEST.* **10**, 140–144.

XIAO Y.H., WANG T.T., ZHAO Q., WANG C.B., LV J.H., NIE L., GAO J.M., MA X.C., HSU W.H. & ZHOU E.M. (2014). Development of Indirect ELISAs for Differential Serodiagnosis of Classical and Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus. *Transbound. Emerg. Dis.,* **61** (4), 341–349.

YOON I.J., JOO H.S., CHRISTIANSON W.T., KIM H.S., COLLINS J.E., MORRISON R.B. & DIAL G.D. (1992). An indirect fluorescent antibody test for the detection of antibody to swine infertility and respiratory syndrome virus in swine sera. *J. Vet. Diagn. Invest.,* **4**, 144–147.

ZHOU L. & YANG H. (2010). Porcine reproductive and respiratory syndrome in China. *Virus Res.* **154** (1–2), 31–37.

ZIMMERMANN J.J., BENFIELD D.A., DEE S.A., MURTAUGH M.P., STADEJEK T., STEVENSON G.W. & Torremorel M. (2012). Porcine reproductive and respiratory syndrome Virus (Porcine Arterivirus). *In:* Diseases of Swine, Tenth Edition, Zimmerman J.J., Karriker L.A., Ramirez A., Schwartz K.J., Stevenson G.W. eds. Iowa State University Press. Ames, Iowa, USA, 461–486.

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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

1 C H A P T E R   2 . 8 . 8 .

2 # ~~SWINE~~ INFLUENZA A VIRUS OF SWINE

3 ## SUMMARY

4 *~~Swine~~ Influenza A viruses of swine (IAV-S) ~~is~~ cause a highly contagious viral infection of*
5 *pigs. ~~Swine influenza virus (SIV)~~ IAV-S infections cause respiratory disease characterised by*
6 *coughing, sneezing, nasal discharge, elevated rectal temperatures, lethargy, breathing difficulty,*
7 *and depressed appetite. In some instances, IAV-S ~~SIV~~ infections are associated with reproductive*
8 *disorders such as abortion. Clinical signs and nasal shedding of virus can occur within 24 hours of*
9 *infection. Morbidity rates can reach 100% with IAV-S ~~SIV~~ infections, while mortality rates are*
10 *generally low. Secondary bacterial infections can exacerbate the clinical signs following infection*
11 *with IAV-S ~~SIV~~. Transmission is through contact with IAV-S ~~SIV~~ -containing secretions such as*
12 *nasal discharges and aerosols created by coughing or sneezing.*

13 ***Identification of the agent:*** *Samples for virus identification should be collected within 24–72 hours*
14 *after development of clinical signs. The animal of choice is an untreated, acutely ill pig with an*
15 *elevated rectal temperature. Virus can readily be detected in lung tissue and nasal swabs. Oral*
16 *fluids collected from cotton ropes hung in a pig pen are becoming more popular as a diagnostic*
17 *specimen due to the ease of sample collection. Virus isolation can be conducted in*
18 *embryonated ~~chicken~~ fowl eggs and on continuous cell lines. Isolated viruses can be subtyped*
19 *using the haemagglutination inhibition (HI) and the neuraminidase inhibition tests, or by reverse*
20 *transcription-polymerase chain reaction assays. Immunohistochemistry can be conducted on*
21 *formalin-fixed tissue ~~and a fluorescent antibody test can be conducted on fresh tissue~~. Enzyme-*
22 *linked immunosorbent assays (ELISA) are commercially available for detection of type A influenza*
23 *viruses.*

24 ***Serological tests:*** *Historically, the primary serological test for detection of IAV-S ~~SIV~~ antibodies is*
25 *the HI test conducted on paired sera. The HI test is subtype specific. The sera are generally*
26 *collected 10–21 days apart. A four-fold or greater increase in titre between the first and second*
27 *sample is suggestive of a recent IAV-S ~~SIV~~ infection. Additional serological tests that have been*
28 *described are the agar gel immunodiffusion test, indirect fluorescent antibody test, virus*
29 *neutralisation, and ELISA. Due to the increasing amount of antigenic diversity in influenza A viruses*
30 *of swine and the need to use multiple H types in HI assays, there is a general trend towards*
31 *increasing use of commercially available ELISAs that are not subtype specific.*

32 ***Requirements for vaccines:*** *Inactivated, adjuvanted IAV-S ~~SIV~~ vaccines are commercially*
33 *available. Vaccines may be in the form of a single IAV-S ~~SIV~~ subtype or may contain multiple IAV-S*
34 *~~SIV~~ subtypes. Vaccines should reflect the current antigenic profile of field viruses, containing*
35 *subtypes and strains that are changed as needed to assure protection. The finished vaccine must*
36 *be shown to be pure, safe, potent, and efficacious.*

37 ## A. INTRODUCTION

38 ~~Swine~~ Influenza A of swine (IAV-S) is a highly contagious viral infection of pigs that can have significant economic
39 impact on an affected herd ~~(Olsen *et al.*, 2005)~~. IAV-S ~~swine influenza virus (SIV)~~ is a type A orthomyxovirus with
40 a segmented RNA genome. The type A ~~swine influenza~~ viruses are further subdivided based on their
41 haemagglutinin and neuraminidase proteins. Subtypes of IAV-S ~~SIV~~ that are most frequently identified in pigs
42 include classical and avian H1N1, human (hu) H1N1 and H1N2, reassortant (r) H3N2, and rH1N2 ~~(Choi *et al.*,~~
43 ~~2004; Gramer, 2007; Marozin *et al.*, 2002; Olsen *et al.*, 2002; Schrader & Süss, 2004)~~. Other subtypes that have
44 been identified in pigs include rH1N7, rH3N1, H2N3, avian (av) H4N6, avH3N3, and avH9N2 ~~(Brown *et al.*, 1997;~~
45 ~~Karasin *et al.*, 2000a; 2004; Ma *et al.*, 2007; Olsen *et al.*, 2005; Peiris *et al.*, 2001)~~. The H1N1, H1N2 and H3N2

46 viruses found in Europe are antigenically and genetically different from those found in America (<u>Brown, 47 2013</u>; ~~Brown *et al.*, 1998; Castrucci *et al.*, 1993; Done & Brown, 1997;~~ Karasin *et al.*, ~~2000a;~~ 2000b; 2002; ~~2004;~~ 48 ~~Noble *et al.*, 1993;~~ Olsen, 2002; ~~Sheerar *et al.*, 1989;~~ <u>Vincent *et al.*, 2009</u>; Webby *et al.*, ~~2000;~~ 2004; Zhou *et al.*, 49 1999). Pigs have receptors in their respiratory tract that will bind influenza A viruses of swine, human, and avian 50 species. Consequently, pigs have been called 'mixing vessels' for the development of new influenza viruses when 51 influenza A viruses of swine, avian, and/or humans ~~influenza viruses~~ undergo genetic reassortment in pigs. <u>IAV-S</u> 52 ~~SIV~~ infections are described as causing respiratory disease characterised by coughing, sneezing, nasal 53 discharge, elevated rectal temperatures, lethargy, breathing difficulty and depressed appetite. Other agents that 54 may cause respiratory disease in pigs include porcine reproductive and respiratory syndrome virus, Aujeszky's 55 disease (pseudorabies) virus, porcine respiratory coronavirus, *Actinobacillus pleuro-pneumoniae, Mycoplasma* 56 *hyopneumoniae* and other bacterial agents. However, most of these have other signs that do not mimic <u>IAV-S</u> 57 <u>infections</u> ~~swine influenza~~. *Actinobacillus pleuropneumoniae,* in the acute form of the infection, has clinical signs 58 most similar to <u>IAV-S infections</u> ~~swine influenza~~, such as dyspnoea, tachypnoea, abdominal breathing, coughing, 59 fever, depression and anorexia. Clinical signs and nasal shedding of ~~SIV~~ <u>influenza A virus</u> can occur within 60 24 hours of infection, and shedding typically ceases by day 7–10 after infection. Two forms of the disease occur in 61 swine, epidemic or endemic. In the epidemic form, the virus quickly moves through all phases of a swine unit with 62 rapid recovery, provided there are not complicating factors such as secondary bacterial infections. In the endemic 63 form, clinical signs may be less obvious and not all pigs may demonstrate traditional clinical signs of infection. 64 Morbidity rates can reach 100% with <u>IAV-S</u> ~~SIV~~ infections, while mortality rates are generally low. The primary 65 economic impact is related to retarded weight gain resulting in an increase in the number of days to reach market 66 weight. Transmission is through contact with <u>IAV-S</u> ~~SIV~~ containing secretions such as nasal discharges and 67 aerosols created by coughing or sneezing. Human infections with <u>IAV-S</u> ~~SIV~~ can occur and a limited number of 68 deaths have been reported (<u>Lindstrom *et al.*, 2012;</u> Myers *et al.*, 2007~~; Olsen *et al.*, 2002~~). Precautions should be 69 taken to prevent human infection as described in Chapter 1.1.3 *Biosafety and biosecurity in the veterinary* 70 *microbiological laboratory and animal facilities*. Conversely, ~~human~~ influenza A viruses can occasionally be 71 transmitted from ~~animal caretakers~~ <u>people</u> to pigs. Similarly, influenza viruses can also be transmitted 72 occasionally from poultry to pigs as well as from pigs to poultry. In the spring of 2009 a newly identified H1N1 73 virus was detected in people in the Western Hemisphere. This novel virus was composed entirely of swine genes 74 the origins of which were of swine, avian, and human lineages. The matrix and neuraminidase were from 75 European H1N1 <u>IAV-S</u> ~~swine influenza viruses~~ of avian lineage and the remaining genes were from North 76 American <u>IAV-S</u> ~~swine influenza viruses~~ of swine, avian, and human lineage. The virus spread rapidly throughout 77 the world through human-to-human transmission. Swine cases in both Northern and Southern Hemispheres 78 <u>occurred simultaneously and subsequently the virus has become endemic in many swine populations worldwide,</u> 79 <u>in addition to continued independent circulation in humans</u> ~~have occurred since the virus was first recognised in~~ 80 ~~humans (http://www.oie.int/eng/en_index.htm). Current information on recent research can be found at a number~~ 81 ~~of websites, including, but not limited to http://www.defra.gov.uk/vla/science/sci_si.htm~~ 82 ~~and http://www.ars.usda.gov/2009H1N1/.~~

83 # B. DIAGNOSTIC TECHNIQUES

84 <u>***Table 1.** Test methods available for diagnosis of IAV-S and their purpose*</u>

Method	**Purpose**					
	<u>Population freedom from infection</u>	<u>Individual animal freedom from infection prior to movement</u>	<u>Contribution to eradication policies</u>	<u>Confirmation of clinical cases</u>	<u>Prevalence of infection – surveillance</u>	<u>Immune status in individual animals or populations post-vaccination</u>
Agent identification[1]						
Virus isolation	<u>±</u>	<u>+++</u>	<u>++</u>	<u>+++</u>	<u>++</u>	<u>–</u>
Real-time RT-PCR	<u>+++</u>	<u>+++</u>	<u>+++</u>	<u>+++</u>	<u>+++</u>	<u>–</u>
Conventional PCR	<u>–</u>	<u>–</u>	<u>–</u>	<u>++</u>	<u>–</u>	<u>–</u>
Detection of immune response[2]						

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1   <u>A combination of agent identification methods applied on the same clinical sample may be needed in some situations.</u>

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
HI	±	±	±	++	++	+++
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; 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.

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 should be considered used 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 viscus 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
forguidance 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 TPCK[3]-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

128		used). The cell culture medium can be supplemented with antibiotics, but is not
129		supplemented with fetal bovine serum.
130	iii)	Inoculate cell cultures with an appropriate amount of tissue suspension, <u>oral fluids,</u> or
131		swab supernatant. Note: The volume of inoculum will vary with the size of the cell culture
132		container. In general, 100–200 µl are inoculated in each well of a 24-well culture plate,
133		1 ml in each Leighton tube, and 1–2 ml into a 25 cm^2 flask.
134	iv)	Incubate inoculated cell cultures for 1–2 hours at 37°C with occasional rocking. When
135		using cell culture containers that are open to the environment, such as culture plates,
136		incubation should be done in a humidified incubator with 5% CO_2.
137	v)	Remove the inoculum and wash the cell monolayer three times with the cell culture
138		medium containing trypsin.
139	vi)	Add an appropriate volume of the cell culture maintenance medium ~~(as noted in ii~~
140		~~above)~~ to all containers and incubate at 37°C for 5–7 days with periodic examination for
141		cytopathic effect (CPE). If CPE is not observed at the end of the incubation period, the cell
142		culture container can be frozen at –70°C <u>or colder</u>, thawed, and blind passaged as
143		described above (step iii). If CPE is observed, an aliquot of the cell culture medium can be
144		tested for haemagglutinating viruses or by reverse transcription-polymerase chain reaction
145		(RT-PCR) for conserved influenza virus genes such as nucleoprotein or matrix, and can be
146		collected and used as inoculum for confirmation by the fluorescent antibody technique
147		(see Section B.1.5 below). Cover-slips (Leighton tube, 24-well cell culture plate) or
148		chamber slides with MDCK (or other appropriate cell) monolayer can be inoculated for this
149		purpose. The isolation procedure is as described above (step iii). In some instances, it
150		may be necessary to make tenfold dilutions of the cell culture virus in order to have
151		appropriate CPE on the cover-slip. Influenza subtypes can be determined by the
152		haemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests, or by RT-PCR
153		with primers validated for sensitive and specific amplification of individual HA and NA
154		genes (<u>Chiapponi *et al.*, 2012;</u> ~~Hoffman *et al.*, 2001;~~ <u>Nagarajan *et al.*, 2010;</u> ~~Phipps *et al.*,~~
155		~~2004~~). <u>However, validation using endemically circulating strains in the region should be</u>
156		<u>done to ensure fitness for purpose of tests since endemic strains of IAV-S may vary</u>
157		<u>genetically between regions.</u>

158		**1.1.3. Egg inoculation (Senne, 1998)**
159	i)	Use 10- to 11-day-old embryonated ~~chicken~~ <u>fowl</u> eggs (Senne, 1998).
160	ii)	Inoculate 0.1–0.3 ml of inoculum into the allantoic cavity and amniotic sac; many
161		laboratories only inoculate via the allantoic route with similar sensitivity. Generally, 3–
162		4 eggs are inoculated per sample.
163	iii)	Incubate eggs at 35–37°C for 3–4 days and candle daily. Eggs with embryos that have
164		died within 24 hours of inoculation are discarded <u>(assumed to be trauma-induced deaths</u>
165		<u>associated with the inoculation process)</u>.
166	iv)	Refrigerate eggs with embryos that have died later than 24 hours after inoculation. Harvest
167		amniotic and allantoic fluids from eggs with dead embryos and from eggs with viable
168		embryos at the end of the incubation period. All egg materials should be considered to be
169		potentially infectious and should be treated accordingly to prevent <u>IAV-S</u> ~~SIV~~ exposure to
170		the laboratory worker.
171	v)	Centrifuge fluids at 1500–1900 ***g*** for 10–20 minutes at 4°C. Transfer the supernatant to
172		another tube for testing.
173	vi)	Fluids are evaluated for the presence of <u>IAV-S</u> ~~SIV~~ with the haemagglutination (HA) test
174		(see below).
175	vii)	Repass <u>(up to 1–2 passages)</u> fluids negative for haemagglutinating activity (negative for
176		<u>IAV-S</u> ~~SIV~~) in eggs or on cell lines as described above. Isolation may be improved by
177		making tenfold dilutions of the fluid in cell culture medium. Antibiotics may be added to the
178		cell culture fluid.

179		**1.1.4. Haemagglutination test**
180	i)	Prepare a 0.5% erythrocyte suspension from male turkey or chicken blood. Dispense
181		whole blood into a tube and add PBS. For example, 10–20 ml whole blood in a 50 ml
182		centrifuge tube to which PBS is added to fill the tube. Gently invert the tube several times
183		to wash the erythrocytes. Centrifuge at 800 ***g*** for 10 minutes in a refrigerated centrifuge.
184		Aspirate PBS and buffy coat (white blood cell layer) from the tube. Refill the tube with fresh

185　　　　PBS and resuspend erythrocytes thoroughly. Repeat the washing and centrifugation cycle
186　　　　two additional times. Once washing is complete, add sufficient erythrocytes to PBS to
187　　　　make a 0.5% solution. Certain virus strains agglutinate turkey rather than chicken
188　　　　erythrocytes to greater or lesser degrees. Therefore, it may be necessary to choose the
189　　　　species of erythrocytes based on the strains circulating in a given area. Washed
190　　　　erythrocytes and 0.5% suspensions of erythrocytes can be stored at 4°C for up to 1 week.
191　　　　Discard if haemolysis is observed.

192　　ii)　　Dispense 50 µl PBS in a row of 8–12 wells on a 96-well V- or U-bottom microtitre plate for
193　　　　each unknown virus. ~~U-bottom plates are generally preferred over V-bottom plates.~~ One
194　　　　additional row of wells should be included for a positive control.

195　　iii)　　Add 50 µl of undiluted isolate to the first well of each corresponding row.

196　　iv)　　Serially dilute the isolate with a micropipette set to deliver 50 µl. The resulting dilutions will
197　　　　range from 1/2 (well 1) to 1/2048 (well 11). Well 12 contains PBS only and serves as a cell
198　　　　control.

199　　v)　　Add 50 µl of 0.5% erythrocyte suspension to each well and agitate the plate to mix
200　　　　thoroughly. Note: keep erythrocytes thoroughly suspended during the dispensing process.

201　　vi)　　Cover the plate with sealing tape and incubate at room temperature (24°C) or 4°C until a
202　　　　distinct button has formed (30–60 minutes) in the control well.

203　　vii)　　Wells with complete haemagglutination (positive HA, IAV-S ~~SIV~~ present) will have
204　　　　erythrocytes spread throughout the well in a 'mat' type appearance. Wells with a distinct
205　　　　button of erythrocytes at the bottom of the well are negative for haemagglutinating activity
206　　　　(negative for IAV-S ~~SIV~~). Incomplete HA activity is demonstrated by partial buttons
207　　　　characterised by fuzzy margins or 'donut-like' appearance. When interpretation between
208　　　　negative and incomplete inhibition is doubtful, tilt the microtitre plate to about a 45-degree
209　　　　angle for 20–30 seconds and look for streaming, which produces a tear-drop appearance
210　　　　and translucency around the cells in wells with negative hemagglutination. Wells with
211　　　　partial inhibition will not produce a tear drop.

212　**1.2. Typing influenza A viruses of swine (IAV-S) ~~SIV~~ isolates**

213　　**1.2.1. Haemagglutination inhibition test**

214　　i)　　Dilute reference HA antigens (H1, H3, etc.) to a concentration of 8 HA units (HAU) per
215　　　　50 µl (4 HAU/25 µl) in 0.01 M PBS, pH 7.2–7.4. Reference antigens should represent what
216　　　　is actively circulating in the region where the pigs are located. For guidance, the OIE
217　　　　Reference Laboratory in the region should be consulted regarding reference antigens.

218　　ii)　　Standardise unknown influenza A viruses to contain 8 HAU in 50 µl.

219　　iii)　　Conduct a back titration (HA test) for all unknown isolates and the H subtype antigens to
220　　　　assure that the correct HAUs are present. The back titration is performed as described in
221　　　　the HA procedures except that six well dilutions are used instead of eleven.

222　　iv)　　Treat each reference serum (specific for an individual HA subtype, and representative of
223　　　　actively circulating viruses in the region) with RDE (receptor-destroying enzyme); add 50 µl
224　　　　serum to 200 µl RDE (1/10 dilution in calcium saline solution equalling 100 units per ml).
225　　　　Incubate overnight (12–18 hours) in a 37°C water bath. Add 150 µl 2.5% sodium citrate
226　　　　solution and heat inactivate at 56°C for 30 minutes. Combine 200 µl treated sample and
227　　　　25 µl PBS. Note: RDE treatment is recommended as it will reduce nonspecific reactions
228　　　　and will enhance the identification of H1N2 and H3N2 isolates.

229　　v)　　Remove natural serum agglutinins from the sera by treating diluted serum with 0.1 ml
230　　　　packed, washed erythrocytes per 1 ml diluted serum. Incubate for 30 minutes at room
231　　　　temperature with occasional mixing to keep the erythrocytes suspended. Centrifuge the
232　　　　treated serum at 800 *g* for 10 minutes and then retain the serum.

233　　vi)　　Dispense 25 µl of standardised antigen (unknown isolate or positive control antigen) into
234　　　　three wells of a 96-well V- or U-bottom microtitre plate. Add 50 µl of PBS to several wells
235　　　　to serve as an erythrocyte cell control. Note: 25 µl of PBS can be used in place of the 25 µl
236　　　　of standardised antigen.

237　　vii)　　Add 25 µl of the appropriate reference serum to the first well of the H subtype being
238　　　　evaluated. Serially dilute the antiserum in 25 µl volumes in the antigen wells with a pipette
239　　　　set to deliver 25 µl. Repeat this procedure for each H subtype being evaluated. Note: If
240　　　　25 µl of PBS was used in place of the 25 µl of standardised antigen in step vi, add 25 µl of
241　　　　standardised antigen to each well containing the reference serum.

242   viii)   Cover plate(s) and incubate at room temperature for 10–30 minutes.

243   ix)   Add 50 µl 0.5% erythrocyte suspension to each well and shake/agitate the plate(s) to mix
244   thoroughly. Keep the erythrocytes thoroughly suspended during the dispensing process.

245   x)   Cover the plate(s) with sealing tape and incubate at room temperature <u>(24°C) or 4°C</u> until
246   a distinct button has formed in the positive control wells (usually 30–60 minutes). Observe
247   the plates after about 20 minutes' incubation for evidence of haemagglutination as some
248   isolates may begin to elute (detach from erythrocytes) in 30 minutes.

249   xi)   Read test results as described above for the HA test. A sample is considered positive for a
250   specific H subtype if haemagglutination is inhibited. The test is considered valid if the
251   positive reference antigen and its homologous antiserum demonstrate the expected HI titre
252   and the back titration of each antigen (unknown and positive control) is 4 or 8 HAUs. If
253   these conditions are not met, the test should be repeated.

254   xii)   If erythrocytes in the cell control wells do not settle into a well-defined button, check the
255   following as possible causes: incorrect formulation of PBS, excessive evaporation from the
256   plates, erythrocytes too old, or incorrect concentration of erythrocytes.

1.2.2. Neuraminidase inhibition test

258   <u>Reliable</u> subtype identification based on the NI test is beyond the scope of many laboratories.
259   Reference laboratories ~~should~~ <u>can</u> be consulted for N typing of isolates.

1.3. Fluorescent antibody test

1.3.1. Test procedure

262   i)   This technique can be used for tissue sections, cover-slips/slides, or 96-well plates of
263   infected cell monolayers (Vincent *et al.*, 1997). Positive and negative controls should be
264   included with all staining procedures.

265   <u>ii)</u>   <u>Note this technique is highly dependent on use of reference reagents representative of</u>
266   <u>circulating viruses in the region and on skilled readers who can differentiate between</u>
267   <u>positive results and background staining (specificity). This method of virus detection is of</u>
268   <u>lower sensitivity compared with other available assays such as PCR.</u>

269   iii)   Inoculated cells are incubated for an appropriate length of time to allow 10–25% of the
270   cells to become productively infected with virus. Rinse the cover-slip or slide once in PBS,
271   place in 100% acetone for 5–10 minutes and air-dry. Acetone should be used in a vented
272   hood.

273   iv)   Prepare frozen tissue sections on glass slides. Fix the glass slides in acetone for 5–
274   10 minutes and air-dry.

275   v)   Apply conjugate (fluorescein-labelled <u>IAV-S</u> ~~swine influenza~~ antibody) and incubate in a
276   humid chamber at 37°C for 30 minutes. Preferably the conjugate contains Evans blue for
277   counter staining.

278   vi)   Rinse in PBS, pH 7.2, soak for 5–10 minutes in fresh PBS, rinse in distilled water, and air-
279   dry.

280   vii)   Place cover-slips on glass slides, cell side down, with mounting fluid. Remove the rubber
281   gasket from chamber slides and add mounting fluid followed by a glass cover-slip.
282   Mounting fluid followed by a glass cover-slip is also placed over tissue sections on the
283   slide. If 96-well plates are used, mounting medium and cover-slips are not required.

284   viii)   Observe stained slides in a darkened room with the use of an ultraviolet microscope. Cells
285   infected <u>IAV-S</u> ~~SIV~~ are identified by the presence of bright apple-green fluorescence. It is
286   recommended that the person examining the slides receive training in reading fluorescein-
287   labelled slides as they can be difficult to interpret. <u>Known positive and negative slides</u>
288   <u>should be included when testing unknowns to verify the test procedure worked and to use</u>
289   <u>as a basis for differentiating between positive (IAV-S) staining and negative (background)</u>
290   <u>staining.</u> It is also important to use an antibody that recognises all possible viruses
291   circulating in the area (e.g. a pan-anti-influenza A nucleoprotein antibody).

292

293 **1.4. Immunohistochemistry (Vincent *et al.,* 1997)**

294   **1.4.1. Test procedure**

295   i) Slice formalin-fixed, paraffin-embedded lung in 4-µm thick sections and place on poly-L-
296   lysine-coated slides (alternatively, commercially available charged slides can be used and
297   are deemed superior to lysine-coated slides by some). Positive and negative control
298   tissues should be included with all tests.

299   ii) Heat slides at 60°C for 15 minutes, deparaffinise, and rehydrate through immersions in
300   decreasing concentrations of ethanol and then in distilled water.

301   iii) Treat samples with 3% hydrogen peroxide for 10 minutes and rinse twice in distilled water.

302   iv) Digest samples with 0.05% protease for 2 minutes and rinse twice for 2 minutes in 0.1 M
303   Tris/PBS buffer, pH 7.2, at room temperature.

304   v) Apply primary mouse anti- IAV-S SIV monoclonal antibody (directed against the viral
305   nucleoprotein) to each slide and incubate at room temperature for 1 hour or overnight at
306   4°C. Rinse slides with Tris/PBS buffer.

307   vi) Apply secondary antibody (biotinylated goat anti-mouse antibody) for 10 minutes at room
308   temperature. Rinse with Tris/PBS buffer.

309   vii) Apply tertiary antibody (peroxidase-conjugated streptavidin) for 10 minutes at room
310   temperature. Rinse with Tris/PBS buffer.

311   viii) Apply diaminobenzidine tetrahydrochloride solution for 5 minutes at room temperature.
312   Rinse twice in distilled water.

313   ix) Counterstain slides in Gill's haematoxylin for 10–30 seconds, wash in water for 2 minutes,
314   dehydrate, clear, and add cover-slips.

315   x) IAV-S SIV -infected tissues are identified by the presence of brown staining in bronchiolar
316   epithelium and pneumocytes.

317 **1.5. Antigen-capture enzyme-linked immunosorbent assays**

318   Type A antigen-capture enzyme-linked immunosorbent assays (ELISAs) are commercially available for
319   detection of human and animal influenza viruses. These types of assays have been used for detection
320   of IAV-S SIV in lung tissue and nasal swabs (Swenson *et al.*, 2001). The assays are generally
321   available through human health and animal health care companies. These assays tend to be of lower
322   sensitivity compared with other assays such as PCR.

323 **1.6. Polymerase chain reaction**

324   RT-PCR tests have been developed for the diagnosis of swine influenza and for hemagglutinin and
325   neuraminidase typing (Landolt *et al.*, 2004). The highly conserved matrix protein is the best target for
326   screening for infection with IAV-S by RT-PCR. Following the identification of the novel (pandemic)
327   H1N1 in 2009, molecular assays based on an avian influenza matrix real-time PCR (Spackman *et al.*,
328   2002) were adapted for use in swine (Brookes *et al.,* 2009; Slomka *et al.,* 2010). Modifications to the
329   assay vary by country and a swine influenza reference laboratory should may be
330   consulted (http://offlu.net) for the most suitable matrix PCR assay.

331   The IAV-S real-time RT-PCR procedure described in this chapter targets the matrix (M) gene of
332   Influenza A viruses. The matrix primer/probe set is a quasi-multiplex real-time RT-PCR that uses a
333   single forward primer, probe and two reverse primers. The two reverse primers can generically detect
334   the Eurasian, North American and pandemic 2009 H1N1 matrix lineages.

335   The real-time RT-PCR uses a one-step procedure. Specific primers are designed to amplify the target
336   region (see Table 2). Non-extendable fluorogenic hydrolysis probes measure the target PCR product
337   formation during each cycle of the PCR reaction. The probes are labelled at the 5' end with a reporter
338   dye, and non-fluorescing quencher at the 3' end. Once the probe hybridises to the target sequence, the
339   5' nuclease activity of Taq polymerase will hydrolyse the probe, and separate the quencher from the
340   reporter dye. This results in the fluorescence of the separated reporter dye, which is detected
341   spectrophotometrically and recorded. The amount of fluorescence recorded and the cycle number of
342   detection is proportional to the amount of target template in the samples.

343   For this procedure, it is critical to have separate preparation areas and equipment for nucleic acid
344 extraction, RNA transfer, and master mix preparation. A "clean" area is needed to prepare reagents
345 used for PCR that is free of amplified c-DNA or sample RNA.

346   ***Table 2.** IAV-S matrix hydrolysis probe and primer sequences*

Specificity	Description	Sequence
Matrix (any influenza A virus)	M+25* 5' Primer	5'-AgA TgA gTC TTC TAA CCg Agg TCg-3'
	M+64* Probe	5'-FAM-TCA ggC CCC CTC AAA gCC gA-BHQ-1 -3'
	M-124* 3' Primer	5'-TgC AAA AAC ATC TTC AAg TCT CTg-3'
	M-124* SIV 3' Primer**	5'- TgC AAA gAC ACT TTC CAg TCT CTg -3'

*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

347 i)   Extract nucleic acid from sample. A positive and negative extraction control (PEC and
348   NEC, respectively) will need to be used to confirm that the extraction was successful.

349 ii)   Prepare RT-PCR master mix in a "clean" PCR room (Table 2).

350 iii)   Aliquot 17 µl of reaction mix to each well in a 96-well plate. Transfer 8 µl of RNA template
351   to each reaction in a designated RNA transfer room. When using a 96-well plate, use a
352   support base to protect the bottom of the plate from scratches, finger prints, or picking up
353   particles that could interfere with the optical system and alter the background
354   fluorescence.

355   a)   The following controls will need to be included in the PCR run to verify that the PCR
356   and RNA extraction were successful: positive extraction control (PEC), negative
357   extraction control (NEC), positive amplification (template) control (PAC), and negative
358   amplification (template) control (NAC). PACs are diluted by each diagnostic lab, and
359   must have a C_t value in the range of 21–29 for the run to be valid.

360 iv)   Place samples in thermocycler and run at appropriate parameters..

361 v)   Analyse results. The PCR run will be valid if:

362   a)   The PAC C_t value is 21–29

363   b)   The PEC is positive

364   c)   Both NEC and NAC are negative

365   d)   All samples and controls that are positive have "sigmoidal curve"

366   e)   If the above conditions are not met, the test will need to be repeated.

367   ***Table 2.** Example Real-time RT-PCR Master Mix for a one step kit*

Component	Final Concentration	Volume per reaction (µl)
H$_2$O	–	0.83
2× RT-PCR buffer	1×	12.5
M+25 5' primer (20 µM)	200 nM	0.25
M-124 3' primer (20 µM)	200 nM	0.25
M-124 SIV 3' primer (20 µM)	200 nM	0.25
25× RT-PCR enzyme mix	1×	1
M+64 probe (6 µM)	60 nM	0.25
Detection enhancer (15×)	1×	1.67
Template	–	8
Total Reaction Volume	**–**	**25**

368
369

370

Table 3. Example thermocycler parameters

Stage	Cycles	Step	Time	Temperature
1	1		10 minutes	45°C
2	1		10 minutes	95°C
3	45	denaturation	1 second	94°C
		annealing*	30 seconds	60°C
		extension	15 seconds	72°C

*Collection of fluorescence

371 Viral isolates can be subtyped using conventional methods or by real-time PCR assays that can
372 differentiate the genetically distinct novel H1 viruses from other known strains (Chiapponi *et al.,* 2012).
373 Increasingly, differential real-time PCRs are being used in many regions conventional H1N1 based on
374 differentiable matrix real-time or N1 real-time assays have also been developed for use in North
375 America. Matrix PCR diagnostic specimens can also be subtyped through use of subtyping PCRs.
376 Samples with high matrix CT's may not be detectable by subtyping PCRs and it may be necessary to
377 attempt virus isolation prior to identifying the subtype. Screening and subtyping PCR reagents are
378 commercially available; however, laboratories need to ensure they will detect currently circulating
379 influenza viruses in their area. In many instances it is necessary to conduct partial or complete gene
380 sequencing of one or more of the IAV-S SIV genes (i.e. matrix, neuraminidase, haemagglutinin) to
381 ascertain the subtype of detected virus. Furthermore, virus genotyping based on gene sequencing
382 several or all gene segments is increasingly being used to determine and monitor virus diversity. Tests
383 should be validated for the region in which they are to be applied given the worldwide variability in IAV-
384 S. Population-wide validation data for these tests are not currently available.

385 ## 2. Serological tests

386 The primary serological test for detection of IAV-S SIV antibodies is the HI test and it is subtype specific.
387 Reference antigens should reflect what is circulating in the region and as broadly cross reactive as possible with
388 the specific subtype. It should be conducted on paired sera collected 10–21 days apart. A four-fold or greater
389 increase in titre between the first and second sample is suggestive of a recent IAV-S SIV infection. Additional
390 serological tests that have been described but not commonly used are the virus neutralisation, agar gel
391 immunodiffusion test, and indirect fluorescent antibody test. ELISA technology for detection of IAV-S antibodies
392 has been described in the literature and commercial kits have been marketed (Barbé *et al.,* 2009; Ciacci-Zanella
393 *et al.,* 2010 Lee *et al.,* 1993).

394 ### 2.1. Haemagglutination inhibition test

395 #### 2.1.1. Test procedure

396   i)   Dilute reference HA antigens (H1, H3, etc.) to a concentration of 4–8 HAU/25 µl in 0.01 M
397   PBS, pH 7.2.

398   ii)   *H1N1 test:* Heat inactivated sera for 30 minutes at 56°C. Dilute 1/10 in PBS. Add 0.1 ml
399   packed, washed erythrocytes to 1 ml of heat-inactivated, diluted serum and mix. Incubate
400   at room temperature for 30 minutes with periodic shaking every 10–15 minutes. Centrifuge
401   at 800 *g* for 10 minutes at 4°C. Note: Sera can be treated with RDE and erythrocytes as
402   described below in step iii as an alternative to heat inactivation and treating with packed
403   erythrocytes. While the use of RDE is encouraged, there may be regional variability in its
404   use for treatment of sera depending on serum specificity for some antigens used in the HI
405   assay.

406   iii)   *H1N2* and *H3N2 test:* Add 50 µl serum to 200 µl RDE (1/10 dilution in calcium saline
407   solution equalling 100 units per ml). Incubate overnight (12–18 hours) in a 37°C water
408   bath. Add 150 µl 2.5% sodium citrate solution and heat inactivate at 56°C for 30 minutes.
409   Combine 200 µl treated sample and 25 µl PBS. Add 50 µl of 50% erythrocytes. Shake and
410   incubate for 30 minutes at room temperature or overnight at 4°C. Centrifuge at 800 *g* for
411   10 minutes at 4°C.

412   iv)   Dispense 50 µl treated serum into two wells of a V- or U-bottom 96-well plate. Dispense
413   25 µl of treated serum into two wells to be used as a serum control. Positive and negative
414   control sera are treated in the same way as the unknown sera.

| 415 | | v) | Dispense 25 µl PBS in the serum control wells and all empty wells except two wells |
| 416 | | | identified as the cell control wells. Add 50 µl PBS in the cell control wells. |

417		vi)	Make serial twofold dilutions of the serum in 25 µl volumes in the plate and then add 25 µl
418			of appropriate antigen to all test wells except the serum control wells and the cell control
419			wells.

| 420 | | vii) | Incubate covered plates at room temperature (24°C) or 4°C for 30–60 minutes. |

421		viii)	Add 50 µl of 0.5% erythrocyte suspension to each well, shake, and incubate at room
422			temperature (24°C) or 4°C for 20–30 minutes until a distinct button forms at the bottom of
423			the cell control wells. Keep erythrocytes thoroughly suspended during the dispensing
424			process.

| 425 | | ix) | Conduct a HA test using the HI test antigens prior to and simultaneously to conducting the |
| 426 | | | HI test to verify that antigen concentrations are appropriate. |

427		x)	For the test to be valid, there should be no haemagglutination in the serum control well, no
428			inhibition of haemagglutination with the negative serum, the positive serum should have its
429			anticipated HI titre and the HA back titration should indicate 4–8 HAU per 25 µl.

2.2. Enzyme-linked immunosorbent assay (<u>Barbé *et al.,* 2009; Ciacci-Zanella *et al.,* 2010</u> ~~Lee *et al.,* 1993~~)

ELISA technology for detection of <u>(IAV-S)</u> ~~SIV~~ 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

<u>IAV-S infections</u> ~~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 <u>reducing virus transmission while</u> minimising the risk of exposure for <u>pigs and people</u> ~~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 <u>IAV-S</u> ~~SIV~~ strains circulating in the field. Haemagglutination inhibition and neutralisation tests demonstrating cross-reactivity between antisera from ~~animals~~ <u>pigs</u> 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~~ <u>virus</u>. All defining characteristics such as haemagglutinin and neuramindase subtype should be established. Haemagglutination inhibition and neuraminidase inhibition by subtype-specific antisera or <u>real-time</u> 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 H1~~N1~~ or H3~~N2~~ <u>IAV-S</u> ~~SIV~~, 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 antiserum or monoclonal antibody against <u>IAV-S</u> <s>SIV</s> 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. <u>IAV-S</u> <s>SIV</s> 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. <u>IAV-S</u> <s>SIV</s> 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 of cells used to propagate the seed, if not of porcine origin, and cell lines for any other species through which the seed has been passaged. Additionally, a cell line highly permissive for bovine viral diarrhoea virus, types 1 and 2, is recommended. Bovine viral diarrhoea virus is a potential contaminant introduced through the use of fetal bovine serum in cell culture systems.

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

515 completed product (see Chapter 1.1.7 *Tests for sterility and freedom from contamination of*
516 *biological materials*).

517 ii) Safety

518 An inactivation kinetics study should be conducted using the approved inactivating agent
519 on a viral lot with a titre greater than the maximum production titre and grown using the
520 approved production method. This study should demonstrate that the inactivation method
521 is adequate to assure complete inactivation of virus. Samples taken at regular timed
522 intervals during inactivation, and then inoculated on to a susceptible cell line or into the
523 allantoic sac of embyonated eggs, should indicate a linear and complete loss of titre by the
524 end of the inactivation process. This is represented as less than one infectious particle per
525 10^4 litres of fluids following inactivation.

526 iii) Batch potency

527 During production, antigen content is measured to establish that minimum bulk titres have
528 been achieved. Antigen content is generally measured before inactivation and prior to
529 further processing. Relative potency ELISA, HA, and HI are among the assays that can be
530 used to determine antigen content in final product. It is necessary to confirm the sensitivity,
531 specificity, reproducibility, and ruggedness of such assays.

532 The potency assay established at the time of the minimum antigen protection study should
533 be used to evaluate new lots for release. The assay needs to be specific and reproducible. It
534 must reliably detect vaccines that are not sufficiently potent. If laboratory animal serology is
535 used instead of swine serology, it should first be demonstrated that vaccination of the
536 laboratory animal induces a specific, sensitive, dose-dependent response as measured in
537 the potency assay and is correlated to protection in swine

538 **2.3. Requirements for authorisation**

539 **2.3.1. Safety requirements**

540 i) Target and non-target animal safety

541 Final container samples of completed product from inactivated vaccines should be tested
542 in young mice for safety. Generally, healthy pigs of weaning age or older and pregnant
543 sows at any stage of gestation may be safely vaccinated with inactivated IAV-S SIV
544 vaccines. Final product may be evaluated in the host animal using two animals of the
545 minimum age recommended for use, according to the instructions given on the label; the
546 animals are observed for 21 days. Field safety studies conducted on vaccinates, in at least
547 three divergent geographical areas, with at least 300 animals per area, are also
548 recommended.

549 ii) Reversion-to-virulence for attenuated/live vaccines

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

555 iii) Environmental consideration

556 Inactivated IAV-S SIV vaccines present no special danger to the user, although accidental
557 inoculation may result in an adverse reaction caused by the adjuvant and secondary
558 components of the vaccine. Modified live virus vaccines may pose a hazard to the user
559 depending on the level of inactivation of the virus.

560 Preservatives should be avoided if possible, and where not possible, should be limited to
561 the lowest concentration possible. The most common preservative is thimerosol, at a final
562 concentration not to exceed 0.01% (1/10,000). Antibiotics may be used as preservatives in
563 IAV-S SIV vaccines but are limited as to kinds and amounts. Also restricted are residual
564 antibiotics from cell culture media that may be present in the final product. For example,
565 the total amount of preservative and residual gentamicin is not to exceed 30 mcg per ml of
566 vaccine.

567 Vaccine bottles, syringes, and needles may pose an environmental hazard for vaccines
568 using adjuvants or preservatives and for modified live virus vaccines. Instructions for
569 disposal should be included within the vaccine packaging information and based on
570 current environmental regulations in the country of use.

571 **2.3.2. Efficacy requirements**

572 i) For animal production

573 A vaccination/challenge study in swine, using homologous and heterologous challenge
574 strains, will indicate the degree of protection afforded by the vaccine. Swine used in
575 vaccination/challenge studies should be free of antibodies against IAV-S ~~SIV~~ at the start of
576 the experiments. Vaccination/challenge studies should be conducted using virus produced
577 by the intended production method, at the maximum viral passage permitted, and using
578 swine of the minimum recommended age listed on the label. Initially, lots are formulated to
579 contain varying amounts of viral antigen. The test lot containing the least amount of
580 antigen that demonstrates protection becomes the standard against which future
581 production lots are measured. The most valuable criterion for blind trial evaluations of
582 treatment groups is a statistically significant reduction of virus (titres and duration of
583 shedding) in the respiratory tract of vaccinated pigs. Differences in clinical observations
584 and lung lesions are also among the criteria used in evaluation of a successful trial. If *in-*
585 *vivo* or *in-vitro* test methods are to be used to determine the potency of each production lot
586 of vaccine, those assays should be conducted concurrent with the minimum antigen
587 studies in order to establish the release criteria. Combination vaccines containing more
588 than one strain of IAV-S ~~SIV~~ are available in some countries. The efficacy of the different
589 components of these vaccines must each be established independently and then as a
590 combination in case interference between different antigens exists.

591 The duration of immunity and recommended frequency of vaccination of a vaccine should
592 be determined before a product is approved. Initially, such information is acquired directly
593 using host animal vaccination/challenge studies. The period of demonstrated protection,
594 as measured by the ability of vaccinates to withstand challenge in a valid test, can be
595 incorporated into claims found on the vaccine label. Once a suitable potency assay has
596 been identified, should antigenic drift require replacement of strains within the vaccine,
597 strains of the same subtype can be evaluated in either the host animal or a correlated
598 laboratory animal model. However, circulating strains may show significant antigenic
599 differences from the vaccine strain, but the vaccine strain may still provide protection. Also,
600 the vaccine may not protect against a new strain that appears to be antigenically similar to
601 the vaccine. Other factors that play a role include the adjuvant and the antigenic dose.
602 Consequently, it would appear that the efficacy of a vaccine will always have to be
603 evaluated in swine.

604 If the vaccine is to be used in swine destined for market and intended for human
605 consumption, a withdrawal time consistent with the adjuvant used (generally 21 days)
606 should be established by such means as histopathological examination submitted to the
607 appropriate food safety regulatory authorities.

608 ii) For control and eradication

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

614 **2.3.3. Stability**

615 Vaccines should be stored with minimal exposure to light at 4°C±2°C, or as approved by the
616 designated regulatory authorities. The shelf life should be determined by use of the approved
617 potency test over the proposed period of viability.

618 **REFERENCES**

619 BARBÉ F., LABARQUE G., PENSAERT M. & VAN REETH K. (2009). Performance of a commercial swine influenza virus
620 H1N1 and H3N2 antibody enzyme-linked immunosorbent assay in pigs experimentally infected with European
621 influenza viruses. *J. Vet. Diagn. Invest.*, **21**, 88–96.

622 BROOKES S.M., NÚÑEZ A., CHOUDHURY B., MATROSOVICH M., ESSEN S.C., CLIFFORD D., SLOMKA M.J., KUNTZ-SIMON
623 G., GARCON F., NASH B., HANNA A., HEEGAARD P.M., QUÉGUINER S., CHIAPPONI C., BUBLOT M., GARCIA J.M., GARDNER
624 R., FONI E., LOEFFEN W., LARSEN L., VAN REETH K., BANKS J., IRVINE R.M. & BROWN I.H. (2010). Replication,
625 pathogenesis and transmission of pandemic (H1N1) 2009 virus in non-immune pigs. *PLoS One*. Feb 5; **5** (2):
626 e9068. doi: 10.1371/journal.pone.0009068

627 BROWN I.H., HARRIS P.A., McCAULEY J.M. & ALEXANDER D.J. (1998). Multiple genetic reassortment of avian and
628 human influenza A viruses in European pigs, resulting in the emergence of the H1N2 virus of novel genotype. *J.
629 Gen. Virol.*, **79**, 2947–2955.

630 BROWN I.H., HILL M.L., HARRIS P.A., ALEXANDER D.J. & McCAULEY J.W. (1997). Genetic characterization of an
631 influenza A virus of unusual subtype (H1N7) isolated from pigs in England. *Arch. Virol.*, **142**, 1045–1050.

632 BROWN I.H. (2013). History and epidemiology of swine influenza in Europe. *Curr. Topics Microbiol. Immunol.*,
633 **370**, 133–146.

634 CASTRUCCI M.R., DONATELLI I., SIDOLI L., BARIGAZZI G., KAWAOKA Y. & WEBSTER R.G. (1993). Genetic reassortment
635 between avian and human influenza A viruses in Italian pigs. *Virology*, **193**, 503–506.

636 CHIAPPONI C., MORENO A., BARBIERI I., MERENDA M. & FONI E. (2012) Multiplex RT-PCR assay for differentiating
637 European swine influenza virus subtypes H1N1, H1N2 and H3N2. *J. Virol. Methods*, **184**, 117–120.

638 CHOI Y.K., LEE J.H., ERICKSON G., GOYAL S.M., JOO H.S., WEBSTER R.G. & WEBBY R. J. (2004). H3N2 influenza virus
639 transmission from swine to turkeys, United states. *Emerg.Infect. Dis.*, **10**, 2156–2160.

640 CIACCI-ZANELLA J.R., VINCENT A.L., PRICKETT J.R., ZIMMERMAN S.M. & ZIMMERMAN J.J. (2010). Detection of anti-
641 influenza A nucleoprotein antibodies in pigs using a commercial influenza epitope-blocking enzyme-linked
642 immunosorbent assay developed for avian species. *J. Vet. Diagn. Invest.*, **22**, 3–9.

643 DONE S.H. & BROWN I.H. (1997). Swine influenza in the United Kingdom, past and present. *Large Anim. Pract.*, **2**,
644 20–28.

645 GRAMER M.R. (2007). SIV: an update on circulating strains, advances in diagnostic tests and interpretation of test
646 results. *In:* Proceedings of the American Association of Swine Veterinarians, 3–6 March 2007, Orlando, Florida,
647 USA.

648 HOFFMAN E., STECH J., GUAN Y., WEBSTER R.G. & PEREZ D.R. (2001). Universal primers set for the full length
649 amplification of all influenza A viruses. *Arch. Virol.*, **146**, 2275–2289.

650 KARASIN A.I., BROWN I.H., CARMAN S. & OLSEN C.W. (2000a). Isolation and characterization of H4N6 avian influenza
651 viruses from pigs with pneumonia in Canada. *J. Virol.*, **74**, 9322–9327.

652 KARASIN A.I., LANDGRAF J., SWENSON S., ERICKSON G., GOYAL S., WOODRUFF M., SCHERBA G., ANDERSON G. & OLSEN
653 C.W. (2002). Genetic characterization of H1N2 influenza A viruses isolated from pigs throughout the United
654 States. *J. Clin. Microbiol.*, **40**, 1073–1079.

655 KARASIN A.I., SCHUTTEN M.M., COOPER L.A., SMITH C.B., SUBBARAO K., ANDERSON G.A., CARMAN S. & OLSEN C.W.
656 (2000). Genetic characterization of H3N2 influenza viruses isolated from pigs in North America, 1977-1999:
657 evidence for wholly human and reassortant virus genotypes. *Virus Res.*, **68**, 71–85.

658 KARASIN A.I., WEST K., CARMAN S. & OLSEN C.W. (2004) Characterization of avian H3N3 and H1N1 influenza A
659 viruses isolated from pigs in Canada. *J. Clin. Microbiol.*, **42**, 4349–4354.

660 LANDOLT G., KARASIN A.I. HOFER C., MAHANEY J., SVAREN J. & OLSEN C.W. (2004). Use of real-time reverse
661 transcriptase polymerase chain reaction assay and cell culture methods for detection of swine influenza A viruses.
662 *Am. J. Vet. Res.*, **66**, 119–124.

663 LEE B.W., BEY R.F., BAARSCH M.J. & EMERY D.A. (1993). Subtype specific ELISA for the detection of antibodies
664 against influenza A H1N1 and H3N2 in swine. *J. Virol. Methods*, **45**, 121–136.

665 LINDSTROM S., GARTEN R., BALISH A., SHU B., EMERY S., BERMAN L., BARNES N., SLEEMAN K., GUBAREVA L.,
666 VILLANUEVA J. & KLIMOV A. (2012). Human infections with novel reassortant influenza A(H2N2)v viruses, United
667 States, 2011. *Emerg. Infect. Dis.*, **18**, 834–837.

668 MA W., WINCENT A.L., GRAMER M.R., BROCKWELL C.B., LAGER K.M., JANKE B.H., GAUGER P.C., PATNAYAK D.P.,
669 WEBBY R.J. & RICHT J.A. (2007). Identification of H23 influenza A viruses from swine in the United States. *Proc.*
670 *Natl. Acad. Sci.,* **104**, 20940–20954.

671 MAROZIN S., GREGORY V., CAMERON K., VALETTE M., AYMARD M., FONI E., BARIGAZZI G., LIN Y. & HAY A. (2002).
672 Antigenic and genetic diversity among swine influenza A H1N1 and H1N2 viruses in Europe. *J. Gen. Virol.,* **83**,
673 735–745.

674 MYERS K.P., OLSEN C.W., & GRAY G.C. (2007). Cases of swine influenza in humans: a review of the literature. *Clin.*
675 *Infect. Dis,* **44**, 1084–1088.

676 NAGARAJAN M.M., SIMARD G., LONGTIN D. & SIMARD C. (2010). Single-step multiplex conventional and real-time
677 reverse transcription polymerase chain reaction assays for simultaneous detection and subtype differentiation of
678 Influenza A virus in swine. *J. Vet. Diagn. Invest.,* **22**, 402–408.

679 NOBLE S., MCGREGOR M.S., WENTWORTH D.E. & HINSHAW V.S. (1993). Antigenic and genetic conservation of the
680 haemagglutinin in H1N1 swine influenza viruses. *J. Gen. Virol.,* **74**, 8243–8251.

681 OLSEN C.W. (2002). Emergence of novel strains of swine influenza virus in North America, *In:* Trends in Emerging
682 Viral Infections of Swine, Morilla A., Yoon K.J. & Zimmerman J.J., eds. Iowa State University Press, Iowa, USA,
683 37–43.

684 OLSEN C.W., BRAMMER L., EASTERDAY B.C., ARDEN N., BELAY E., BAKER I. & COX N. J. (2002). Serologic evidence of
685 H1 swine influenza virus infection in swine farm residents and employees. *Emerg. Infect. Dis.,* **8**, 814–815.

686 OLSEN C.W., BROWN I., EASTERDAY B.C. & VAN REETH K. (2005). Swine Influenza. *In:* Diseases of Swine, Straw B.,
687 D'Allaire S., Zimmerman J. & Taylor D., eds. Iowa State University Press, Iowa, USA, 469–482.

688 PEIRIS J. S. M., GUAN Y., MARKWELL D., GHOSE P., WEBSTER R. G. & SHORTRIDGE K. F. (2001). Cocirculation of avian
689 H9N2 and contemporary "human" H3N2 influenza A viruses in pigs in southeastern China: potential for genetic
690 reassortment. *J. Virol.,* **75**, 9679–9686.

691 PHIPPS L.P., ESSEN S.C. & BROWN I.H. (2004) Genetic subtyping of influenza A viruses using RT/PCR with a single
692 set of primers based on conserve sequences within the HA2 coding region. *J. Virol. Methods,* 122, 119–122.

693 SCHRADER C. & SÜSS J. (2004). Molecular epidemiology of porcine H3N2 influenza viruses isolated in Germany
694 between 1982 and 2001. *Intervirolgy,* **47**, 72–77.

695 SENNE D.A. (1998). Virus propagation in embryonating eggs. *In:* A Laboratory Manual for the Isolation and
696 Identification of Avian Pathogens, Fourth Edition, Swayne D.E., Glisson J.R., Jackwood M.W., Pearson J.E. &
697 Reed W.M., eds. American Association of Avian Pathologists, Kennett Square, Pennsylvania, USA, 235–240.

698 SHEERAR M.G., EASTERDAY B.C. & HINSHAW V.S. (1989). Antigenic conservation of H1N1 swine influenza viruses.
699 *J. Gen. Virol.,* **70**, 3297–3303.

700 SLOMKA M.J., DENSHAM A.L., COWARD V.J., ESSEN S., BROOKES S.M., IRVINE R.M., SPACKMAN E., RIDGEON J.,
701 GARDNER R., HANNA A., SUAREZ D.L. & BROWN I.H. (2010). Real time reverse transcription (RRT)-polymerase chain
702 reaction (PCR) methods for detection of pandemic (H1N1) 2009 influenza virus and European swine influenza A
703 virus infections in pigs. *Influenza Other Respir. Viruses,* **4**, 277–293.

704 SPACKMAN E., SENNE D.A., MYERS T.J., BULAGA L.L., GARBER L.P., PERDUE M.L., LOHMAN K., DAUM L.T. & SUAREZ
705 D.L. (2002) Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian
706 H5 and H7 hemagglutinin subtypes. *J. Clin. Microbiol.,* **40**, 3256–3260.

707 SWENSON S.L., VINCENT L.L, LUTE B.M., JANKE B.H., LECHTENBERG K.E., LANDGRAF J.G., SCHMITT B.J., KINKER D.R. &
708 MCMILLEN J.K. (2001). A comparison of diagnostic assays for the detection of type A swine influenza virus from
709 nasal swabs and lungs. *J. Vet. Diagn. Invest.,* **13**, 36–42.

710 VINCENT L.L., JANKE B.H., PAUL P.S. & HALBUR P.G. (1997). A monoclonal-antibody-based immunohistochemical
711 method for the detection of swine influenza virus in formalin-fixed, paraffin-embedded tissues. *J. Vet. Diagn.*
712 *Invest.,* **9**, 191–195.

713 VINCENT A.L., MA W., LAGER K.M., GRAMER M.R., RICHT J.A. & JANKE B.H.(2009). Characterization of a newly
714 emerged genetic cluster of H1N1 and H1N2 swine influenza virus in the United States. *Virus Genes,* **39** (2), 176–
715 185. doi: 10.1007/s11262-009-0386-6

716 WEBBY R.J., ROSSOW K., ERICKSON G., SIMS Y. é WEBSTER R. (2004). Multiple lineages of antigenically and
717 genetically diverse influenza A virus co-circulate in the United States swine population. *Virus Res.*, **103**, 67–73.

718 ~~WEBBY R.J., SWENSON S.L., KRAUSS S.L., GERRISH P.J., GOYAL S.M. & WEBSTER R.G. (2000). Evolution of swine~~
719 ~~H3N2 influenza viruses in the United States. *J. Virol.,* **74**, 8243–8251.~~

720 ZHOU N.N., SENNE D.A., LANDGRAF J.S., SWENSON S.L., ERICKSON G., ROSSOW K., LIU L., YOON K.J., KRAUSS S. &
721 WEBSTER R.G. (1999). Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. *J.*
722 *Virol.*, **73**, 8851–8856.

723   *

724   *   *

725   **NB:** There are OIE Reference Laboratories for Swine influenza
726   (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date
727   list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
728   Please contact the OIE Reference Laboratories for any further information on
729   diagnostic tests, reagents and vaccines for swine influenza

730

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. Syncytial endothelial cells containing viral antigen are common in capillaries and arterioles. HeV infection of horses is not uniformly fatal and some horses manifesting clinical signs survive infection. 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. Immunohistochemical lesions are found in either or both the respiratory system (tracheitis and bronchial and interstitial pneumonia) and the brain (meningitis) of infected animals. Syncytial cells containing viral antigen are seen in small blood vessels, lymphatic vessels and the respiratory epithelium.

Both viruses affect companion animals. 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 rates; there is serological evidence that some dogs survive infection. Experimentally NiV causes a similar disease to HeV in cats. Syncytial endothelial cells containing viral antigen were demonstrated in both HeV and NiV infections in cats and in NiV infection in dogs.

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

47   *Malaysia and Singapore, but <u>limited</u> human-to-human transmission is suspected*
48   *in ~~recent~~ outbreaks of NiV in Bangladesh.*

49   *HeV and NiV are closely related members of the ~~family Paramyxoviridae. Differences between~~*
50   *~~them and other family members have led to their classification in a new~~ genus, Henipavirus, in*
51   *~~the~~ subfamily Paramyxovirinae, <u>family</u> Paramyxoviridae. HeV and NiV are <u>dangerous human</u>*
52   *<u>pathogens such as are designated in laboratory risk management analyses as requiring</u> biosafety*
53   *level 4 (<u>BSL4</u>) ~~agents~~ <u>containment</u>. It is important that samples from suspect animals be*
54   *transported to authorised laboratories only under biologically secure conditions according to*
55   *international regulations.*

56   ***Identification of the agent:*** *Both HeV and NiV may be propagated in a range of cultured cells.*
57   *Virus isolation from unfixed field samples should be attempted, but only in situations where operator*
58   *safety can be assured. Identification procedures following virus isolation include immunostaining of*
59   *infected cells, neutralisation with specific antisera and molecular characterisation. Real-time*
60   *<u>reverse-transcriptase</u> polymerase chain reaction (RT-PCR) is now available as a diagnostic test.*

61   *Viral antigen is present in vascular endothelium, and in the case of NiV in pigs, the respiratory*
62   *epithelium. A wide range of formalin-fixed tissues can be examined to detect HeV and NiV*
63   *antigens. Submissions for immunohistochemistry should include samples of brain at various levels*
64   *including meninges, lung, spleen and kidney. In pregnant animals or in cases of abortion, uterus,*
65   *placenta and fetal tissues should be included as appropriate. Specimens for virus isolation and*
66   *molecular detection of virus should be fresh tissues from the same organs, <u>and/</u>or urine<u>,</u> ~~or~~ throat or*
67   *nasal swabs.*

68   ***Serological tests:*** *Virus neutralisation tests (VNT) and enzyme-linked immunosorbent assay*
69   *(ELISA) are available. VNT is currently accepted as the reference procedure. The ability of antisera*
70   *to HeV and NiV to cross-neutralise to a limited degree means that a single VNT using either virus*
71   *does not provide definitive identification of antibody specificity. Neutralising antibodies to HeV and*
72   *NiV can be differentiated by the greater capacity to neutralise the homologous compared with the*
73   *heterologous virus. This may not be a major impediment in outbreak situations where the causative*
74   *agent is known, but serum samples from suspect cases or from areas of the world other than*
75   *Australia and Malaysia should be subjected to VNT analyses with both HeV and NiV. The*
76   *serological relationship between HeV and NiV ensures that ELISAs using HeV or NiV antigen can*
77   *be used to detect antibodies to both viruses.*

78   ***Requirements for vaccines and diagnostic biologicals:*** *There <u>is a vaccine available for HeV,</u>*
79   *<u>registered for use in horses in Australia</u>. There ~~are~~ no vaccine~~s~~ currently available for ~~either HeV~~*
80   *~~or~~ NiV.*

A. INTRODUCTION

82   Hendra virus (HeV) and Nipah virus (NiV) <u>are classified in the family *Paramyxoviridae* subfamily *Paramyxovirinae*,</u>
83   <u>genus *Henipavirus*. They have morphological and physicochemical properties typical of paramyxoviruses. The</u>
84   <u>viruses are pleomorphic in shape and enveloped, with herringboned nucleocapsids. Virions are 40–600 nm in</u>
85   <u>diameter. Glycoprotein and fusion protein spikes project through a lipid envelope. HeV and NiV have a non-</u>
86   <u>segmented, single-stranded, negative-sense RNA genome (18.2 kb) consisting of six genes which code for six</u>
87   <u>major structural proteins, namely: N (nucleocapsid protein), P (phosphoprotein), M (matrix protein), F (fusion</u>
88   <u>protein), G (glycoprotein) and L (large protein).</u>

89   <u>HeV and NiV</u> occur naturally as viruses of fruit bats commonly known as 'flying foxes'. <u>These are</u> members of the
90   genus *Pteropus,* <u>family *Pteropodidae*</u>. Antibodies to HeV are found in <u>all</u> ~~approximately 50% of the~~ four Australian
91   pteropus species <u>with seroprevalence varying over time and location</u> ~~(Young *et al.*, 1996). Serological surveys of~~
92   ~~antibodies to NiV show seroprevalances up to 20% in Malaysian pteropid bats (Epstein *et al.*, 2006; Johara *et al.*,~~
93   <u>In one bat colony, seroprevalence steadily increased from 45% to 69% over a 2-year period supporting a model of</u>
94   <u>endemic infection in the populations.</u> Antibodies to NiV or putative closely related viruses have subsequently been
95   detected in pteropid bats in Bangladesh ~~(Hsu *et al.*, 2004)~~, Cambodia (Olson *et al.*, 2002; Reynes *et al.*, 2005),
96   Indonesia (Sendow *et al.*, 2006), Madagascar (Iehle *et al.*, 2007) and Thailand (Wacharapluesadee *et al.*, 2005).
97   HeV has been isolated from Australian flying foxes (Halpin *et al.*, 2000), and NiV from flying foxes from Malaysia
98   and Cambodia (Chua *et al.*, 2002; Reynes *et al.*, 2005). NiV RNA has been detected by polymerase chain
99   reaction (PCR) in pteropid bat urine, saliva and blood in Thailand (Wacharapluesadee & Hemachudha, 2007;
100   Wacharapluesadee *et al.*, 2005). <u>In Ghana, 39% of *Eidolon helvum,* a non-pteropus fruit bat, had NiV reactive</u>

101   antibodies (Hayman *et al.*, 2008). Henipavirus-like sequences were also obtained from *Eidolon helvum* in Ghana.
102   The detection of antibodies to and sequences of henipaviruses in African bats suggests that the range of potential
103   NiV infections may be wider than previously thought, though no human cases of NiV have been reported from any
104   region other than South-East Asia.

105   HeV disease emerged in Brisbane, Australia, in September 1994 in an outbreak of acute respiratory disease that
106   killed 13 horses and a horse trainer (Murray *et al.*, 1995). The virus was initially called equine morbillivirus, but
107   subsequent genetic analyses indicated that it did not resemble morbilliviruses sufficiently closely ~~enough to merit~~
108   for inclusion in that genus. There have been other instances of fatal HeV infection of horses in northern
109   Queensland and further instances of infection of people. Two horses developed an acute disease and died almost
110   1 month before the Brisbane outbreak, but HeV was determined to be the cause of death only after the horse
111   owner, who probably acquired HeV during necropsy of the horses, died 13 months later with HeV-mediated
112   encephalitis (Rogers *et al.*, 1996). Since then there have been more than 40 outbreaks involving more than
113   75 horses but with only two outbreaks involving more than three horses. The seven human cases have resulted in
114   four deaths (57%). All infected people have had very close contact with infected body fluids from horses through
115   performing invasive procedures and/or have not worn fully protective PPE. ~~A third horse died in January 1999~~
116   ~~with no associated human disease (Field *et al.*, 2000). Two further equine cases occurred in 2004, one confirmed~~
117   ~~and the other unconfirmed, the latter identified by an associated human infection (Hanna *et al.*, 2006). In 2006~~
118   ~~Australia reported two further cases in horses, one in Southern Queensland and one in northern New South~~
119   ~~Wales. Detailed reports of the three most recent human cases have not been published, but all three people were~~
120   ~~infected by contact with horses. One of the two cases reported in 2008, as a well as a case the occurred in~~
121   ~~August 2009, were fatal.~~

122   In Malaysia, retrospective studies of archival histological specimens indicate that NiV has caused low mortality in
123   pigs since 1996, but remained unknown until 1999 when it emerged as the causative agent of an outbreak of
124   encephalitis in humans that had commenced in 1998 (Chua *et al.*, 2000; Nor *et al.*, 2000). Unlike respiratory
125   disease caused by HeV in horses, which was frequently fatal but characterised by poor transmissibility
126   (Williamson *et al.*, 1998), respiratory disease caused by NiV in pigs was often subclinical but highly contagious
127   (Hooper *et al.,* 2001), properties that led to rapid virus dispersal through the Malaysian pig population and forced
128   authorities to choose culling as the primary means to control spread (Nor *et al.*, 2000). Over one million pigs were
129   destroyed; 106 of 267 infected humans, mostly pig farmers in Malaysia and abattoir workers in Singapore who
130   had direct contact with live pigs, died of encephalitis (Chua *et al.*, 2000; Paton *et al.*, 1999). Dogs and horses
131   were also infected on infected pig farms during that outbreak (Hooper *et al.*, 2001) but the infections were not
132   epidemiologically significant.

133   New foci of human NiV disease have subsequently been identified on an annual basis in Bangladesh, with a few
134   outbreaks in West Bengal, in neighbouring ~~and~~ India. In outbreaks in 2001 and 2003 an animal source of the
135   human infections was not identified ~~(Hsu *et al.*, 2004)~~, but pteropid bats, *Pteropus giganteus*, were present and
136   had antibodies capable of neutralising Nipah virus. Clustering of cases and time–sequence studies indicated that
137   there is human-to-human transmission but at low levels ~~(Hsu *et al.*, 2004)~~. In another outbreak in 2004 in which
138   27 of 36 infected humans died, epidemiological evidence indicated person-to- person transmission and
139   serological studies identified seropositive fruit bats at the location ~~(Anonymous, 2004)~~. Drinking fresh date palm
140   sap contaminated by fruit bat saliva, urine or excreta has been identified as ~~one possible~~ the likely route of
141   transmission from the wildlife reservoir to humans (Luby *et al.*, 2006). As a result of these ongoing outbreaks it is
142   estimated that across Malaysia, Singapore, Bangladesh and India there have now been up more than 585 ~~to 400~~
143   cases of NiV in humans, with approximately 334 ~~200~~ deaths.

144   ~~NiV and HeV are classified taxonomically as paramyxoviruses in the subfamily Paramyxovirinae, and have been~~
145   ~~grouped in a separate and new genus, the henipaviruses (Eaton *et al.*, 2006).~~

146   Diagnosis of disease caused by henipaviruses is by virus isolation, detection of viral RNA in clinical or post-
147   mortem specimens or demonstration of viral antigen in tissue samples taken at necropsy (Daniels *et al.*, 2001).
148   Detection of specific antibody can also be useful particularly in pigs where NiV infection may go unnoticed.
149   Identification of HeV antibody in horses is less useful because of the high case fatality rate of infection in that
150   species. Human infections of both HeV and NiV have been diagnosed retrospectively by serology. Demonstration
151   of specific antibody to HeV or NiV in either animals or humans is of diagnostic significance because of the rarity of
152   infection and the serious zoonotic implication of transmission of infection.

153   The henipavirus genus is expanding, with new viruses recently identified. Cedar virus was isolated from the urine
154   of pteropus bats in Australia, but it remains to be seen if it has the capacity to spill over to other species, and if so,
155   cause disease (Marsh *et al.*, 2012). A number of other henipa-like viruses have been detected by PCR and
156   sequencing, but have not yet been isolated by traditional virus isolation techniques (Wu *et al.*, 2014).

157

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of henipaviruses and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification[1]						
Virus isolation	–	±	–	+++	–	–
Real-time RT-PCR	+	±	++	+++	++	–
IHC	–	–	–	++	–	–
IFA	–	–	–	++	–	–
Detection of immune response[2]						
ELISA	+++	+++	+++	++	+++	+++
VNT	+++	+++	+++	++	+++	+++
Luminex	+++	+++	+++	++	+++	+++

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).

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[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.

1.1.1. Sampling and submission of ~~samples~~ specimens

Diagnostic ~~samples~~ specimens should be submitted to designated laboratories in specially designed containers. ~~The International Air Transport Association (IATA), Dangerous Goods Regulations (DGR) for shipping specimens from a suspected zoonotic disease must be followed (International Air Transport Association, 2002).~~ Recommendations made by the United Nations Committee of Experts on the Transport of Dangerous Goods (UNCETDG), a committee of the United Nations Economic and Social Council must be followed (http://www.unece.org/trans/danger/publi/unrec/rev17/17files_e.html) The requirements are summarised in Chapter 1.1.~~1~~ 2 *Transport of specimens of animal origin ~~Collection and shipment of diagnostic specimens~~*. The range of tissues yielding virus in natural and experimental cases has been summarised (Daniels *et al.*, 2000). ~~Brain, lung, kidney and spleen should always be submitted.~~ Swabs (nasal and/or oronasal) and serum should always be submitted. Urine can also be useful, if it can be collected. If appropriate biosafety precautions can be taken during their collection then brain, lung, kidney and spleen can also be submitted. ~~Samples~~ Specimens should be transported at 4°C if they can arrive at the laboratory within 48 hours; if shipping time will be over 48 hours, the samples should be sent frozen on dry ice or nitrogen vapours should be used. ~~Samples~~ specimens should not be held at –20°C ~~for long periods~~.

1.1.2. Isolation in cultured cells

Virus propagation should be conducted under BSL4 conditions. Strict adherence to this guideline would limit the handling of diagnostic specimens where the presence of HeV or NiV may be suspected but not confirmed to laboratories with BSL4 facilities. Primary virus isolation from suspect samples may of necessity be conducted under BSL3 conditions. However, if this is to be attempted, stringent local guidelines must be developed to ensure operator safety and applied if a 'paramyxovirus-like' cytopathic effect (CPE) develops in infected cultures. Such guidelines will emphasise good laboratory practice, the use of class II safety cabinet with appropriate personal protective equipment or a class III cabinet and may require acetone fixation of infected cells, to destroy infectious virus, followed by immunofluorescent detection of henipavirus antigen. The culture medium from henipavirus-positive cells should be transferred to a BSL4 laboratory

At the recipient laboratory tissues are handled under sterile conditions, and 10% (w/v) suspensions are generated by grinding the tissues in a ~~closed~~ homogenisation system, preferably a closed system ~~e.g. stomacher/bag mixer using plastic bag or mixer mills using autoclavable steel balls in closed metal cylinders~~. All processes should be carried out in a Class III cabinet or a Class II cabinet with appropriate personal protective equipment for the operator ~~with the stomacher operated in the cabinet~~. Tubes should have O-rings, and they should have an external thread ~~the centrifuge pots, with aerosol covers, loaded and unloaded in the cabinet~~. Following clarification of the homogenate by centrifugation at 300 g, the supernatant is added to cultured cell monolayers. Virus isolation is aided by the fact that HeV and NiV grow rapidly to high titre in many cultured cells. African green monkey kidney (Vero) and rabbit kidney (RK-13) cells have been found to be particularly susceptible. HeV also replicates in suckling mouse brain and in embryonated hens eggs, and although the former may represent a viable method of primary isolation, there are no data on the relative susceptibility of *in-vivo* systems such as these compared with the more convenient cell culture systems. A CPE usually develops within 3 days, but two 5-day passages are recommended before judging the attempt unsuccessful. After low multiplicity of infection, the CPE is characterised by formation of syncytia that may, after 24–48 hours, contain over 60 or more nuclei. Syncytia formed by NiV in Vero cell monolayers are significantly larger than those created by HeV in the same time period. Although the distribution of nuclei in NiV-induced syncytia early in infection resembles that induced by HeV, with nuclei aggregated in the middle of the syncytia, nuclei in mature NiV-induced syncytia are distributed around the outside of the giant cell ~~(Hyatt *et al.*, 2001)~~.

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 fulfil this function both in primary identification of the virus~~

245	~~upon isolation and for use in immunohistochemical examination of tissues from suspect~~
246	~~cases.~~

247   **a)**   **Test procedure**

248   Under BSL4 <u>laboratory conditions</u> monolayers of Vero or RK-13 cells grown on glass
249   cover-slips or <u>in</u> chamber slides are infected with the isolated virus, and the
250   monolayers are examined for the presence of syncytia after incubation for 24–
251   48 hours at 37°C. It is recommended that a range of virus dilutions (undiluted, 1/10,
252   1/100) be tested because syncytia are more readily observed after infection at low
253   multiplicity. Once visible syncytia are detected, infected cells are fixed by immersion
254   in a vessel filled completely with acetone. The vessel is sealed and surface sterilised
255   prior to removal to a less secure laboratory environment, for example BSL2, where
256   the slides are air-dried. Viral antigen is detected using anti-HeV or anti-NiV antiserum
257   and standard immunofluorescent procedures. A characteristic feature of henipavirus-
258   induced syncytia is the presence of large polygonal structures containing viral
259   antigen. ~~These are observed most readily with monospecific and MAbs to the~~
260   ~~nucleocapsid protein N and phosphoprotein P.~~

261   ii)   Immunoelectron microscopy

262   The high titres generated by HeV and NiV in cells in vitro permits their visualisation in the
263   culture medium by negative-contrast electron microscopy without a centrifugal
264   concentration step. Detection of virus–antibody interactions by immunoelectron
265   microscopy provides valuable information on virus structure and antigenic reactivity, even
266   during primary isolation of the virus. Other ultrastructural techniques, such as grid cell
267   culture (Hanna *et al.*, 2006), in which cells are grown, infected and visualised on electron
268   microscope grids, and identification of replicating viruses and inclusion bodies in thin
269   sections of fixed, embedded cell cultures and infected tissues complement the diagnostic
270   effort. The details of these techniques and their application to the detection and analysis of
271   HeV and NiV have been described ~~(Hyatt *et al.*, 2001)~~.

1.2. Virus neutralisation: differentiation of HeV and NiV

273 Neutralisation tests rely on quantification methods ~~and three procedures are available to titre HeV and~~
274 ~~NiV~~. In the ~~traditional plaque and~~ microtitre assay procedure~~s~~, the titre is calculated as ~~plaque forming~~
275 ~~units (PFU) or~~ the tissue culture infectious dose capable of causing CPE in 50% of replicate wells
276 ($TCID_{50}$) ~~respectively~~.

277 In an alternative procedure, the viruses are titrated on Vero cell monolayers in 96-well plates and after
278 18–24 hours, foci of infection are detected immunologically in acetone-fixed cells using anti-viral
279 antiserum ~~(Crameri *et al.*, 2002)~~. The virus titre is expressed as focus-forming units (FFU)/ml.

280 ~~Neutralisation assays using these three methods are described below.~~ A virus isolate that reacts with
281 anti- HeV and/or anti-NiV antisera in an immunofluorescence assay is considered to be serologically
282 identical to either HeV or NiV if it displays the same sensitivity to neutralisation by anti-HeV and anti-
283 NiV antisera as HeV or NiV. Anti-HeV antiserum neutralises HeV at an approximately four-fold greater
284 dilution than that which neutralises NiV to the same extent. Conversely, anti-NiV antiserum neutralises
285 NiV approximately four times more efficiently than HeV (Chua *et al.*, 2000). Virus quantification
286 procedures should be conducted at BSL4. A new version of the differential neutralisation test has been
287 recently described, which avoids the use of infectious virus by the use of ephrin-B2-bound biospheres
288 (Bossart *et al.*, 2007). Although the test has yet to be formally validated, it appears to have the potential
289 to be a screening tool for use in countries without BSL4 facilities.

1.2.1. <u>Microtitre neutralisation</u> ~~Plaque reduction~~

291 <u>This procedure is dependent on the availability of anti-serum, specific for HeV and NiV, as well</u>
292 <u>as stock viruses.</u> Stock HeV and NiV and the unidentified henipavirus are diluted ~~in media and~~
293 ~~replicates of each virus containing approximately 100 PFU in 50–100 µl are mixed with an equal~~
294 ~~volume of either Eagle's minimal essential media (EMEM) or a range of dilutions of anti-HeV or~~
295 ~~anti-NiV antiserum in EMEM. The virus–antiserum mixtures are incubated at 37°C for 45~~
296 ~~minutes, adsorbed to monolayers of Vero cells at 37°C for 45 minutes and the number of~~
297 ~~plaques determined by traditional plaque assay procedures after incubation at 37°C for 3 days.~~

~~1.2.2. Microtitre neutralisation~~

299 ~~Stock HeV and NiV and the unidentified~~ and replicates of each virus containing approximately
300 100 TCID$_{50}$ in 50 µl are added to the test wells of a flat bottom 96-well microtitre plate. The
301 viruses are mixed with an equal volume of either EMEM or a range of dilutions of anti-HeV or
302 anti-NiV antiserum in EMEM. The mixtures are incubated at 37°C for 45 minutes and
303 approximately 2.4 × 10^{4} cells are added to each well to a final volume of approximately 200 µl.
304 After 3 days at 37°C, the test is read using an inverted microscope and wells are scored for the
305 degree of CPE observed. Those that contain cells only or cells and antiserum should show no
306 CPE. In contrast, wells containing cells and virus should show syncytia and cell destruction. A
307 positive well is one where all or a proportion of cells in the monolayer form large syncytia typical
308 of henipavirus infection.

309 **1.3. Molecular methods – detection of nucleic acid**

310 ~~iii)   Immune plaque assay~~

311 ~~Vero cells (2 × 10⁴ in 200 µl medium/well) are added to flat-bottom microtitre plates grown overnight at~~
312 ~~37°C. Stock HeV and NiV and the unidentified Henipavirus are diluted and replicates containing about~~
313 ~~60 FFU/50 µl are mixed with an equal volume of either EMEM or a range of dilutions of anti-HeV and~~
314 ~~anti-NiV antisera diluted in EMEM. Virus–antiserum mixtures are incubated for 45 minutes at 37°C and~~
315 ~~adsorbed to Vero cell monolayers for 45 minutes at 37°C with 5% CO₂. Virus–antiserum mixtures are~~
316 ~~removed, 200 µl EMEM is added to each well and incubation is continued at 37°C. After 18–24 hours~~
317 ~~the culture medium is discarded and plates are immersed in cold, absolute acetone for 10 minutes and~~
318 ~~then placed in plastic bags, which are filled with acetone, heat-sealed and surface sterilised with 4%~~
319 ~~(v/v) lysol during removal from the BSL4 laboratory. Gluteraldehyde can also be used for sterilisation at~~
320 ~~concentrations as low as 0.1% for 24 hours. It is recommended that each laboratory determine the~~
321 ~~concentration of gluteraldehyde required for sterilisation within the time frame required. Acetone-fixed~~
322 ~~plates are air-dried, the wells are blocked with phosphate buffered saline (PBS) containing 0.05%~~
323 ~~Tween 20 and 2% skim milk powder, and incubated for 30 minutes at 37°C with antiserum to either~~
324 ~~HeV or NiV or a monospecific antiserum to a virus protein. Anti-viral antibody binding to syncytia can~~
325 ~~be detected using alkaline phosphatase-conjugated species-specific antibody and the substrate 5-~~
326 ~~bromo-4-chloro-3-indolyl phosphate and p-nitro blue tetrazolium substrate (NBT/BCIP; Promega,~~
327 ~~Catalog number S3771). When purple plaques appear against a clear background (10–30 minutes),~~
328 ~~the substrate is removed and the plates are rinsed with distilled water and air-dried. Plaques are~~
329 ~~counted using a magnifying glass.~~

330 ~~**1.3. Nucleic acid based recognition methods**~~

331 The complete genomes of both HeV and NiV have been sequenced ~~(Wang *et al.*, 2001)~~, and <u>as more</u>
332 <u>isolates come to hand their sequences have been deposited on Genbank.</u> PCR-based methods <u>are</u>
333 <u>commonly</u> ~~have been~~ used to detect virus. <u>They have the biosafety advantage of not propagating live</u>
334 <u>infectious virus</u> and <u>they have been</u> ~~are being~~ validated in a number of laboratories. <u>They are also</u>
335 <u>highly sensitive and specific.</u>

336 **1.3.1. Real-time reverse transcription polymerase chain reaction**

337 A particularly sensitive and useful approach to the detection of henipavirus genome in
338 specimens is real-time <u>RT-</u>PCR <u>(see Table 2)</u> ~~This method has the biosafety advantage of not~~
339 ~~propagating live infectious virus~~. Test methods and primers used depend on the technology
340 platform and associated chemistry being used in individual laboratories (Mungall *et al.*, 2006;
341 Wacharapluesadee & Hemachudha, 2007). <u>The HeV M gene (Smith *et al.*, 2001) and N gene</u>
342 <u>(Feldman *et al.*, 2009) TaqMan assays are the primary tests for Hendra virus disease</u>
343 <u>diagnosis.</u> ~~The virus-specific reagents used in one such assay (Mungall *et al.*, 2006) based on~~
344 ~~Taqman chemistry are as follows:~~

345 *Table 2.* <u>Real-time RT PCR (Taqman) assays for the detection of HEV and NIV</u>

Assay	**Oligo**	**Name**	**Primer sequence (5'–3')**	**Probe label (5'–3')**
<u>HeV_TQM_M</u>	<u>Forward</u>	<u>HeV M 5755F</u>	<u>CTT-CGA-CAA-AGA-CGG-AAC-CAA</u>	
	<u>Reverse</u>	<u>HeV M 5823R</u>	<u>CCA-GCT-CGT-CGG-ACA-AAA-TT</u>	
	~~(HENDRA N1433F)~~ <u>Probe</u>	HeV M 5778P	<u>TGG-CAT-CTT-TCA-TGC-TCC-ATC-TCG-G</u> ~~5' TCA GAT CCA GAT TAG CTG CAA 3'~~	FAM-TAMRA

Assay	Oligo	Name	Primer sequence (5'–3')	Probe label (5'–3')
<u>HeV_TQM_N</u> ~~Primer # 2~~	~~(HENDRA-N1572R)~~ <u>Forward</u>	HeV N119F	<u>GAT-ATI-TTT-GAM-GAG-GCG-GCT-AGT-T</u> ~~5' ATC ATT TTG GGC AGG GG 3'~~	
	~~(HENDRA-N1510T-FAM)~~ <u>Reverse</u>	HeV N260R	<u>CCC-ATC-TCA-GTT-CTG-GGC-TAT-TAG</u> ~~5' 6FAM AAC CGC CCT CAG GCA GAC TCA GGA TAMRA 3'~~	
	Probe	HeV N198-220P	CTA-CTT-TGA-CTA-CTA-AGA-TAA-GA	FAM-MGBNFQ
<u>NiV_TQM_N</u> ~~Primer # 1~~	<u>Forward</u> ~~(Nipah-N1198F)~~	NiV_N_1198F	5' <u>TCA-GCA-GGA-AGG-CAA-GAG-AGT-AA</u> 3'	
	<u>Reverse</u> ~~(Nipah-N1297R)~~	NiV_N_1297R	5' <u>CCC-CTT-CAT-CGA-TAT-CTT-GAT-CA</u> 3'	
	Probe	~~(Nipah-~~ <u>NiV_N_</u>1247comp ~~-FAM~~	5' ~~6FAM~~ <u>CCT-CCA-ATG-AGC-ACA-CCT-CCT-GCA-G</u> ~~TAMRA 3'~~	FAM-TAMRA

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*

<u>Target</u>	<u>Assay</u>	<u>Type</u>	<u>Name</u>	<u>Primer Sequence (5'-3')</u>	<u>PCR product</u>
<u>M gene</u>	<u>Primary PCR</u>	<u>Forward</u>	<u>HeV M 5481F</u>	GCC-CGC-TTC-ATC-ATC-TCT-T	<u>300 bp</u>
		<u>Reverse</u>	<u>HeV M 5781R1</u>	CCA-CTT-TGG-TTC-CGT-CTT-TG	
	<u>Semi-nested PCR</u>	<u>Forward</u>	<u>HeV M 5481F</u>	GCC-CGC-TTC-ATC-ATC-TCT-T	<u>211 bp</u>
		<u>Reverse</u>	<u>HeV M 5691R2</u>	TGG-CAT-CTT-TCA-TGC-TCC-ATC-TCG-G	
<u>P gene</u>	<u>Primary PCR</u>	<u>Forward</u>	<u>HeV P 4464F1</u>	CAG-GAG-GTG-GCC-AAT-ACA-GT	<u>335 bp</u>
		<u>Reverse</u>	<u>HeV P 4798R</u>	GAC-TTG-GCA-CAA-CCC-AGA-TT	
	<u>Semi-nested PCR</u>	<u>Forward</u>	<u>HeV P 4594F2</u>	TCA-ACC-ATT-CAT-AAA-CCG-TCA-G	<u>205 bp</u>
		<u>Reverse</u>	<u>HeV P 4798R</u>	GAC-TTG-GCA-CAA-CCC-AGA-TT	

<u>i)</u> <u>PCR conditions</u>

a) **Primary RT-PCR**

<u>1×</u> <u>48°C for 30 minutes, 94°C for 2 minutes</u>

<u>40×</u> <u>95°C for 30 seconds, 53°C for 30 seconds, 68°C for 45 seconds</u>

<u>1×</u> <u>68°C for 7 minutes</u>

b) **Semi-nested PCR**

<u>1×</u> <u>95°C for 5 minutes</u>

<u>30×</u> <u>95°C for 30 seconds, 55°C for 30 seconds, 72°C for 45 seconds</u>

361   1~~x~~   72°C for 7 minutes

362   A range of conventional PCRs for NiV have been described, most of which target the N gene.
363   For more details see the following publication – Wacharapluesadee & Hemachudha (2007). A
364   hemi-nested PCR targeting the L gene has been described by Feldman *et al.*, 2009.

365   Laboratories wishing to establish molecular detection methods should refer to published
366   protocols or consult the OIE Reference Laboratory.

367   **1.3.3. HTS analysis of Hendra virus**

368   High throughput sequencing (HTS) technology has been used for whole genome analysis of
369   Hendra virus. Hendra virus infected Vero cells (e.g. 75 cm culture flask) are used for HTS
370   analysis. The infected cells with CPE are clarified at low speed (4000–5000 rpm). The
371   supernatant is then purified through centrifugation on a 15% sucrose cushion at approximately
372   100,000 *g* for 2–3 hours. The pellet is resuspended with RLT buffer from RNeasy Mini Kit
373   (QIAGEN) and RNA is then extracted following manufacturer's instructions. The first-strand
374   cDNA was performed using Superscript III RT (Invitrogen, Carlsbad, CA, USA) and random
375   octamers linked to a defined arbitrary, 17-mer primer sequence tail (5'-GTT TCC CAG TAG
376   GTCTCN NNN NNN N-3') (Palacios *et al.,* 2007). The second-strand cDNA synthesis was
377   performed with the addition of DNA polymerase I Large (Klenow) fragment (Promega) to the
378   first-strand cDNA. Random PCR amplification is performed using primer 5'-CGC CGT TTC CCA
379   GTA GGT CTC-3′ (Palacios *et al.,* 2007). The resultant products are used for library preparation
380   and sequencing following the standard protocols for specified HTS platforms, e.g. Ion Torrent
381   PGM and Illumina MiSeq (Glenn, 2011). The same method could be used for Nipah virus.

382   **1.4. Henipavirus antigen detection in fixed tissue — immunohistochemistry**

383   ~~Immunohistochemistry is also a useful test in HeV and NiV detection. Performed on formalin-fixed~~
384   ~~tissues or formalin-fixed cells, it is safe and has allowed retrospective investigations on archival~~
385   ~~material. As virus replication and the primary pathology occur in the vascular endothelium (Hooper *et*~~
386   ~~*al.*, 2001), there is a wide range of tissues in which HeV and NiV antigen can be detected (Daniels *et*~~
387   ~~*al.*, 2001). It is thought that HeV antigens may be cleared from lung tissue early in the course of~~
388   ~~infection and so the sample submitted should include a range of tissues, not just lung. HeV antigen has~~
389   ~~been detected in the kidney of a horse 21 days post-infection (Williamson *et al.*, 1998) and so this~~
390   ~~organ should always be submitted. Ideally a submission for immunohistochemistry would include~~
391   ~~samples of the brain at various levels, lung, mediastinal lymph nodes, spleen and kidney. In pregnant~~
392   ~~animals the uterus, placenta and fetal tissues should be included.~~

393   ~~A range of antisera to HeV and NiV may be used in immunohistochemical investigations of HeV- and~~
394   ~~NiV-infected tissues, but rabbit antisera to plaque-purified HeV and NiV have been found to be~~
395   ~~particularly useful. Some MAbs are also available. The Nipah Virus Pathology Working Group has~~
396   ~~described a detection system (Wong *et al.*, 2002). A biotin–streptavidin peroxidase-linked detection~~
397   ~~system has also been used successfully (Hooper *et al.*, 2001). The following detection system is an~~
398   ~~anti-rabbit/anti-mouse dextran polymer-linked reagent conjugated with alkaline phosphatase.~~

399   Immunohistochemistry is a powerful tool that allows the visualisation of viral antigen within cell and
400   tissue structures. Nucleoprotein viral antigen in usually located within particulate structures of variable
401   size and form within the cytoplasm. Because of the morphological aspect to the interpretation, colour
402   signal can be effectively evaluated for its specificity. The test is done on formalin-fixed tissues, allowing
403   the procedure to be done safely under non-microbiologically-contained conditions.

404   Henipavirus antigen replicates in a range of cell types, including endothelium, vascular smooth muscle,
405   lung parenchyma, kidney glomeruli, neuron cell bodies, lymphoid tissues and connective tissues
406   (Hooper *et al.,* 2001; Marsh *et al.,* 2011; Middleton *et al.,* 2002; Mungall *et al.,* 2006). Antigen is
407   particular dense in syncytia and in macrophages within lesions. Therefore, suitable tissues for
408   diagnosis of henipavirus infection include lung, brain, lymph nodes, spleen and kidney. In the absence
409   of these tissues, it is worthwhile examining any tissue type, as antigen can be found in occasional
410   blood vessels throughout the vascular bed. Unless full protective clothing can be worn and suitable
411   disinfection protocols be implemented, it is safer to remove only small pieces of tissue through
412   'keyhole' sampling. Lung tissue and sub-mandibular lymph nodes are good tissues to remove in this
413   manner.

414   Rabbit polyclonal antisera raised against recombinant henipavirus nucleoprotein are highly reliable for
415   use as primary antibodies for diagnostic immunohistochemistry. Detection of phosphoprotein antigens
416   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-Process 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 controls, if appropriate.

ii) Dewax the slides by immersion in three times in consecutive xylene baths for 3 1 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 time.

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_2O_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' diamino-benzidine (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.

ii) 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.

iii) Lay slides flat in a humid chamber and rinse with PBS for 5 minutes. Add 200 µl 3% aqueous H_2O_2 to each slide for 20 minutes at room temperature to block endogenous peroxidase. Rinse slides in PBS for 5 minutes.

iv) Add 200 µl of an appropriate dilution of rabbit anti-Nipah or anti-Hendra antibody in PBS containing 0.1% (w/v) skim milk powder to test tissue slides and positive and negative control slides. To a duplicate set of test and positive and negative control slides add rabbit antibody to an unrelated pathogen. Cover the slides and incubate at 37°C for 1 hour.

v)   Rinse slides in PBS for 5 minutes and apply 2–3 drops of Envision™ solution (anti-rabbit Ig conjugated to peroxidase-labelled dextran polymer [DAKO Corporation, 6392 Via Real, Carpinteria, CA 03013]). Incubate at 37°C for 20 minutes.

vi) Prepare the substrate by dissolving 2 mg 3-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_2O_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.

vii) Counterstain the slides in haematoxylin for 1–3 minutes, rinse in tap water, add Scott's solution (0.04 M sodium bicarbonate, 0.3 M magnesium sulphate), and wash well in

469   ~~running tap water. Rinse the slides in distilled water and mount with a cover-slip using~~
470   ~~aqueous mounting medium.~~

471   All the above test methods should be considered as a guide only; each test parameter will need to be optimised
472   for each testing laboratory, as they will vary according to specific laboratory conditions.

473   ## 2.   Serological tests

474   In laboratories doing serological testing, particularly in outbreak situations, several strategies have been adopted
475   to reduce the risk of exposure of laboratory personnel to HeV and NiV. Sera may be gamma-irradiated (6
476   kiloGreys) or diluted 1/5 in PBS containing 0.5% Tween 20 and 0.5% Triton-X100 and heat-inactivated at 56°C for
477   30 minutes. The process used will be based on a risk assessment. Specimens for surveillance testing and testing
478   for animal movement certification may be considered a lesser biosafety risk than those for disease investigation.
479   In some circumstances heat inactivation may be adopted as a sufficient precaution. However there is value in
480   having a standardised approach for all samples in managing a test, rather than be maintaining multiple test
481   methods.

482   ### 2.1.   Virus neutralisation tests

483   Henipaviruses can be quantified by plaque, microtitre or immune plaque assays and these assays can
484   be modified to detect anti-virus antibody (see above). The virus neutralisation test (VNT) (Kaku *et al.*,
485   2009; Tamin *et al.*, 2009) is accepted as the reference standard. In the most commonly used microtitre
486   assay, which is performed under BSL4 conditions, sera are incubated with virus in the wells of 96-well
487   microtitre plates prior to the addition of Vero cells. Sera are screened starting at a 1/2 dilution although
488   this may lead to problems with serum-induced cytotoxicity. Where sample quality is poor or sample
489   volumes are small, as may be the case with flying fox or microbat sera, an initial dilution of 1/5 may be
490   used. Cultures are read at 3 days, and those sera that completely block development of CPE are
491   designated as positive. If cytotoxicity is a problem the immune plaque assay described above has merit
492   because the virus/serum mixtures are removed from the Vero cell monolayers after the adsorption
493   period, thereby limiting their ~~CPE~~ toxic effect.

494   ### 2.2.   Enzyme-linked immunosorbent assay

495   Henipavirus antigens derived from tissue culture for use in the enzyme-linked immunosorbent assay
496   (ELISA) are irradiated with 6 kiloGreys prior to use, a treatment that has negligible effect on antigen
497   titre. In the indirect ELISA developed in response to the initial outbreak at Hendra in 1994, antigen was
498   derived from HeV-infected cells subjected to several cycles of freezing and thawing and treatment with
499   0.1% (w/v) sodium dodecyl sulphate ~~(P. Selleck, unpublished data)~~. More recently, the use of a
500   recombinant expressed soluble form of the Hendra G protein (Bossart *et al.*, 2005) has been applied
501   for improvements in Hendra immunoassays (McNabb *et al.*, 2014). ~~In the national swine surveillance~~
502   ~~programme in Malaysia in 1999 (Daniels *et al.*, 2000) a similar~~ In Australia, the introduction of equine
503   vaccination against Hendra virus has affected the diagnostic application of assays that detect antibody
504   to the G protein. An earlier version of the Nipah indirect antibody ELISA (Daniels *et al.*, 2000) an
505   indirect ELISA format was used in which antigen was derived by non-ionic detergent treatment of NiV-
506   infected cells. Subsequently, to control for high levels of nonspecific binding activity in some porcine
507   antisera, a modified ELISA was developed based on the relative reactivity of sera with NiV antigen and
508   a control antigen derived from uninfected Vero cells. At the Centers for Disease Control (CDC), Atlanta,
509   USA, the approach has been to not only have an indirect ELISA for detection of IgG but also to use a
510   capture ELISA for detection of IgM. For NiV, an ELISA using a recombinant nucleocapsid antigen has
511   also been described (Yu *et al.*, 2006), which is also configured to detect either IgG or IgM.

512   The specificity of the indirect NiV ELISA (98.4%) (Ong *et al.*, 2000) means that in surveillance
513   programmes the test will yield false positives. This may not be a significant problem in the face of a NiV
514   outbreak where a high proportion of pigs are infected and the purpose of the surveillance is to detect
515   infected farms. However, this level of test specificity creates a problem in the absence of an outbreak
516   or if the number of samples to be tested is limited. If a positive ELISA result was indicative of a bona
517   fide infection, failure to respond may lead to virus spread and human fatalities. In contrast, initiating
518   control measures in response to a false positive ELISA result would be wasteful of resources (Daniels
519   *et al.,* 2001). The current approach is to test all ELISA reactive sera by VNT, with sera reacting in the
520   VNT considered to be positive. Confirmatory VNT should be done under BSL4 conditions and this may
521   entail sending the samples to an internationally recognised laboratory.

522   The following procedure for the NiV ELISA has been developed at Australian Animal Health Laboratory
523   (AAHL) for porcine sera and standardised after collaborative studies in the Veterinary Research
524   Institute, Ipoh, Malaysia.

525 **2.2.1. Test procedure**

526 i) Preparation of NiV antigens

527 a) Grow Vero cells until confluent in roller bottles in EMEM containing 10% (v/v) fetal
528   calf serum (FCS). To infect with virus, pour off all but 5 ml of the medium from each
529   roller bottle and, in a BSL4 laboratory, add low passage, plaque-purified NiV to a
530   multiplicity of infection of 0.1 TCID50/cell.

531 b) Rotate roller bottles for 30 minutes at 33°C to adsorb virus, add 60 ml EMEM
532   containing 10% FCS to each bottle and roll for a further 48 hours at 33°C. The
533   multiplicity of infection, incubation time and temperature are chosen so that although
534   the majority of cells become infected and are incorporated into syncytia within
535   48 hours, few cells detach into the culture medium. The culture medium of cells
536   infected under these conditions is an excellent source of virus for further purification.

537 c) Wash monolayers of virus-infected cells once with cold 0.01 M PBS and, using a
538   large scraper, scrape cells from each roller bottle into 5–10 ml ice-cold PBS.

539 d) Pool scraped cells into 50 ml tubes kept in ice and pellet the cells at 300 ***g*** for
540   5 minutes at 4°C. Pour off PBS and resuspend cells in ice cold TNM (10 mM Tris,
541   10 mM NaCl, 1.5 mM MgCl$_2$, pH 7.2), approximately 0.5 ml TNM per roller bottle.

542 e) Add NP40 (non-ionic detergent, Nonidet P40) to 1% (by addition of 1/10 volume of
543   10% [v/v] NP-40 in water) and lyse cells using 5–10 strokes of a Dounce
544   homogeniser. This also releases from the cytoskeleton viral antigen that would
545   otherwise be removed by centrifugation (step i.f).

546 f) Pellet the nuclei at 600 ***g*** for 10 minutes at 4°C. The nuclei will not lyse under these
547   conditions and should form a tight white pellet.

548 g) Gently remove the supernatant cytoplasmic extract into a clean tube and add
549   ethylene diamine tetra-acetic acid to 1.5 mM. Make up to 10 ml with TNE, aliquot in
550   small amounts, freeze at –80°C and gamma-irradiate with 6 kiloGreys. Store aliquots
551   at –80°C.

552 ii) Preparation of control, uninfected Vero cell antigen

553 a) Grow Vero cells in roller bottles in EMEM containing 10% FCS. When confluent,
554   wash monolayers once with cold PBS and scrape the cells from each roller bottle into
555   5–10 ml ice-cold PBS. Proceed as described for virus-infected cells in steps i.d–i.g
556   above.

557 Detailed methodology for production and/or supply of irradiated NiV and uninfected Vero cell
558 antigens are available from the Australian Animal Health Laboratory.

559 i) Preparation of test sera

560 a) In a biological class II safety cabinet with appropriate personal protective equipment
561   or a class III cabinet, dilute test serum 1/5 in PBS containing 0.5% (v/v) Triton X-100
562   and 0.5% (v/v) Tween 20 in the wells of a 96-well microtitre plate. Seal the microtitre
563   plate. Laboratory personnel should wear gowns and gloves and spray both their
564   hands and the sealed microtitre plate with suitable disinfectant (e.g. 1% Virkon)
565   before removing the microtitre plate from the biosafety cabinet to heat at 56°C for
566   30 minutes.

567 b) Mix 22.5 µl heat-inactivated serum with an equal volume of uninfected Vero cell
568   antigen diluted 1/100 in PBS. Mix thoroughly and incubate at 18–22°C for
569   30 minutes.

570 c) Add 405 µl blocking solution (PBS containing 5% chicken serum and 5% skim milk
571   powder) to give a final serum dilution of 1/100 and incubate at 18–22°C for
572   30 minutes. Aliquots of 100 µl are added to two wells containing NiV antigen and two
573   wells containing uninfected Vero cell control antigen as described in step vi.

574 ii) ELISA procedure

575 a) Dilute Vero cell control and NiV antigens in PBS to ensure that control and virus
576   antigen wells are coated with a similar concentration of protein. Antigen is usually
577   diluted 1/1000 to 1/4000, but a specific dilution factor must be determined for each
578   batch of antigen. Add 50 µl virus and cell control antigen to the wells of a Nunc

579   Maxisorp 96-well microtitre plate as follows: virus antigen in columns 1, 3, 5, 7, 9 and
580   11 and cell control antigen in columns 2, 4, 6, 8 10 and 12 (Fig. 1). Incubate at 37°C
581   for 1 hour with shaking. Plates can be also incubated at 4°C overnight.

582   b)   Wash ELISA plates three times with PBS containing 0.05% Tween 20 (PBST)
583   (250 µl/well) and block with PBS containing 5% chicken serum and 5% skim milk
584   powder (100 µl/well) for 30 minutes at 37°C on a shaker.

585   c)   Wash plates three times with PBST and add 100 µl of inactivated, absorbed sera
586   from step iii to each well as indicated in the format below. Add 100 µl PBS containing
587   5% chicken serum and 5% skim milk powder to conjugate and substrate control
588   wells. Incubate the plates without shaking for 1 hour at 37°C and wash three times
589   with PBST.

590   d)   Dilute protein A/G-horseradish peroxidase conjugate (Protein-A/G-
591   Conjugate ~~Supplied by Pierce, through Progen Biosciences~~ Thermo Scientific Product
592   No. 32490,) in PBST containing 1% (w/v) skim milk powder. The dilution factor is
593   approximately 1/50,000. Mix well and add 100 µl protein A-conjugate to all wells
594   except the substrate control wells. Add 100 µl PBST containing 1% skim milk powder
595   to the substrate control wells. Incubate the plates for 1 hour at 37°C without shaking
596   and wash four times with PBST.

597   e)   Prepare the substrate (3,3',5,5'-tetramethylbenzidine; TMB; Sigma, catalogue
598   number T 3405) by dissolving one tablet (1 mg) in 10 ml of 0.05 M phosphate citrate
599   buffer, pH 5.0, and add 2 µl of fresh 30% (v/v) H_2O_2. Add 100 µl of the TMB
600   substrate to each well. Incubate for 10 minutes at 18–22°C and stop the test by
601   adding 100 µl 1 M sulphuric acid to each well.

602   f)   Read plates after blanking on a substrate control well. The optical density (OD) at
603   450 nm on NiV antigen and control Vero cell antigen are used to calculate an OD
604   ratio for each serum (OD on NiV antigen/OD on Vero control antigen).

605   ~~v)~~   ~~Interpretation of results~~

606   ~~a)~~   ~~An OD ratio >2.0 with an OD on NiV antigen >0.20 is considered positive.~~

607   ~~b)~~   ~~An OD ratio >2.0 with an OD on NiV antigen <0.20 is considered negative.~~

608   ~~c)~~   ~~Sera displaying an OD ratio between 2.0 and 2.2 should be considered doubtful.~~

609   ~~**Fig. 1.** ELISA plate format and result sheet.~~

	~~Ni~~	~~Ni~~	~~U~~	~~U~~	~~Ni~~	~~Ni~~	~~U~~	~~U~~	~~Ni~~	~~Ni~~	~~U~~	~~U~~
	~~1~~	~~2~~	~~3~~	~~4~~	~~5~~	~~6~~	~~7~~	~~8~~	~~9~~	~~10~~	~~11~~	~~12~~
~~A~~	~~Test serum #1~~	~~Test serum #1~~	~~Test serum #1~~	~~Test serum #1~~								
~~B~~	~~Test serum #2~~	~~Test serum #2~~	~~Test serum #2~~	~~Test serum #2~~								
~~C~~	~~Test serum #3~~	~~Test serum #3~~	~~Test serum #3~~	~~Test serum #3~~								
~~D~~	~~Test serum #4~~	~~Test serum #4~~	~~Test serum #4~~	~~Test serum #4~~								
~~E~~	~~Test serum #5~~	~~Test serum #5~~	~~Test serum #5~~	~~Test serum #5~~					~~H+~~	~~H+~~	~~H+~~	~~H+~~
~~F~~	~~Test serum #6~~	~~Test serum #6~~	~~Test serum #6~~	~~Test serum #6~~					~~L+~~	~~L+~~	~~L+~~	~~L+~~
~~G~~	~~Test serum #7~~	~~Test serum #7~~	~~Test serum #7~~	~~Test serum #7~~					~~N–~~	~~N–~~	~~N–~~	~~N–~~
~~H~~	~~Test serum #8~~	~~Test serum #8~~	~~Test serum #8~~	~~Test serum #8~~					~~CC~~	~~TMB~~	~~CC~~	~~TMB~~

610   ~~Nunc Maxisorp 96 well plate; Ni: Nipah virus infected cell antigen; U: uninfected Vero antigen (control antigen);~~
611   ~~H+: High positive control sera e.g. LAF pig 6 sera; N: Negative control sera e.g. LAF pig 6 sera; L+: Low positive control sera~~
612   ~~e.g. LAF Pig 6 sera 1:800.~~

613   ii)   Interpretation of results

614   Samples with NiV antigen O.D. value less than 0.20 are negative. Samples with NiV
615   antigen OD value greater than 0.2 are assessed by OD ratio value accordingly as:

616   a)   an OD ratio >2.0 (with an OD on NiV antigen > 0.20) are considered positive

617   b)   an OD ratio between 2.0 and 2.2 should be considered doubtful

618   Doubtful and positive sera should be tested by VNT.

2.3. Bead-based assays

620   Bead-based assays can be used. The methods below are examples of such assays.

621   Two multiplexed bead-based serological assays have been developed using Luminex technology and
622   incorporate identification of antibodies to both HeV or NiV in a single test (Bossart *et al.*, 2007; McNabb
623   *et al.*, 2014). Both assays measure antibodies to recombinant expressed soluble glycoprotein (sG) of
624   HeV and NiV. One assay measures antibodies that bind directly to sG (binding assay) and the other
625   assay measures the ability of antibodies to block the henipavirus receptor EphrinB2 binding to sG
626   (blocking assay). The recombinant HeV or NiV sG proteins are first coupled to individually identifiable
627   magnetic beads. The coupled beads are then mixed with test sera. For the binding assay, bound sera
628   are then detected using a biotinylated protein A/G secondary conjugate and Streptavidin-phycoerythrin
629   (S-PE). For the blocking assay, sera must compete with biotinylated ephrinb2 for binding to the sG and
630   S-PE is again used to quantify the reaction. The beads are then interrogated by lasers in a Luminex
631   machine and the results recorded as the median fluorescent intensity (MFI) of 100 beads. The assays
632   use completely recombinant reagents and can be performed at PC2, whereas the traditional ELISA
633   requires PC4 containment to produce antigen. Similar to the approach taken with ELISA, any suspect
634   positive sera are then tested by VNT at BSL4 for confirmation.

2.3.1. Bead-coupling procedure

636   i)   Bead activation

637   a)   Bring the bead activation buffer (0.1 M NaH_2PO_4, pH6.2) to room temperature prior
638   to use.

639   NOTE: Be careful to protect the beads from light as they photobleach (cover tubes
640   with foil where possible).

641   b)   Select the MagPlex carboxylated beads (Luminex corp., supplied as 1.25 ×
642   10^7 beads/ml) for the protein coupling reaction (usually HeV: Bead#29 & NiV:
643   Bead#30). Vortex the beads for 30 seconds at medium speed, then sonicate the
644   beads by bath sonication for ~30–60 seconds. It is important that the beads are
645   completely resuspended as single monodisperse particles.

646   c)   Transfer 300 µl of MagPlex carboxylated beads #28 & #30 (3.75 × 10^6 beads) into
647   2 ml sarstedt tubes. Place the tubes into a magnetic separator and allow separation
648   to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator,
649   remove the supernatant with a pipette; take care not to disturb the bead pellet.

650   d)   Wash beads by adding 300µl of PBS-T to the tubes and vortexing. Place the tubes
651   into a magnetic separator and allow separation to occur for 30–60 seconds. With the
652   tubes still positioned in the magnetic separator, remove the supernatant with a
653   pipette; take care not to disturb the bead pellet. Repeat.

654   e)   Add 600 µl of bead activation buffer to the tubes and vortex. Place the tubes into a
655   magnetic separator and allow separation to occur for 30–60 seconds. With the tubes
656   still positioned in the magnetic separator, remove the supernatant with a pipette; take
657   care not to disturb the bead pellet. Repeat.

658   f)   Add 240 µl of bead activation buffer to the tubes, cover with foil and shake for
659   3 minutes.

660   g)   Prepare EDC (Pierce) and S-NHS (Pierce) in bead activation buffer immediately prior
661   to use to a concentration of 50 mg/ml (20 µl buffer/mg powder). Add 30 µl of the
662   freshly made 50 mg/ml EDC into the tubes, closely followed by 30 µl of the freshly
663   made 50 mg/ml S-NHS into the tubes. NOTE: Discard unused portion and make
664   fresh each time.

665   h)   Cover the tubes with aluminum foil and shake the beads at room temperature for
666   20 minutes.

667   i)   While beads are incubating, prepare sG proteins. Use 90 µg each of HeV sG & NiV
668   sG and use PBS (do not use PBS-T, as it blocks carboxy groups) to bring proteins up
669   to a final volume of 300 µl.

670   j)   After incubation, the beads are now activated and ready for coupling. Place the tubes
671   into a magnetic separator and allow separation to occur for 30–60 seconds. With the

672				tubes still positioned in the magnetic separator, remove the supernatant with a
673				pipette; take care not to disturb the bead pellet.

674   ii)   Protein coupling

675   a)   Wash beads by adding 300 µl of PBS to the tubes and vortexing (do not use PBS-T
676   as it blocks carboxy groups). Place the tubes into a magnetic separator and allow
677   separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic
678   separator, remove the supernatant with a pipette; take care not to disturb the bead
679   pellet.

680   b)   Add all of the 300 µl of prepared protein, above, to the activated beads.

681   c)   Cover the tubes with aluminium foil and shake the beads moderately at room
682   temperature for 2 hours.

683   d)   The protein is now coupled to the beads. Place the tubes into a magnetic separator
684   and allow separation to occur for 30–60 seconds. With the tubes still positioned in the
685   magnetic separator, remove the supernatant with a pipette; take care not to disturb
686   the bead pellet.

687   e)   Wash the beads twice with 300 µl of PBS-T as described above. Place the tubes into
688   a magnetic separator and allow separation to occur for 30–60 seconds. With the
689   tubes still positioned in the magnetic separator, remove the supernatant with a
690   pipette; take care not to disturb the bead pellet.

691   f)   Resuspend the coupled beads in 1.8 ml bead storage buffer (10 ml PBS, 1% BSA,
692   0.05% sodium azide and 1 protease inhibitor tablet (Roche) and store at 4°C.

693   NOTES: Check reactivity of sG with panel of henipavirus sera before use. Use 1 µl of coupled
694   beads per well for henipavirus binding and blocking serological assays (this procedure couples
695   enough beads to test around 1800 sera). Coupled beads are able to be stored at 4°C for at
696   least 1 year and maintain reactivity.

697   **2.3.2. Henipavirus luminex binding assay procedure**

698   i)   Test procedure

699   a)   Select previously coupled HeV and NiV sG beads. Vortex the beads for 30 seconds
700   at maximum speed, then sonicate the beads by bath sonication for ~30–60 seconds.

701   b)   Dilute beads in blocker (2% skim milk in PBS-T) at an appropriate concentration for
702   the number of sera to be tested (1 µl of each bead set/well).

703   c)   Add 100 µl of diluted beads to appropriate wells of a 96-well NUNC TC flat bottom
704   plate.

705   d)   Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

706   e)   Place plate on magnetic holder and allow separation to occur for 30–60 seconds.
707   With the plate still in the magnetic holder, flick contents into the sink and gently blot
708   on paper towel, remove plate from magnetic holder.

709   f)   Wash twice with PBST or alternatively, use automated magnetic plate washer.

710   g)   Add 100 µl of control and test sera diluted 1/100 in PBS-T to the wells (bat sera dilute
711   1/50).

712   NOTE: All sera should be heat-inactivated for 35 minutes at 56°C before testing.

713   h)   Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

714   i)   Place plate on magnetic holder and allow separation to occur for 30–60 seconds.
715   With the plate still in the magnetic holder, flick contents into the sink and gently blot
716   on paper towel, remove plate from magnetic holder.

717   j)   Wash twice with PBST or alternatively, use automated magnetic plate washer.

718   k)   Dilute biotinylated protein A (Pierce) 1/500 (2 ug/ml) and biotinylated protein G
719   (Pierce) 1/250 (2 µg/ml) in the same tube in PBS-T and add 100 µl to the wells.

720   l)   Cover plate in foil and shake at RT for 30min on a plate shaker.

721   m)   Place plate on magnetic holder and allow separation to occur for 30–60 seconds.
722   With the plate still in the magnetic holder, flick contents into the sink and gently blot
723   on paper towel, remove plate from magnetic holder.

724   n)   Wash twice with PBST or alternatively, use automated magnetic plate washer.

725   o)   Add 100 µl of Streptavidin R-PE (QIAGEN) diluted 1/1000 (1 ug/ml) in PBS-T to the
726   wells.

727   p)   Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

728   q)   Read plate using an appropriate Luminex machine and software.

729   ii)   Interpretation of results

730   The results can be interpreted from the raw MFI values or can be transformed into a
731   percentage relative to the MFI for the positive control (%P) using the following formula:

732   (MFI test serum/MFI positive control) × 100

733   A sample giving an MFI >1000 or %P >5 should be first retested in the Binding assay and
734   Blocking assay. If the sample is still positive it should be tested further by VNT for
735   confirmation.

2.3.3. Henipavirus luminex blocking assay procedure

737   i)   Test procedure

738   a)   Select previously coupled HeV and NiV sG beads. Vortex the beads for 30 seconds
739   at max speed, then sonicate the beads by bath sonication for ~30–60 seconds.

740   b)   Dilute beads in blocker (2% skim milk in PBS-T) at an appropriate concentration for
741   the number of sera to be tested (1 µl of each bead set/well).

742   c)   Add 100 µl of diluted beads to appropriate wells of a 96 well NUNC TC flat-bottom
743   plate.

744   d)   Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

745   e)   Place plate on magnetic holder and allow separation to occur for 30–60 seconds.
746   With the plate still in the magnetic holder, flick contents into the sink and gently blot
747   on paper towel, remove plate from magnetic holder.

748   f)   Wash twice with PBST. Or, alternatively, use automated magnetic plate washer.

749   g)   Add 100 µl of control and test sera diluted 1/50 in PBS-T to the wells (bat sera dilute
750   1/25).

751   NOTE: All sera should be heat-inactivated for 35 minutes at 56°C before testing.

752   h)   Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

753   i)   Place plate on magnetic holder and allow separation to occur for 30–60 seconds.
754   With the plate still in the magnetic holder, flick contents into the sink and gently blot
755   on paper towel, remove plate from magnetic holder.

756   j)   Wash twice with PBST or alternatively, use automated magnetic plate washer.

757   k)   Dilute biotinylated ephrinB2 (RnD Systems) 1/1000 (50 ng/ml) in PBS-T and add
758   100 µl to the wells.

759   l)   Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

760   m)   Place plate on magnetic holder and allow separation to occur for 30–60 seconds.
761   With the plate still in the magnetic holder, flick contents into the sink and gently blot
762   on paper towel, remove plate from magnetic holder.

763   n)   Wash twice with PBST or alternatively, use automated magnetic plate washer.

764   o)   Add 100 µl of streptavidin R-PE (QIAGEN) diluted 1/1000 (1 ug/ml) in PBS-T to the
765   wells.

766   p)   Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

767   q)   Read plate using an appropriate Luminex machine and software.

768   ii)   Interpretation of results

769   For the blocking assay, the raw MFI readings are converted into percentage inhibition (%I)
770   using the following formula: (1 - [MFI test serum/MFI NSC]) × 100

771   A sample giving a %I >15 should be first retested in the binding assay and blocking assay.
772   If the sample is still positive it should be tested further by VNT for confirmation.

773 **2.4. DIVA**

774   Now that a vaccine exists for Hendra virus for use in horses, the ability to differentiate vaccinated
775   horses from unvaccinated naturally infected horses may seem desirable to some parties. Traditionally
776   DIVAs have been applied on the premise that vaccinated animals will only have antibodies to the viral
777   protein(s) used in the vaccine (in the case of HeV, that is the G protein) whereas naturally infected
778   animals will have antibodies to all viral proteins, both structural and non-structural. However, caution
779   must be exerted when interpreting such serological results. Experimentally it has been shown in ferrets
780   that not all individuals mount an immune response to all of the viral proteins of Hendra virus that are
781   detectable using conventional assay systems (Middleton D., unpublished data). In particular,
782   serological profiles of unvaccinated infected animals may be indistinguishable from those that are
783   vaccinated.

784 # C. REQUIREMENTS FOR VACCINES

785 **Veterinary vaccines for henipaviruses**

786 **1. Background**

787 ~~**1.1. Rationale and intended use of the product**~~

788 ~~There is no commercially produced vaccine against Hendra and/or Nipah virus available.~~ The original outbreak of
789 NiV in Malaysia and Singapore was linked to transmission of the virus from pigs to humans, and all of the human
790 infections with Hendra virus in Australia have been linked to contact with sick horses. Development of veterinary
791 vaccines against henipaviruses is important both to protect susceptible domestic animal species (i.e. porcine,
792 equine, feline, and canine) and to reduce transmission from domestic animals to humans. ~~The original outbreak of~~
793 ~~NiV in Malaysia and Singapore was linked to transmission of the virus from pigs to humans, and all of the human~~
794 ~~infections with Hendra virus in Australia have been linked to contact with sick horses.~~ This was the rationale for
795 development of the vaccine for HeV which is currently available for use in horses in Australia.

796   ~~A vaccine that protects against both NiV and HeV, and could be used in a number of species (i.e.~~
797   ~~equine, swine, feline, canine) would be desirable. In addition, a vaccine for wildlife may be~~
798   ~~advantageous to assist in outbreak control. Henipaviruses are considered to be bioterrorism and~~
799   ~~agroterrorism threats, which increase the need for development and production of safe and effective~~
800   ~~vaccines for domestic animals.~~

801 ~~**2. Outline of production and minimum requirements for conventional vaccines**~~

802 ~~The desired profile for henipavirus vaccines includes a manufacturing process that is safe under low containment~~
803 ~~conditions, yielding a large number of doses at a reasonable cost. The vaccine should be highly efficacious with a~~
804 ~~quick onset of immunity following a single dose. The vaccine should ideally cross-protect against both NiV and~~
805 ~~HeV and be safe in a wide range of species, and across all ages. Vaccination should prevent virus transmission~~
806 ~~to susceptible animals and people, and should prevent virus entry into the brain. The vaccine should allow~~
807 ~~detection of vaccinated animals which become infected (differentiation of infected from vaccinated animals~~
808 ~~[DIVA]) and should have duration of immunity of at least 1 year.~~

809 ~~Vaccine production techniques which require growing large quantities of henipaviruses have not been considered~~
810 ~~to date because of the requirement for biosafety level 4 containment, and subsequent extensive safety testing.~~
811 ~~This precludes the development of conventional killed, split, split-subunit or live attenuated vaccines.~~

812 ~~**3. Vaccines based on biotechnology**~~

813 ~~**3.1. Vaccines available and their advantages**~~

814 The use of biotechnology presents opportunities for production of safe and effective henipavirus
815 vaccines. Live vectored vaccines expressing the fusion (F) and/or attachment proteins (G) and subunit
816 vaccines containing recombinant F and/or G proteins have been produced and shown to be safe and
817 effective under experimental conditions.

818 The vaccine currently under development for commercialisation is a recombinant canarypox virus
819 expressing Nipah virus F and/or G proteins. The ALVAC canarypox vector has been successfully used
820 to produce licensed, commercially available live vectored vaccines in equine, feline, and canine
821 species. This vector will infect mammalian cells and produce viral-encoded protein, but does not
822 replicate in mammalian hosts or cells. These properties provide an acceptable safety and efficacy
823 profile and provide a vaccine that is DIVA compatible.

824 **3.2. Special requirements for biotechnological vaccines, if any**

825 Live recombinant vectored vaccines must meet all requirements for safety, efficacy, potency, and purity
826 required of all vaccines. In addition they must be shown to stably express the recombinant proteins
827 upon passage *in vitro* and growth in cell culture to produce vaccine virus. A risk assessment should be
828 conducted before biotechnology-derived vaccines are released into the environment. The risk
829 assessment should include information on the design, construction, and testing of the biotechnology-
830 derived vaccine. Detailed information should be provided about the documented genetic characteristics
831 and history of the organisms used to construct the final recombinant biological agent and its
832 survivability in the environment.

833 **3.3. Experimental vaccines based on biotechnology**

834 The data on experimental vaccines published by mid-2009 indicate that experimental henipavirus
835 vaccines can prevent clinical disease, elicit systemic and mucosal immunity, and prevent viral
836 replication in target tissues in several mammalian animal species In addition there are indications that
837 the HeV vaccine formulations can cross protect against NiV.

838 **3.3.1. Canarypox-vectored NiV vaccines**

839 The ALVAC canarypox virus-based recombinant vaccine vector was used to construct two
840 experimental NiV vaccines, carrying the gene for NiV glycoprotein (ALVAC-G) or the fusion
841 protein (ALVAC-F). The efficacy of both the ALVAC-G and ALVAC-F were tested in 10-week old
842 pigs, either as monovalent vaccine or in combination (ALVAC-G/F) in a pilot protection study
843 (Weingartl *et al.,* 2006).

844 The vaccination regimen was two doses administered intramuscularly 14 days apart, each of
845 them containing 10^8 PFU. Both non-vaccinated controls and vaccinated pigs were challenged
846 intra-nasally with $10^{5.4}$ PFU of NiV 2 weeks later.

847 The combined ALVAC-F/G vaccine induced the highest levels of neutralising antibodies, and
848 stimulated both type 1 and type 2 cytokine responses. Virus was not isolated from the tissues of
849 any of the vaccinated pigs post-challenge, and no virus shedding was detected in vaccinated
850 animals, in contrast to challenge control pigs. Histopathological findings indicated that there was
851 no enhancement of lesions in the challenged vaccinates. Based on the data generated in this
852 one study, the combined ALVAC-F/G vaccine appears in particular to be a very promising
853 vaccine candidate. The canarypox vaccine vector has been licensed for commercially available
854 vaccines for dogs, cats, and horses. The canarypox (ALVAC) vaccine vectors induce antibody
855 and cytotoxic T cell responses in a range of mammalian species, and the replication of
856 canarypox viral vectors is abortive in mammalian cells, eliminating some of the safety concerns.

857 **3.3.2. Vaccinia-vectored NiV vaccine**

858 The NYVAC vaccinia virus-based recombinant vaccine vector was used to construct
859 experimental NiV vaccines where the vaccinia virus expresses either NiV G or F glycoprotein
860 (Guillaume *et al.,* 2004). The recombinant vaccines were used for subcutaneous immunisation
861 of hamsters, either individually or in combination, using 10^7 PFU/animal in two doses (1 month
862 apart). Both of the NiV glycoproteins G and F vaccinia virus recombinants induced an immune
863 response in hamsters that protected against a lethal intraperitoneal challenge with 10^3 PFU of
864 NiV/animal. This team also demonstrated that passive transfer of antibody induced by the
865 glycoproteins protected the animals against NiV, and also against HeV (Guillaume *et al.,* 2009).

866 ~~Although NYVAC vaccinia-based vector is highly attenuated, it still has the potential to infect~~
867 ~~people, creating safety concerns for use of the vaccine in domestic animals (or humans).~~
868 ~~However vaccinia vectors were successfully used in wildlife rabies vaccination campaigns in~~
869 ~~Europe with the advantage for lending themselves to oral immunisation.~~

2. Soluble G henipavirus vaccine

871 Studies using NiV in cats (Mungall *et al.*, 2006;) and monkeys (Bossart *et al.*, 2012) and HeV in ferrets (Pallister
872 *et al.*, 2011) provided strong evidence that a HeV soluble G (HeVsG) glycoprotein subunit–based vaccine could
873 prevent not only disease but often infection in animals exposed to otherwise lethal doses of NiV or HeV. The
874 horse vaccine has been formulated using a proprietary adjuvant (Zoetis). The henipavirus surface-expressed G
875 glycoprotein has the critical role of initiating infection by binding to receptors on host cells, and antibodies directed
876 against this protein can neutralize virus. The vaccine, Equivac HeV (Zoetis) was released under a Minor Use
877 Permit for use in Australia in November 2012, and is only available for administration by accredited veterinarians.
878 For primary immunisation two doses of vaccine should be administered 3 weeks apart in horses four months of
879 age or above. For continued effect, a booster dose every 6 months is currently recommended by the
880 manufacturer.

3. Experimental vaccines

882 ~~In preliminary evaluation of experimental subunit vaccine formulations containing either soluble~~
883 ~~forms of HeV sG or NiV sG glycoprotein in a NiV challenge study in cats, good crossreactivity~~
884 ~~was demonstrated, with HeV sG possibly providing better protection (Mungall *et al.*, 2006). In a~~
885 ~~subsequent study, a subunit formulation containing CpG as an adjuvant and HeV sG with its~~
886 ~~cytoplasmic tail and transmembrane domains replaced by an immunoglobulin kappa leader~~
887 ~~sequence coupled with an S-peptide tag to facilitate purification, was evaluated as a potential~~
888 ~~NiV vaccine. Intramuscularly vaccinated cats developed varying levels of NiV-specific Ig~~
889 ~~systemically and importantly, all vaccinated cats possessed antigen-specific IgA on the mucosa.~~
890 ~~Upon oronasal challenge with NiV ($10^{4.7}$ TCID$_{50}$), all vaccinated animals were protected from~~
891 ~~disease although virus was detected on day 21 post-challenge in one animal. However~~
892 ~~shedding was detected at 6 and 8 days based on virus genome detection at about the same~~
893 ~~levels in both vaccinated and control challenged animals (McEachern *et al.*, 2008). In addition~~
894 ~~NiV genome was detected in the brain of several vaccinated challenged animals compared to~~
895 ~~challenge control animals raising some concerns about significance of this phenomenon in the~~
896 ~~light of the observed late henipavirus encephalitis in humans (Paterson *et al.*, 1998; Tan *et al.*,~~
897 ~~2002). Beside the potential efficacy concerns, cost of production may prohibit development of~~
898 ~~this vaccine for veterinary application.~~

899 Current experimental vaccines for protection from NiV infection have focused on the use of NiV glycoprotein (G)
900 and/or fusionprotein (F) as immunogens in various platforms, including DNA vaccines, subunit vaccines, non-
901 replicating vectors, as well as replicating vectors. Efficacy of most candidates required a prime/boost(s) approach,
902 which would not favour their use in an emergency situation for rapid dissemination during an outbreak. A live-
903 attenuated vaccine vector based on recombinant vesicular stomatitis viruses (rVSV) expressing NiV glycoproteins
904 (G or F) or nucleoprotein (N) has been evaluated. Vaccination of Syrian hamsters with a single dose of the rVSV
905 vaccine vectors resulted in strong humoral immune responses with neutralizing activities found only in those
906 animals vaccinated with rVSV expressing NiV G or F proteins suggesting that these may be prime candidates for
907 emergency vaccines to be utilised in NiV outbreak management. A similar construct consisting of a replication-
908 defective vesicular stomatitis virus (VSV)-based vaccine vectors expressing either the NiV fusion (F) or
909 attachment (G) glycoproteins protected hamsters from over 1000 times LD$_{50}$ NiV challenge when vaccinated with
910 a single dose.

REFERENCES

912 ~~ANONYMOUS (2004). Nipah encephalitis outbreak over wide area of western Bangladesh, 2004. *Health Sci. Bull.,* **2**,~~
913 ~~7–11.~~

914 BOSSART K.N., CRAMERI G., DIMITROV A.S., MUNGALL B.A., FENG Y.R., PATCH J.R., CHOUDHARY A., WANG L.F., EATON
915 B.T. & BRODER C.C. (2005). Receptor binding, fusion inhibition, and induction of cross-reactive neutralizing
916 antibodies by a soluble G glycoprotein of Hendra virus. *J. Virol.*, **79**, 6690–6702.

917 BOSSART K.N., MCEACHERN J.A., HICKEY A.C., CHOUDHRY V., DIMITROV D.S., EATON B.T. & WANG L.F. (2007).
918 Neutralization assays for differential henipavirus serology using Bio-Plex protein array systems. *J. Virol. Methods*,
919 **142**, 29–40.

Bossart K.N., Rockx B., Feldmann F., Brining D., Scott D., LaCasse R., Geisbert J.B., Feng Y.R., Chan Y.P., Hickey A.C., Broder C.C., Feldmann H. & Geisbert T.W. (2012). A Hendra virus G glycoprotein subunit vaccine protects African green monkeys from Nipah virus challenge. *Sci. Transl. Med.*, **4** (146).

Chua K.B., Bellini W.J., Rota P.A., Harcourt B.H., Tamin A., Lam S.K., Ksiazek T.G., Rollin P.E., Zaki S.R., Shieh W.J., Goldsmith C.S., Gubler D.J., Roehrig J.T., Eaton B., Gould A.R., Olson J., Field H., Daniels P., Ling A.E., Peters C.J., Anderson L.J. & Mahy B.W.J. (2000). Nipah virus: A recently emergent deadly paramyxovirus. *Science,* **288**, 1432–1435.

Chua K.B., Koh C.L., Hooi P.S., Kong, F.W., Khong J.H., Chua B.H., Chan Y.P, Lim M.E. & Lam S.K. (2002). Isolation of Nipah virus from Malaysian Island flying foxes. *Microbes and Infection*, **4**, 145–151.

~~Crameri G., Wang L.-F., Morrissy C.W.J.R. & Eaton B.T. (2002). A rapid immune plaque assay for the detection of Hendra and Nipah viruses and anti-virus antibodies. *J. Virol. Methods,* **99**, 41–51.~~

Daniels P., Aziz J., Ksiazek T.G., Ong B.L., Bunning M.J.B., Field H., Olson J., Hoffman D., Bolou J. & Ozawa Y. (2000). Nipah virus – developing a regional approach. *In:* Comprehensive Reports on Technical Items presented to the International Committee or Regional Commissions. OIE, Paris, France, 2207–2217.

Daniels P., Halpin K., Hyatt A. & Middleton D. (2007). Infection and disease in reservoir and spillover hosts: determinants of pathogen emergence. *Curr. Top. Immunol. Microbiol.*, **315**, 113–131.

Daniels P., Ksiazek T. & Eaton B.T. (2001). Laboratory diagnosis of Nipah and Hendra virus infections. *Microbes Infect.,* **3**, 289–295.

~~Eaton B.T., Broder C.C., Middleton D. & Wang L.F. (2006). Hendra and Nipah viruses: different and dangerous. *Nat. Rev. Microbiol.,* **4** (1), 23–35.~~

~~Epstein J.H., Rahman S.A., Zambriski J.A., Halpin K., Meehan G., Jamaluddin A.A., Hassan S.S., Field H.E., Hyatt A.D. & Daszak P. (2006). Feral cats and risk for Nipah virus transmission. *Emerg. Infect. Dis.,* **12**, 1178–1179.~~

Feldman K.S., Foord A., Heine H.G., Smith I.L., Boyd V., Marsh G.A., Wood J.L.N., Cunningham A.A. & Wang L.F. (2009). Design and evaluation of consensus PCR assays for henipaviruses. *J. Virol. Methods,* **161**, 52–57.

~~Field H.E., Barratt P.C., Hughes R.J., Shield J. & Sullivan N.D. (2000). A fatal case of Hendra virus infection in a horse in north Queensland: clinical and epidemiological features. *Aust. Vet. J.,* **78**, 279–280.~~

Glenn TC. (2011). Field guide to next-generation DNA sequencers. *Mol. Ecol. Resour.,* **11**, 759–769.

~~Guillaume V., Contamin H., Loth P., Georges-Courbot M.-C., Lefeuvre A., Marianneau P., Chua K.B., Lam S.K., Buckland R., Deubel V. & Wild T.F (2004). Nipah virus: Vaccination and passive protection studies in a hamster model. *J. Virol.,* **78**, 834–840.~~

~~Guillaume V, Wong KT, Looi RY, Georges-Courbot MC, Barrot L, Buckland R, Wild TF, Horvat B. (2009) Acute Hendra virus infection: Analysis of the pathogenesis and passive antibody protection in hamster model. *Virology,* **387**, 459–465.~~

Halpin K., Young P.L., Field H.E. & Mackenzie J.S. (2000). Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. *J. Gen. Virol.,* **81**, 1927–1932.

Hanna, J.N., McBride W.J., Brookes D.L., Shield J., Taylor C.T., Smith I.L., Craig S.B. & Smith G.A. (2006). Hendra virus infection in a veterinarian. *MJA,* **185**, 562–564.

Hayman D.T., Suu-Ire R., Breed A.C., McEachern J.A., Wang L., Wood J.L. & Cunningham A.A. (2008). Evidence of henipavirus infection in West African fruit bats. *PLoS ONE,* **3**, e2739.

Hooper P., Zaki S., Daniels P. & Middleton D. (2001). Comparative pathology of the diseases caused hy Hendra and Nipah viruses. *Microbes Infect.,* **3**, 315–322.

~~Hsu V.P., Hossain M.J., Parashar U.D., Ali M.M., Ksaizek T.G., Kuzmin I., Niezgoda M., Rupprecht C., Bresee J. & Brieman R.F. (2004). Nipah virus encephalitis reemergence, Bangladesh. *Emerg. Infect. Dis.,* **10**, 2082–2087.~~

~~Hyatt A.D., Zaki S.R., Goldsmith C.S., Wise T.G. & Hengstberger S.G. (2001). Ultrastructure of Hendra virus and Nipah virus within cultured cells and host animals. *Microbes Infect. ,* **3**, 297–306.~~

965 IEHLE C., RAZAFITRIMO G., RAZAINIRINA J., ANDRIAHOLINIRINA N., GOODMAN S.M., FAURE C., GEORGES-CORBOT M-C.,
966 ROUSSET D. & REYNES J-M. (2007). Henipavirus and Tioman virus antibodies in pteropid bats, Madagascar.
967 *Emerg. Infect Dis.,* **13**, 159–161.

968 ~~INTERNATIONAL AIR TRANSPORT ASSOCIATION (2002). Dangerous Goods Regulations, 43rd edition. International Air~~
969 ~~Transport Association (IATA), 800 Place Victoria, P.O. Box 113, Montreal, Quebec H4Z 1M1, Canada.~~

970 ~~JOHARA M.Y., FIELD H., RASHDI A.M., MORRISSY C., VANDERHEIDE B., ROTA P., BINADZHAR A., WHITE J., DANIELS P.,~~
971 ~~JAMALUDDIN A. & KSIAZEK T. (2001). Nipah virus infection in bats (order Chiroptera) in peninsular Malaysia. *Emerg.*~~
972 ~~*Infect. Dis.,* **7**, 439–441.~~

973 KAKU Y., NOGUCHI A., MARSH G.A., MCEACHERN J.A., OKUTANI A., HOTTA K., BAZARTSEREN B., FUKUSHI S., BRODER
974 C.C., YAMADA A., INOUE S., WANG L.F. (2009). A neutralization test for specific detection of Nipah virus antibodies
975 using pseudotyped vesicular stomatitis virus expressing green fluorescent protein. *J. Virol. Methods*, **160**, 7–13.

976 LUBY S.P., RAHMAN M., HOSSAIN M.J., BLUM L.S., HUSAIN M.M., GURLEY E., KHAN R., AHMED B.N., RAHMAN S., NAHAR
977 N., KENAH E., COMER J.A. & KSIAZEK T.G. (2006). Foodborne transmission of Nipah virus, Bangladesh. *Emerg.*
978 *Infect. Dis.,* **12**, 1888–1894.

979 MARSH G.A., HAINING J., HANCOCK T.J., ROBINSON R., FOORD A.J., BARR J.A., RIDDELL S., HEINE H.G., WHITE J.R.,
980 CRAMERI G., FIELD H.E., WANG L.F. & MIDDLETON D. (2011). Experimental infection of horses with Hendra
981 virus/Australia/horse/2008/Redlands. *Emerg. Infect. Dis.,* **7**, 2232–2238.

982 MARSH G.A., de JONG C., BARR J.A., TACHEDJIAN M., SMITH C., MIDDLETON D., YU M., TODD S., FOORD A.J., HARING V.,
983 PAYNE J., ROBINSON R., BROZ I., CRAMERI G., FIELD H.E. & WANG LF. (2012). Cedar virus: a novel Henipavirus
984 isolated from Australian bats. *PLoS Pathog,* **8**, e1002836.

985 ~~MCEACHERN J.A., BINGHAM J., CRAMERI G., GREEN D.J., HANCOCK T.J., MIDDLETON D., FENG Y.R., BRODER C.C.,~~
986 ~~WANG L.F. & BOSSART K.N. (2008). A recombinant subunit vaccine formulation protects against lethal Nipah virus~~
987 ~~challenge in cats. *Vaccine,* **26** (31), 3842–3852.~~

988 MCNABB L., BARR J., CRAMERI G., JUZVA S., RIDDELL S., COLLING A., BOYD V., BRODER C., WANG L.F. & LUNT R.
989 (2014). Henipavirus microsphere immuno-assays for detection of antibodies against Hendra virus. *J. Virol.*
990 *Methods*, **200**, 22–28.

991 MIDDLETON D.J., WESTBURY H.A., MORRISSY C.J., van der HEIDE B.M., RUSSELL G.M., BRAUN M.A. & HYATT A.D.
992 (2002). Experimental Nipah virus infection in pigs and cats. *J. Comp. Pathol.*, **126**, 124–136.

993 MUNGALL B.A., MIDDLETON D., CRAMERI G., BINGHAM J., HALPIN K., RUSSELL G., GREEN D., MCEACHERN J., PRITCHARD
994 L.I., EATON B.T., WANG L.F., BOSSART K.N. & BRODER C.C. (2006). Feline model of acute nipah virus infection and
995 protection with a soluble glycoprotein-based subunit vaccine.*J. Virol.*, **80**, 12293–12302.

996 MURRAY K., SELLECK P., HOOPER P., HYATT A., GOULD A., GLEESON L., WESTBURY H., HILEY L., SELVEY L. & RODWELL
997 B. (1995). A morbillivirus that caused fatal disease in horses and humans. *Science,* **268**, 94–97.

998 NOR M.N.M., GAN C.H. & ONG B.L. (2000). Nipah virus infection of pigs in peninsular Malaysia. *Rev. sci. tech. Off.*
999 *int. Epiz.,* **19**, 160–165.

1000 OLSON J.G., RUPPRECHT C., ROLLIN P.E., UNG S.A., NIEZGODA M., CLEMINS T., WALSTON J. & KSIAZEK T.G. (2002).
1001 Antibodies to Nipah-like virus in bats (Pteropus lylei), Cambodia. *Emerg. Infect. Dis.,* **8**, 987–988.

1002 ONG B.L., DANIELS P.W., BUNNING M., AZIZ J., WHITE J., MUNIANDY M., OLSON J., CHANG K.W., MORRISSY C., LIM Y.S.,
1003 KSIAZEK T. & NORDIN M.N. (2000). A surveillance program for the detection of pig herds exposed to Nipah virus
1004 infections in peninsular Malaysia. *In:* Proceedings of the 9th International Symposium on Veterinary Epidemiology
1005 and Economics (ISVEE). Abstract ID 63. Colorado, USA.

1006 PALACIOS G., QUAN P.L., JABADO O.J., CONLAN S., HIRSCHBERG D.L., LIU Y., ZHAI J., RENWICK N., HUI J., HEGYI H.,
1007 GROLLA A., STRONG J.E., TOWNER J.S., GEISBERT T.W., JAHRLING P.B., BÜCHEN-OSMOND C., ELLERBROK H., SANCHEZ-
1008 SECO M.P., LUSSIER Y., FORMENTY P., NICHOL M.S., FELDMANN H., BRIESE T. & LIPKIN W.I. (2007). Panmicrobial
1009 oligonucleotide array for diagnosis of infectious diseases. *Emerg. Infect. Dis.,* **13**, 73–81.

1010 PALLISTER J., MIDDLETON D., WANG L.F., KLEIN R., HAINING J., ROBINSON R., YAMADA M., WHITE J., PAYNE J., FENG
1011 Y.R., CHAN Y.P. & BRODER C.C. (2011). A recombinant Hendra virus G glycoprotein-based subunit vaccine
1012 protects ferrets from lethal Hendra virus challenge. *Vaccine,* **29**, 5623–56230.

1013 PATERSON D.L., MURRAY P.K. & MCCORMACK J.G. (1998). Zoonotic disease in Australia caused by a novel member
1014 of the paramyxoviridae. *Clin. Infect. Dis.*, **27**, 112–118.

1015 PATON N.I., LEO Y.S., ZAKI S.R., AUCHUS A.P., LEE K.E., LING A.E., CHEW S.K., ANG B., ROLLIN P.E., UMAPATHI T.,
1016 SNG I., LEE C.C., LIM E. & KSIAZEK T.G. (1999). Outbreak of Nipah-virus infection among abattoir workers in
1017 Singapore. *Lancet,* **354**, 1253–1256.

1018 PLOTKIN S.A., CADOZ M., MEIGNIER B., MERIC C., LEROY O., EXCLER J.L., TARTAGLIA J, PAOLETTI E., GONCZOL E. &
1019 CHAPPUIS G. (1995). The safety and use of canarypox vectored vaccines. *Dev. Biol. Stand.*, **84**, 165–170.

1020 REYNES J., COUNOR D., ONG S., FAURE C., SENG V., MOLIA S., WALSTON J., GEORGES-COURBOT M.C., DEUBEL V. &
1021 SARTHOU J. (2005). Nipah virus in Lyle's flying foxes, Cambodia. *Emerg. Infect. Dis.,* **11**, 1042–1047.

1022 ROGERS R.J., DOUGLAS I.C., BALDOCK F.C., GLANVILLE R.J., SEPPANEN K.T., GLEESON L.J., SELLECK P.N. & DUNN K.J.
1023 (1996). Investigation of a second focus of equine morbillivirus infection in coastal Queensland. *Aust. Vet. J.,* **74**,
1024 243–244.

1025 RUPPRECHT C.E., STÖHR K. & MEREDITH C. (2001). Rabies. *In:* Infectious Diseases of Wild Animals, Williams E.S. &
1026 Barker I.K., eds. Iowa Sate University Press, USA, 3–36.

1027 SENDOW I., FIELD H.E., CURRAN J., DARMINTO, MORRISSY C.J., MEEHAN G., BUICK T. & DANIELS P.W. (2006).
1028 Henipavirus in *Pteropus vampyrus* bats, Indonesia. *Emerg. Infect. Dis.*, **12**, 711–712.

1029 SMITH I.L., HALPIN K., WARRILOW D. & SMITH G.A. (2001). Development of a fluorogenic RT-PCR assay (TaqMan)
1030 for the detection of Hendra virus. *J. Virol. Methods,* **98**, 33-40.

1031 TAMIN A., HARCOURT B.H., LO M.K., ROTH J.A., WOLF M.C., LEE B., WEINGARTL H., AUDONNET J.C., BELLINI W.J. &
1032 ROTA P.A. (2009). Development of a neutralization assay for Nipah virus using pseudotype particles. *J. Virol.
1033 Methods*, **160**, 1–6.

1034 TAN C.T., GOH K.J., WONG K.T., SARJI S.A., CHUA K.B., CHEW N.K., MURUGASU P., LOH Y.L., CHONG H.T., TAN K.S.,
1035 THAYAPARAN T., KUMAR S. & JUSOH M.R. (2002). Relapsed and late-onset Nipah encephalitis. *Ann. Neurol.,* **51**,
1036 703–708.

1037 WACHARAPLUESADEE S. & HEMACHUDHA T. (2007). Duplex nested RT-PCR for detection of Nipah virus RNA from
1038 urine specimens of bats. *J. Virol. Methods*, **141**, 97–101.

1039 WACHARAPLUESADEE S., LUMLERTDACHA B., BOONGIRD K., WANGHONGSA S., CHANHOME S., ROLLIN P., STOCKTON P.,
1040 RUPPRECHT C.E., KSIAZEK T.G. & HEMACHUDHA T. (2005). Bat Nipah Virus, Thailand, *Emerg. Infect. Dis.*, **11**, 1949–
1041 1951.

1042 WANG L.F., HARCOURT B.H., YU M., TAMIN A., ROTA P.A., BELLINI W.J. & EATON B.T. (2001). Molecular biology of
1043 Hendra and Nipah viruses. *Microbes Infect.,* **3**, 279–287.

1044 WEINGARTL H.M., BERHANE Y., CASWELL J.L., LOOSMORE S., AUDONNET J-C., ROTH J.A. & CZUB M. (2006).
1045 Recombinant nipah virus vaccines protect pigs against challenge. *J. Virol.*, **ss**, 7929–7938.

1046 WILLIAMSON M.M., HOOPER P.T., SELLECK P.W., GLEESON L.J., DANIELS P.W., WESTBURY H.A. & MURRAY P.K. (1998).
1047 Transmission studies of Hendra virus (equine morbillivirus) in fruit bats, horses and cats. *Aust. Vet. J.,* **76**, 813–
1048 818.

1049 WORLD HEALTH ORGANIZATION (WHO) (2004). Laboratory Biosafety Manual, Third Edition. WHO, Geneva,
1050 Switzerland. http://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

1051 WONG K.T., SHIEH W.J., KUMAR S., NORAIN K., ABDULLAH W., GUARNER J., GOLDSMITH C.S., CHUA K.B., LAM S.K., TAN
1052 C.T., GOH K.J., CHONG H.T., JUSOH R., ROLLIN P.E., KSIAZEK T.G., ZAKI S.R. & NIPAH VIRUS PATHOLOGY WORKING
1053 GROUP. (2002). Nipah virus infection: pathology and pathogenesis of an emerging paramyxoviral zoonosis. *Am. J.
1054 Pathol.,* **161** (6), 2153–2167.

1055 WU Z., YANG L., YANG F., REN X., JIANG J., DONG J., SUN L., ZHU Y., ZHOU H. & JIN Q. (2014). Novel Henipa-like
1056 virus, Mojiang Paramyxovirus, in rats, China, 2012. *Emerg. Infect. Dis.,* **20**, 1064–1066.

1057 YOUNG P.L., HALPIN K., SELLECK P.W., FIELD H., GRAVEL J.L., KELLY M.A. & MACKENZIE J.S. (1996). Serologic
1058 evidence for the presence in Pteropus bats of a paramyxovirus related to equine morbillivirus. *Emerg. Infect. Dis.,*
1059 **2**, 239–240.

1060   Yu F., Khairullah N.S., Inoue S., Balasubramaniam V., Berendam S.J., Teh L.K., Ibrahim N.S., Abdul Rahman S.,
1061   Hassan S.S., Hasebe F., Sinniah M. & Morita K. (2006). Serodiagnosis using recombinant Nipah virus
1062   nucleocapsid protein expressed in *Escherichia coli. J. Clin. Microbiol.,* **44**, 3134–3138.

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1065   **NB:** There is an OIE Reference Laboratory for Hendra and Nipah virus diseases
1066   (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date
1067   list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
1068   Please contact the OIE Reference Laboratories for any further information on
1069   diagnostic tests, reagents and vaccines for Hendra and Nipah virus diseases

ZOONOSES TRANSMISSIBLE FROM NON-HUMAN PRIMATES

SUMMARY

For standards for testing non-human primates, please consult the following document:

Health monitoring of non-human primate Colonies. Recommendations of the Federation of European Laboratory Animal Science Associations (FELASA) Working Group on Non-Human Primate Health accepted by the FELASA Board of Management, 21 November 1998.
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"[1] 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).

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[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 1. *Microorganisms and parasites of current concern in non-human primates* (*from FELASA* [*1998*])

(1) Viruses	
B virus, *Herpesvirus simiae,* Cercopithecine herpesvirus 1	Marburg virus
Herpesvirus cercopithecus, (SA 8), *Cercopithecine herpesvirus 2*	Ebola-Reston virus
Herpesvirus papio 2 (HVP/2), *Cercopithecine herpesvirus 12*	Simian immunodeficiency virus (SIV)
Herpes T, Herpesvirus platyrrhinae, Saimiriine herpesvirus 1	Simian T-cell lymphotropic virus-1 (STLV-1)
Herpesvirus saimiri, Saimiriine herpèsvirus 2	Simian retrovirus, type D (SRV/D)
Hepatitis A virus	Foamy virus
Hepatitis B virus	Monkeypox virus
SV 40	Lyssa virus (rabies)
Simian haemorrhagic, fever virus	Yellow fever virus
(2) Bacteria	
Campylobacter jejuni	*Salmonella typhimurium*
Campylobacter fetus	*Salmonella enteritidis*

Leptospira interrogans (various serovars)	*Shigella flexneri*
Mycobacterium africanum	*Yersinia pseudotuberculosis*
Mycobacterium bovis	*Pseudomonas pseudomallei* (*Burkolderia pseudomallei*)
Mycobacterium tuberculosis	
(3) 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 • Lite
(4) Dermatomycosis	
Trichophyton	

REFERENCES

68 ARRAUSI-SUBIZA M., IBABE J., ATXAERANDIO R., JUSTE R.A. & BARRAL M. (2014). Evaluation of different enrichment
69 methods for pathogenic *Yersinia* species detection by real time PCR. *BMC Vet. Res.*, **10**, 192 (29 August 2014).
70 available at: http://www.biomedcentral.com/1746-6148/10/192

71 COGSWELL F. (2007). Chapter 21: Parasites of Non-human Primates. *In:* Flynn's Parasites of Laboratory Animals,
72 2nd Edition, Baker D.G., ed. Blackwell, Ames, Iowa, USA, pp. 693–743.

73 FELASA (1998), Health monitoring of non-human primate Colonies. Recommendations of the Federation of
74 European Laboratory Animal Science Associations (FELASA) Working Group on Non-Human Primate Health
75 (*http://lan.sagepub.com/content/33/suppl_1/3.full.pdf*)

76 FREDRIKSSON-AHOMAA M. (2007). *Yersinia enterocolitica* and *Yersinia pseudotuberculosis. In:* Infectious Disease:
77 Foodborne Diseases. Humana Press, Totowa, New Jersey, USA, pp. 79–113.
78 available at: http://eknygos.lsmuni.lt/springer/655/79-113.pdf

79 KRAJDEN M., MCNABB G. & PETRIC M. (2005). The laboratory diagnosis of hepatitis B virus. *Can. J. Infect. Dis. Med.*
80 *Microbiol.,* 16 (2), 65–72. (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2095015/)

81 LAUKKANEN R., HAKKINEN M., LUNDÉN J., FREDRIKSSON-AHOMAA M., JOHANSSON T. & KORKEALA H. (2010). Evaluation
82 of isolation methods for pathogenic *Yersinia enterocolitica* from pig intestinal content. J. *Appl. Microbiol.*, **108** (3),
83 956–964.

84 SMITH P.H., WILES S.E., MALONE J.B. & MONAHAN C.M. (2007). Chapter 1: Collection, Preservation, and Diagnostic
85 Methods. *In:* Flynn's Parasites of Laboratory Animals, 2nd Edition, Baker D.G., ed. Blackwell, Ames, Iowa, USA,
86 pp. 1–13.

87 WHO (2003). Manual for the laboratory identification and antimicrobial susceptibility testing of bacterial pathogens
88 of public health concern in the developing world.
89 (http://www.who.int/csr/resources/publications/drugresist/WHO_CDS_CSR_RMD_2003_6/en/)

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